Title
Multiple roles of PDZ ligands in the membrane trafficking of G protein-coupled receptors

Author
Gage, Robert Michael

Publication Date
2005

Peer reviewed|Thesis/dissertation

Multiple Roles of PDZ Ligands in the Membrane Trafficking of G Protein-Coupled Receptors

by

Robert Michael Gage

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Pharmaceutical Chemistry

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

Dedication

This thesis is dedicated to the memories of Moses Horwitz, Louis Fienberg, and Jerome Lester Horwitz whose timeless comedy has sustained me through both happy and rough times.

Acknowledgements

I would like to thank my parents for all their support and helping me to enjoy life. My grandparents for their wishes and hopes that have helped me aspire to do things I didn't think possible. For all my relatives who have wished me well and supported me through my long road from high school to finally graduate school.

I'd like to thank Mark and all the members of the von Zastrow lab for advice and the fun times. Kyung for starting the investigations and providing help with the β_2AR recycling sequence. Michael and Aaron for invaluable advice on experimental protocol and for scientific discussions. Gabriel for advice on flow cytometry.

I would also like to thank my committee, Dr. R. Kip Guy and Dr. Henry Bourne for their advice and help.

Special thanks for Elena A. Matveeva for a rewarding collaboration on binding between the β_2AR and NSF.

I'd also like to thank Denise for sharing three great years when we first started graduate school. You brought joy and happiness into my life and I hope that we can stay close friends wherever life takes us. It was fun to work with you throughout these six years and I wish you all the best.

I could not have finished by May if not for the tireless efforts of my new wife Chandreyee. You set a great example by working hard to accomplish your goals and have helped me in numerous ways to be able to finish on time. The best way that you have helped motivate me to finish was to start your post-doc in Boston. I love you more than anyone I have ever known and will work very hard to meet you there as soon as possible.

Portions of this thesis have been published previously:

Chapter 2 was published previously in the Journal of Biological Chemistry with contributions from Kyung-Ah Kim and Tracy Cao.

J Biol Chem (2001), "A transplantable sorting signal that is sufficient to mediate rapid recycling of G protein-coupled receptors.", Nov 30;276(48):44712-20. Epub 2001 Sep 17.

Chapter 3 was previously published in the Journal of Biological Chemistry with contributions from Elena A Matveeva in Sidney W. Whiteheart's laboratory at the University of Kentucky.

J Biol Chem (2005), "Type I PDZ ligands are sufficient to promote rapid recycling of G Protein-coupled receptors independent of binding to N-ethylmaleimide-sensitive factor.", Feb 4;280(5):3305-13. Epub 2004 Nov 17.

Appendix 1 contains significant contributions from Naoaki Fuji working in the laboratory of R. Kip Guy at UCSF.

Multiple roles of PDZ ligands in the membrane trafficking of G protein-coupled receptors

Robert Michael Gage

Performed in the laboratory of Mark von Zastrow

Abstract

Many membrane proteins and most lipids rapidly return to the plasma membrane following endocytosis without the need for a specific sorting signal. However, the β_2 adrenergic receptor (β_2AR) as well as several other G protein-coupled receptors (GPCRs) requires a specific sequence present in their respective carboxyl terminal tails in order to efficiently recycle back to the plasma membrane following agonist induced endocytosis. While necessary for the proper routing of its cognate receptor, the sufficiency of these "recycling signals" was unknown as well as the mechanism behind their action. We first sought to determine whether the recycling motif present in the β_2AR was sufficient to re-route a heterologous GPCR, the δ opioid receptor (δ OR), which is normally trafficked to lysosomes into a rapidly recycling pathway. We used fluorescent light microscopy to visualize tagged versions of a chimeric δ OR to which had been appended the carboxyl terminal β_2AR derived recycling sequence. We also used biochemical and radioligand techniques to show that the distal carboxyl terminal tail of the β_2AR is sufficient to greatly enhance the recycling efficiency of the δ OR.

The distal region of the β_2AR binds to both the PDZ (PSD-95, discs large, zona occludens) domain containing protein hNHERF1 / EBP50 (Na^+/H^+ exchanger regulatory factor / ezrin moesin radixin binding phospho-protein of 50 kDa) and the ATPase NSF (N-ethyl maleimide sensitive factor). Binding to both of these proteins has been

suggested to play a role in the recycling of the β_2AR. By using point mutations to the sequence derived from the β_2AR as well as novel PDZ ligand sequences from other membrane proteins, we were able to correlate the enhanced recycling efficiency of these sequences to PDZ domain mediated protein interactions. In the course of this investigation, it was noted that receptors which bound solely to PDZ domain containing proteins showed a marked decrease in the ability of the receptor to internalize in response to agonist. This decreased internalization rate could be countered by engineering the ability to bind NSF into the sequences. Thus it appears that NSF may play a role in membrane protein internalization.

Table of contents

List of Tables

List of Figures

Chapter 1: Introduction

Robert Michael Gage

Chapter 1: Introduction

G protein-coupled receptors

G protein coupled receptors (GPCRs) represent the largest family of receptors in the body and over 1000 receptors have been identified so far (1,2). These receptors are responsible for many physiological responses to a myriad of different stimuli including, but not limited to biogenic amines, peptides, lipids, nucleotides, ions and large proteins such as proteases (1). In fact the senses of sight, taste, and smell depend critically on G protein-coupled receptors (1). Structurally, GPCRs span the plasma membrane seven times, have their amino terminus directed into the extracellular space, and have a cytoplasmic directed carboxyl terminus. GPCRs can be grouped based upon several characteristics. So called family A or Rhodopsin-like receptors are identified by a large intracellular third loop, a palmitoylated cysteine in the cytoplasmic tail and sequence motifs in the intracellular 2^{nd} loop (DRY) and 7^{th} transmembrane alpha helix (NPXXY) (1,3,4). Family B or Glucagon/VIP/Calcitonin receptor-like GPCRs also have a large 3^{rd} intracellular loop and contain a large amino terminus containing multiple cysteines and lack both the palmitoylated cysteine present in the carboxyl terminal tail and sequence motifs of family A receptors (1). A third group, the family C or Metabotropic neurotransmitter/Calcium receptors, have an extended amino terminus largely free of cysteines and a relatively small intracellular 3^{rd} loop (1). Family C receptors also lack the palmitoylation site in the carboxyl terminal tail and do not contain the sequence elements of family A receptors (1).

G protein-coupled receptors are so named because following agonist binding the activated receptor promotes guanine nucleotide exchange on a heterotrimeric G protein (1,5). These G proteins bind and hydrolyze guanosine tri-phosphate and consist of three subunits (5). The alpha subunit, which contains the catalytic GTPase activity, binds to the beta/gamma subunit dependent on the phosphorylation state of the bound nucleotide (1,4-6). In the GDP bound form, the G protein consists of an alpha subunit and a beta/gamma subunit. After GDP is exchanged for GTP, the alpha subunit dissociates from the beta/gamma subunit. The beta/gamma (β/γ) subunits are tightly and essentially irreversibly bound together (7-10). Following nucleotide exchange, both the alpha subunit and the beta/gamma subunit can go on to act as second messengers in a signaling network (1,6,11). For example, the stimulatory alpha subunit (α_s) when in the GTP bound form stimulates adenylyl cyclase and causes an increase in intracellular cAMP (1,6,11).

The β_2 adrenergic receptor

A prototypical family A G protein-coupled receptor, the β_2 adrenergic receptor (β_2AR), is activated by several biogenic catecholamines such as epinephrine and norepinephrine (7,12). Activation of the β_2AR leads to guanine nucleotide exchange of $G_{\alpha s}$, activation of adenylyl cyclase, and an increase in cellular cAMP levels (7). Interestingly, the β_2AR can also activate a second class of G protein, $G_{\alpha i}$, which has the opposite functional effect on adenylyl cyclase and cAMP levels (13-17). The receptor is believed to activate the G proteins sequentially such that there is an initial rise in cAMP levels and then a return to a level below that of unstimulated cells (13-17).

Adrenergic signaling is involved in the important physiological processes governing cardiac contraction rate and vasodilation. In the body there are three different β adrenergic receptors (β_1AR, β_2AR, and β_3AR) (18,19). There are differences among the three receptors in their tissue distribution and function. The β_1AR plays a large role in relaxing peripheral arteries (20,21), although the β_2AR also contributes to vasodilation (22). In mice genetically engineered to lack both the β_1AR and β_2AR, the gene for the β_3AR is upregulated to compensate for the lack of the other adrenergic receptors and can contribute to vasodilation (21). In the heart, the β_1AR constitutes approximately 75-80 % of the complement of β adrenergic receptors (12). The vast majority of adrenergic receptors present in the myocardium are βAR's where the ratio of β:α adrendoreceptors is about 10:1 (12,23). During periods of exertion, cardiac output and contraction rate are increased through enhanced βAR signaling in cardiac myocytes (12).

The adrenergic receptors have been implicated in heart disease as expected from their central role in modulating cardiac and vascular activity. Cardiac failure has many etiological features often including increased levels of catecholamine agonists of the adrenergic receptors (12,24). Chronically elevated levels of these compounds invariably lead to the desensitization and downregulation of both β_1AR and β_2AR (24). Desensitization can be defined simply as the necessity for an increased amount of adrenergic agonist to cause half maximal biological effect (7). Downregulation is a process whereby after prolonged exposure to agonist, receptors are either decreased in number through a variety of mechanisms or modified in such a way that further signaling is dramatically reduced over a prolonged period (7). Long term reduction of adrenergic receptor signaling can lead to remodeling in cardiac myocytes and a reduced capacity for

exercise (12). Eventually, these effects lead to the inability to properly perfuse the body and heart with oxygen laden blood and cardiac arrest occurs.

Receptor number is carefully coordinated and regulated at many levels. For many GPCRs, including the β_1AR (25-27), the gene encoding the receptor can be induced or repressed to maintain an appropriate surface receptor number. Following transcription, the stability of the mRNA which codes for receptors can be altered in a homeostatic process to maintain cell surface receptor number by modulating the efficiency of protein translation(28-35). Modulation of the protein synthesis of new receptors through multiple mechanisms can lead to a long lasting alteration in cell surface receptor number.

Regulation of those receptors already present in the cell surface is equally complex and important, especially for rapidly modulating receptor signaling. Post-translational modification of the adrenergic receptors, specifically phosphorylation on cytoplasmic ser/thr residues, has been shown to adversely affect the receptor's ability to promote guanine nucleotide exchange of their cognate G protein (36-42). Following phosphorylation, ligand-activated adrenergic receptors bind to arrestin molecules which further decouples the receptor from G proteins (37,43-55). Arrestin molecules (which can directly interact with clathrin, adapter molecules, and receptors) are thought to concentrate receptors into clathrin coated structures from which the receptors can be internalized into the cell (37,43-55).

Membrane trafficking of GPCRs such as the β_2AR can help determine the signaling output from the receptor following prolonged agonist exposure. Internalization into the cell and separation of receptor from activating ligand is an effective means of decreasing receptor signaling. In fact, internalization is often the first step for a receptor along a

degradative pathway (56-58). Degradation of G protein-coupled receptors, leading to a desensitization of agonist response, can occur via both lysosome dependent and independent mechanisms (56-60). Conversely, there is evidence that for the β_2AR, a trip through the endocytic pathway is required to return functional naive receptors to the plasma membrane in a process termed "resensitization" (61,62). Ultimately, the proper regulation of adrenergic receptor number and signaling through multiple mechanisms is essential for the proper operation of the cardio-vascular system. Disease states can arise from alterations in the regulation of receptor number and signaling.

Membrane trafficking of G protein-coupled receptors

Many membrane proteins and bulk lipid traverse the endocytic pathway and rapidly recycle back to the plasma membrane without the need for a specific cytosolic signal or protein interaction (63-65). For example, the transferrin (tfn) receptor rapidly recycles to the plasma membrane even when stripped of all cytoplasmic amino acid residues (66,67). Certain fluorescently labeled lipids also recycle rapidly to the plasma membrane with similar kinetics to those exhibited by the transferrin receptor (68). A simple model which readily explains the recycling rates and ratios of surface to cargo molecules was described as "iterative fractionation" (69). In this model, endocytosed receptors and cargo are internalized into spherical vesicles. Fusion between early endosomes and endocytic vesicles increases the endosome's content of both receptor and cargo. Fission of the early endosome proceeds through tubulation which has a larger surface to volume ratio than the parent spherical endosome. Over a number of rounds of fusion and fission, proportionally more receptors are removed from the endosome than

cargo molecules since the receptors are bound to the membrane (surface) while the cargo molecules are present in the endosomal lumen (volume). By measuring the rates at which receptors and cargo are accumulated into endosomes, the authors calculated the efficiencies of sorting via this mechanism. By this model after 20 rounds of fission and fusion, 93% of tfn receptors were recycled while 83% of the tfn cargo was retained within the lumen of the endosome (69). From kinetic studies of the tfn receptor and low density lipoprotein (LDL) receptor (70,71), it is estimated that each receptor internalizes and recycles 150 or 300 times respectively. This gives an estimate of >99% recycling efficiency for the receptors using the "iterative fractionation" model (69). The recycling efficiency of the tfn and LDL receptors can be adequately described using the simple physical sorting model described here.

Often, endocytosis of cell surface receptors is utilized as a mechanism to bring cargo into the cytoplasm of the cell. Such is the case for the transferrin and low density lipoprotein receptors. In other cases, such as the epidermal growth factor receptor (EGF R) internalization serves to decrease receptor signaling by removing the receptor from the source of agonist (extracellular milieu) and/or directing the receptor to an intracellular compartment for degradation (72-74). Many G protein coupled receptors undergo similar processes.

Following agonist stimulation, many GPCRs are rapidly phosphorylated by G protein-coupled receptor kinases (GRKs) or other kinases on specific cytoplasmic residues (36-42). Phosphorylation of GPCRs can function to uncouple receptors from their cognate G proteins and diminish signaling (46,61,62,75). In most cases GRK phosphorylated receptors are substrates for arrestin binding. Arrrestin molecules serve to further

decouple receptors from the signaling pathway and also act as an adapter to recruit receptors into clathrin coated pits where the receptors are concentrated prior to endocytosis (37,43-55). Endocytosis of GPCRs has been implicated in receptor desensitization (37,43-55), the process by which greater amounts agonist are required to produce half-maximal biological effect.

Endocytosis can also serve a number of other functions. It may be the first step in the endocytic pathway of a receptor targeted for degradation. Being membrane proteins, receptors must reach lysosomes for degradation. To reach the lysosomes for processing, however, the receptors must first be taken up from the cell surface and enter the cytoplasm in clathrin coated vesicles. Many rounds of vesicle fusion and fission with ever larger endocytic structures deposit the receptors first in early endosomes then later in multi-vesicular bodies / late endosomes. Finally the receptors reach the lysosome, whose contents are kept at low pH and contains proteases which together act to degrade the receptor. From the early endosome, GPCRs can also recycle back to the plasma membrane in a process that dephosphorylates the receptor and returns it to a naïve state (76). In the process of "resensitization", the recycled receptors are ready to undergo a further round of agonist induced signaling. G protein coupled receptors can also exit the early endosome and traffick to recycling endosomes from which they return to the plasma membrane. A further possibility, exhibited by the V2 vasopressin receptor (V2R), is to remain within the cell for prolonged periods before returning to the cell surface (77). This may facilitate further signaling from the endocytosed V2R via signaling pathways distinct from G proteins (78). Evidence suggests that signaling may occur through the arrestin adapter molecule, which normally quickly dissociates from endosomes but

remains bound to V2R containing endosomes for prolonged periods (78). Endocytosis of activated receptors can thus serve to dampen signaling, conversely to resensitize receptors, or to facilitate non-G protein based signaling.

In contrast to many single transmembrane receptors and bulk lipid, several G protein-coupled receptors (GPCRs) including the β_2AR, β_1AR, and μ OR (beta 2 and 1 adrenergic receptors and the mu opioid receptor respectively), require a specific amino sequence in their cytoplasmic carboxyl terminus for their proper membrane trafficking following agonist induced endocytosis (79-82). The adrenergic receptors bind to PDZ domain containing proteins via a carboxyl terminal motif that is critical for their rapid recycling (83-86). The μ opioid receptor also contains a sequence in its carboxyl terminal tail that is both necessary and sufficient to promote rapid recycling (82) although it is not present at the extreme carboxyl terminus and does not constitute a canonical class I PDZ ligand. Deletion of the recycling signal sequence or point mutation is sufficient to cause impaired receptor recycling for these GPCRs (79-82).

Summary of Research

The discovery that certain G protein-coupled receptors require a sorting signal to be efficiently recycled to the plasma membrane was unexpected since other membrane proteins and bulk lipids seem to recycle without the need for such a signal (63-65). In the work presented herein, we show that the recycling motifs from both the β_1AR and the β_2AR are sufficient to enhance the recycling efficiency of a heterologous GPCR, the δ OR. While the δ OR normally internalizes, trafficks to lysosomes and degrades following agonist exposure (56-58), chimeric δ OR which contain the carboxyl terminal

class I PDZ ligands from either β_1AR or β_2AR recycle back to the plasma membrane efficiently following agonist treatment. We also present evidence that the recycling sequences from these receptors function through interaction with PDZ domain containing proteins. We also uncovered another role PDZ domain mediated interactions in the membrane trafficking of GPCRs. We found that interaction with certain class I PDZ domain containing proteins inhibits internalization of the receptor. This has been documented before in the case of the β_1AR (85,86), but we uncovered a novel internalization inhibition motif present in the carboxyl terminus of the CFTR. We demonstrate that the sequence is sufficient to inhibit the internalization of the δ opioid receptor. Since membrane trafficking of GPCRs plays a large role in the long term processes of resensitization and down-regulation modulation of these processes could be exploited pharmacologically. We attempted to disrupt the recycling of the β_2AR using a small molecule inhibitor of PDZ domain mediated interactions. The initial data generated from this effort has been encouraging and could lead to a long-acting β blocker drug.

We first sought to determine whether the recycling motif present in the β_2AR was sufficient to re-route a heterologous GPCR, the δ opioid receptor (δ OR), which is normally trafficked to lysosomes into a rapidly recycling pathway. We used fluorescent light microscopy to visualize tagged versions of a chimeric δ OR to which had been appended the carboxyl terminal β_2AR derived recycling sequence. We also used biochemical and radioligand techniques to show that the distal carboxyl terminal tail of the β_2AR is sufficient to greatly enhance the recycling efficiency of the δ OR and also to diminish the degradation of the opioid receptor.

The V2 vasopressin receptor (V2R) endocytoses in response to agonist and does not recycle rapidly to the plasma membrane (77). It neither recycles rapidly nor degrades in lysosomes, but instead remains in a peri-nuclear compartment for prolonged periods (77). After a significant time, the V2R does recycle to the plasma membrane. The intracellular trafficking of the V2R was found to be dependent on the carboxyl terminal tail and specifically on a ser/thr cluster present in the tail (87,88). We sought to determine whether the recycling sequence from the β_2AR was capable of conferring a rapid recycling phenotype on the V2R with its unique trafficking itinerary. Appending the last ten residues of the β_2AR to the carboxyl terminus of the V2R did not affect the plasma membrane localization of the vasopressin receptor or alter its internalization rate. Addition of the β_2AR derived recycling sequence did not increase the recycling rate of the V2R. Even after allowing 2 hours for recycling to occur, most of the chimeric V2R/β_2AR was present in an internal peri-nuclear compartment. Thus it appears that while the recycling sequence from the β_2AR is capable of rerouting the δ OR from a degradative to a rapidly recycling pathway, it is incapable of performing the same feat when appended to the V2R. The slow recycling phenotype of the wild-type vasopressin 2 receptors appears to be dominant to the rapid recycling sequence from the β_2AR. Perhaps in order for the PDZ-ligand mediated rapid recycling sequence to function correctly, it must have unimpeded access to the receptor tail. By enhancing binding to β-arrestin, the ser/thr cluster in the V2R tail may also preclude interaction with other proteins such as hNHERF / EBP50 after internalization. Thus the inability of the β_2 adrenergic receptor derived recycling sequence to function in the context of the full

length vasopressin 2 receptor may be explained by a competition between β-arrestin and the PDZ domain containing protein responsible for the rapid recycling of the β_2AR.

The distal region of the β_2AR binds to both the PDZ (PSD-95, discs large, zona occludens) domain containing protein hNHERF1 / EBP50 (Na^+/H^+ exchanger regulatory factor / ezrin moesin radixin binding phospho-protein of 50 kDa) and the ATPase NSF (N-ethyl maleimide sensitive factor) (79,83,84,89). Binding to both of these proteins has been suggested to play a role in the recycling of the β_2AR (79-81,89). By using point mutations to the sequence derived from the β_2AR as well as novel PDZ ligand sequences from other membrane proteins, we were able to correlate the enhanced recycling efficiency of these sequences to PDZ domain mediated protein interactions. In the course of this investigation, it was noted that receptors which bound solely to PDZ domain containing proteins showed a marked decrease in the ability of the receptor to internalize in response to agonist. This decreased internalization rate could be countered by engineering the ability to bind NSF into the sequences. Thus it appears that direct binding between a membrane receptor and NSF may play a role in the process of internalization. Studies conducted using TIRF microscopy suggest that direct binding of a receptor to certain class I PDZ domain containing proteins may decrease the rate at which receptors cluster in Clathrin coated structures. Receptors which directly bind to NSF in addition to PDZ domain containing proteins seem to have a normal clustering and internalization rate. The receptor does not seem to be a substrate for the NSF ATPase, so interaction with NSF may be competitive with the PDZ domain mediated "endocytic brake."

The mechanism by which binding to the PDZ domain containing protein hNHERF1 / EBP50 leads to the rapidly recycling of the β_2AR is presently unknown. hNHERF1 / EBP50 contains an ERM binding domain which links it to the actin cytoskeleton. It is possible that simply binding to the cortical actin network may promote the local retention of vesicles containing the β_2AR and recycling of the receptor. We tested whether there were any visible differences in the localization or motility of vesicles containing the β_2AR, δ OR, or the chimeric receptors δ OR/β_2AR and δ OR/β_2AR-Ala (δ OR with the last ten residues from the β_2AR and a terminal alanine). Using live cell imaging we followed the motion of receptor containing vesicles following endocytosis on-stage. In general, vesicles containing receptors which have the PDZ ligand from the β_2AR and thus are able to bind hNHERF / EBP50 have a much reduced velocity compared to the δ OR or δ OR/β_2AR-Ala. In all cases, receptor containing vesicles were seen throughout the cell and no definitive differences in vesicle location were seen based on receptor type. It is possible that linking a receptor to the actin cytoskeleton, either directly or indirectly through a PDZ domain containing protein, can restrict the mobility of receptor containing endosomes. Actin binding may also form a basis for receptor sorting and concentration into a recycling pathway.

PDZ domain mediated interaction results from the recognition of a relatively small three to four amino acid motif usually present at the carboxyl terminus of the ligand protein (90). This well defined and small interaction motif makes it possible to design and chemically construct inhibitors to PDZ domain mediated function (91,92). A chemical inhibitor to the PDZ domain mediated recycling of the β_2AR could act as a long acting β blocker drug. We investigated whether a small molecule designed to mimic the

PDZ ligand of the β_2AR could indeed inhibit the recycling of the receptor. We further determined whether it would alter the degradation rate and downregulation of the β_2AR. While the PDZ inhibitor did significantly inhibit the recycling of the β_2AR, it did not alter the degradation rate as expected. Further refinement of the chemical inhibitor may produce one that is capable of acting as a long term β blocker.

References

1. Gether, U. (2000) *Endocr Rev* **21**, 90-113
2. Kolakowski, L. F., Jr. (1994) *Receptors Channels* **2**, 1-7
3. Mitchell, R., McCulloch, D., Lutz, E., Johnson, M., MacKenzie, C., Fennell, M., Fink, G., Zhou, W., and Sealfon, S. C. (1998) *Nature* **392**, 411-414
4. Schoneberg, T., Schultz, G., and Gudermann, T. (1999) *Mol Cell Endocrinol* **151**, 181-193
5. Bourne, H. R., Sanders, D. A., and McCormick, F. (1991) *Nature* **349**, 117-127
6. Marinissen, M. J., and Gutkind, J. S. (2001) *Trends Pharmacol Sci* **22**, 368-376
7. Kobilka, B. (1992) *Annu Rev Neurosci* **15**, 87-114
8. Ferguson, S. S. (2001) *Pharmacol Rev* **53**, 1-24
9. Neer, E. J. (1995) *Cell* **80**, 249-257
10. Surya, A., Stadel, J. M., and Knox, B. E. (1998) *Trends Pharmacol Sci* **19**, 243-247
11. Hamm, H. E. (1998) *J Biol Chem* **273**, 669-672
12. Rockman, H. A., Koch, W. J., and Lefkowitz, R. J. (2002) *Nature* **415**, 206-212
13. Daaka, Y., Luttrell, L. M., and Lefkowitz, R. J. (1997) *Nature* **390**, 88-91
14. Xiao, R. P., Ji, X., and Lakatta, E. G. (1995) *Mol Pharmacol* **47**, 322-329
15. Xiao, R. P., Avdonin, P., Zhou, Y. Y., Cheng, H., Akhter, S. A., Eschenhagen, T., Lefkowitz, R. J., Koch, W. J., and Lakatta, E. G. (1999) *Circ Res* **84**, 43-52
16. Communal, C., Singh, K., Sawyer, D. B., and Colucci, W. S. (1999) *Circulation* **100**, 2210-2212

17. Devic, E., Xiang, Y., Gould, D., and Kobilka, B. (2001) *Mol Pharmacol* **60**, 577-583

18. Benovic, J. L., Bouvier, M., Caron, M. G., and Lefkowitz, R. J. (1988) *Annu Rev Cell Biol* **4**, 405-428

19. Bylund, D. B., Eikenberg, D. C., Hieble, J. P., Langer, S. Z., Lefkowitz, R. J., Minneman, K. P., Molinoff, P. B., Ruffolo, R. R., Jr., and Trendelenburg, U. (1994) *Pharmacol Rev* **46**, 121-136

20. Wellstein, A., Belz, G. G., and Palm, D. (1988) *J Pharmacol Exp Ther* **246**, 328-337

21. Chruscinski, A., Brede, M. E., Meinel, L., Lohse, M. J., Kobilka, B. K., and Hein, L. (2001) *Mol Pharmacol* **60**, 955-962

22. Chruscinski, A. J., Rohrer, D. K., Schauble, E., Desai, K. H., Bernstein, D., and Kobilka, B. K. (1999) *J Biol Chem* **274**, 16694-16700

23. Hoffman BB, L. R. (1996) in *The Pharmocological Basis of Therapeutics* (Hardman JG, G. A., Limbird LE, ed), pp. 199-248, McGraw-Hill, New York

24. Bristow, M. R. (1998) *Lancet* **352 Suppl 1**, SI8-14

25. Kizaki, K., Momozaki, M., Akatsuka, K., Fujimori, Y., Uchide, T., Temma, K., and Hara, Y. (2004) *Biol Pharm Bull* **27**, 1130-1132

26. Wong, D. L., Tai, T. C., Wong-Faull, D. C., Claycomb, R., and Kvetnansky, R. (2004) *Ann N Y Acad Sci* **1018**, 387-397

27. Kimura, H., Miyamoto, A., and Ohshika, H. (1993) *Life Sci* **53**, PL171-176

28. Headley, V. V., Tanveer, R., Greene, S. M., Zweifach, A., and Port, J. D. (2004) *Mol Cell Biochem* **258**, 109-119

29. Dunigan, C. D., Hoang, Q., Curran, P. K., and Fishman, P. H. (2002) *Biochemistry* **41**, 8019-8030

30. Kirigiti, P., Bai, Y., Yang, Y. F., Li, X., Li, B., Brewer, G., and Machida, C. A. (2001) *Mol Pharmacol* **60**, 1308-1324

31. Blaxall, B. C., Pellett, A. C., Wu, S. C., Pende, A., and Port, J. D. (2000) *J Biol Chem* **275**, 4290-4297

32. Mitchusson, K. D., Blaxall, B. C., Pende, A., and Port, J. D. (1998) *Biochem Biophys Res Commun* **252**, 357-362

33. Tittelbach, V., Volff, J. N., Giray, J., Ratge, D., and Wisser, H. (1998) *Biochem Pharmacol* **56**, 967-975

34. Danner, S., Frank, M., and Lohse, M. J. (1998) *J Biol Chem* **273**, 3223-3229

35. Danner, S., and Lohse, M. J. (1997) *Eur J Pharmacol* **331**, 73-78

36. Sibley, D. R., Strasser, R. H., Caron, M. G., and Lefkowitz, R. J. (1985) *J Biol Chem* **260**, 3883-3886

37. Benovic, J. L., Kuhn, H., Weyand, I., Codina, J., Caron, M. G., and Lefkowitz, R. J. (1987) *Proc Natl Acad Sci U S A* **84**, 8879-8882

38. Benovic, J. L., Strasser, R. H., Caron, M. G., and Lefkowitz, R. J. (1986) *Proc Natl Acad Sci U S A* **83**, 2797-2801

39. Gurevich, V. V., and Benovic, J. L. (1997) *Mol Pharmacol* **51**, 161-169

40. Gurevich, V. V., and Benovic, J. L. (2000) *Methods Enzymol* **315**, 422-437

41. Gurevich, V. V., Pals, R. R., Benovic, J. L., Hosey, M. M., and Onorato, J. J. (1997) *J Biol Chem* **272**, 28849-28852

42. Gurevich, V. V., and Benovic, J. L. (1993) *J Biol Chem* **268**, 11628-11638

43. DeGraff, J. L., Gagnon, A. W., Benovic, J. L., and Orsini, M. J. (1999) *Journal of Biological Chemistry* **274**, 11253-11259

44. Ferguson, S. S., Barak, L. S., Zhang, J., and Caron, M. G. (1996) *Can J Physiol Pharmacol* **74**, 1095-1110

45. Goodman, O. J., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1996) *Nature* **383**, 447-450

46. Goodman, O. B., Jr., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1998) *Advances in Pharmacology* **42**, 429-433

47. Krupnick, J. G., Goodman, O. J., Keen, J. H., and Benovic, J. L. (1997) *J Biol Chem* **272**, 15011-15016

48. Krupnick, J. G., and Benovic, J. L. (1998) *Annual Review of Pharmacology and Toxicology* **38**, 289-319

49. Laporte, S. A., Oakley, R. H., Holt, J. A., Barak, L. S., and Caron, M. G. (2000) *J Biol Chem* **275**, 23120-23126

50. Lohse, M. J., Benovic, J. L., Codina, J., Caron, M. G., and Lefkowitz, R. J. (1990) *Science* **248**, 1547-1550

51. Miller, W. E., and Lefkowitz, R. J. (2001) *Curr Opin Cell Biol* **13**, 139-145.

52. Oakley, R. H., Laporte, S. A., Holt, J. A., Barak, L. S., and Caron, M. G. (1999) *Journal of Biological Chemistry* **274**, 32248-32257

53. Pippig, S., Andexinger, S., Daniel, K., Puzicha, M., Caron, M. G., Lefkowitz, R. J., and Lohse, M. J. (1993) *J Biol Chem* **268**, 3201-3208

54. Zhang, J., Ferguson, S. S., Barak, L. S., Aber, M. J., Giros, B., Lefkowitz, R. J., and Caron, M. G. (1997) *Receptors and Channels* **5**, 193-199

55. Zhang, J., Barak, L. S., Winkler, K. E., Caron, M. G., and Ferguson, S. S. (1997) *J Biol Chem* **272**, 27005-27014

56. Law, P.-Y., Hom, D. S., and Loh, H. H. (1984) *J. Biol. Chem.* **259**, 4096-4104

57. Law, P. Y., and Loh, H. H. (1999) *Journal of Pharmacology and Experimental Therapeutics* **289**, 607-624

58. Tsao, P., Cao, T., and von Zastrow, M. (2001) *Trends Pharmacol Sci* **22**, 91-96.

59. Shenoy, S. K., McDonald, P. H., Kohout, T. A., and Lefkowitz, R. J. (2001) *Science* **294**, 1307-1313

60. Jockers, R., Angers, S., Da Silva, A., Benaroch, P., Strosberg, A. D., Bouvier, M., and Marullo, S. (1999) *J Biol Chem* **274**, 28900-28908

61. Lefkowitz, R. J., Pitcher, J., Krueger, K., and Daaka, Y. (1998) *Advances in Pharmacology* **42**, 416-420

62. Ferguson, S. S., Zhang, J., Barak, L. S., and Caron, M. G. (1998) *Life Sci* **62**, 1561-1565

63. Trowbridge, I. S., Collawn, J. F., and Hopkins, C. R. (1993) *Annual Review of Cell Biology* **9**, 129-161

64. Gruenberg, J., and Maxfield, F. R. (1995) *Current Opinion in Cell Biology* **7**, 552-563

65. Gruenberg, J. (2001) *Nat Rev Mol Cell Biol.* **2**, 721-730

66. Jing, S. Q., Spencer, T., Miller, K., Hopkins, C., and Trowbridge, I. S. (1990) *J Cell Biol* **110**, 283-294

67. McGraw, T. E., and Maxfield, F. R. (1990) *Cell Regul* **1**, 369-377

68. Mayor, S., Presley, J. F., and Maxfield, F. R. (1993) *Journal of Cell Biology* **121**, 1257-1269

69. Dunn, K. W., McGraw, T. E., and Maxfield, F. R. (1989) *Journal of Cell Biology* **109**, 3303-3314

70. Goldstein, J. L., Brown, M. S., Anderson, R. G., Russell, D. W., and Schneider, W. J. (1985) *Annu Rev Cell Biol* **1**, 1-39

71. Omary, M. B., and Trowbridge, I. S. (1981) *J Biol Chem* **256**, 12888-12892

72. Le Roy, C., and Wrana, J. L. (2005) *Nat Rev Mol Cell Biol* **6**, 112-126

73. Haglund, K., Di Fiore, P. P., and Dikic, I. (2003) *Trends Biochem Sci* **28**, 598-603

74. Haglund, K., Sigismund, S., Polo, S., Szymkiewicz, I., Di Fiore, P. P., and Dikic, I. (2003) *Nat Cell Biol* **5**, 461-466

75. von Zastrow, M., and Kobilka, B. K. (1994) *J Biol Chem* **269**, 18448-18452.

76. Hausdorff, W. P., Caron, M. G., and Lefkowitz, R. J. (1990) *FASEB J* **4**, 2881-2889

77. Innamorati, G., Sadeghi, H., and Birnbaumer, M. (1999) *Journal of Receptor and Signal Transduction Research* **19**, 315-326

78. Shenoy SK, L. R. (2003) *Biochem J.* **375(Pt 3)**, 503-515

79. Cao, T. T., Deacon, H. W., Reczek, D., Bretscher, A., and von Zastrow, M. (1999) *Nature* **401**, 286-290.

80. Gage, R. M., Kim, K. A., Cao, T. T., and von Zastrow, M. (2001) *J Biol Chem* **276**, 44712-44720.

81. Gage, R. M., Matveeva, E. A., Whiteheart, S. W., and von Zastrow, M. (2005) *J. Biol. Chem.* **280**, 3305-3313

82. Tanowitz, M., and von Zastrow, M. (2003) *J. Biol. Chem.* **278**, 45978-45986

83. Hall, R. A., Premont, R. T., Chow, C. W., Blitzer, J. T., Pitcher, J. A., Claing, A., Stoffel, R. H., Barak, L. S., Shenolikar, S., Weinman, E. J., Grinstein, S., and Lefkowitz, R. J. (1998) *Nature* **392**, 626-630

84. Hall, R. A., Ostedgaard, L. S., Premont, R. T., Blitzer, J. T., Rahman, N., Welsh, M. J., and Lefkowitz, R. J. (1998) *Proceedings of the National Academy of Sciences of the United States of America* **95**, 8496-8501

85. Hu, L. A., Tang, Y., Miller, W. E., Cong, M., Lau, A. G., Lefkowitz, R. J., and Hall, R. A. (2000) *J. Biol. Chem.* **275**, 38659-38666

86. Xu, J., Paquet, M., Lau, A. G., Wood, J. D., Ross, C. A., and Hall, R. A. (2001) *J. Biol. Chem.* **276**, 41310-41317

87. Innamorati, G., Sadeghi, H. M., Tran, N. T., and Birnbaumer, M. (1998) *Proceedings of the National Academy of Sciences of the United States of America* **95**, 2222-2226

88. Innamorati, G., Le Gouill, C., Balamotis, M., and Birnbaumer, M. (2001) *Journal of Biological Chemistry* **276**, 13096-13103

89. Cong, M., Perry, S. J., Hu, L. A., Hanson, P. I., Claing, A., and Lefkowitz, R. J. (2001) *J. Biol. Chem.* **276**, 45145-45152

90. Harris, B. Z., and Lim, W. A. (2001) *J Cell Sci* **114**, 3219-3231

91. Fujii, N., Haresco, J. J., Novak, K. A., Stokoe, D., Kuntz, I. D., and Guy, R. K. (2003) *J Am Chem Soc* **125**, 12074-12075

Chapter 2: A transplantable sorting signal that is sufficient to mediate rapid recycling of G protein-coupled receptors

All work was performed by Robert Michael Gage with the exceptions noted below:

(1) The work presented in Figure 1 (epifluorescence and confocal microscopy, microscopic quantification) was performed by Kyung-Ah Kim

(2) The work presented in Figure 6 (biotinylation and quantification of surface receptors) was performed by Tracy T. Cao

Summary

The beta-2 adrenergic receptor (β_2AR) and delta opioid receptor (δOR) represent distinct G protein-coupled receptors that undergo agonist-induced endocytosis via clathrin-coated pits but differ significantly in their postendocytic sorting between recycling and degradative membrane pathways, respectively. Previous results indicate that a distal portion of the carboxyl-terminal cytoplasmic domain of the β_2AR engages in a PDZ domain-mediated protein interaction required for efficient recycling of receptors after agonist-induced endocytosis. Here we demonstrate that a four residue sequence (DSLL) comprising the core of this protein interaction domain functions as a transplantable endocytic sorting signal that is sufficient to re-route endocytosed δOR into a rapid recycling pathway, to inhibit proteolytic downregulation of receptors, and to mediate receptor-autonomous sorting of mutant receptors from the wild type allele when co-expressed in the same cells. To our knowledge these observations provide the first demonstration of a transplantable signal mediating rapid recycling of any GPCR, and they suggest that rapid recycling of certain membrane proteins does not occur by bulk membrane flow but is instead mediated by a specific endocytic sorting mechanism.

Introduction

Many G protein-coupled receptors (GPCRs) undergo agonist-induced endocytosis via clathrin-coated pits (1-4). However, GPCRs endocytosed by this highly conserved mechanism can follow divergent downstream membrane pathways that serve distinct physiological functions (5). For example, both the beta-2 adrenergic receptor (β_2AR) and delta opioid receptor (δ OR) endocytose in HEK293 cells via clathrin-coated pits within several minutes after agonist-induced activation (1,6,7). Most β_2ARs are recycled back to the plasma membrane within 30 minutes after endocytosis, whereas most internalized δORs do not recycle but instead traverse a divergent membrane pathway leading to lysosomes (8). Rapid recycling of the β_2AR is well established to play an important role in promoting functional resensitization of signal transduction (3,4), whereas trafficking of δOR to lysosomes contributes to the functionally opposite process of agonist-induced downregulation of receptors (9-11). Recent studies have identified additional functions of endocytosis in mediating signal transduction and suggest the existence of additional complexity in the post-endocytic membrane trafficking of certain GPCRs (12-16). However, little is known about mechanisms that determine the specificity with which GPCRs are sorted between distinct membrane pathways after endocytosis.

In general it is thought that cytoplasmic domains of membrane proteins contain structural elements that function as sorting "signals" to control specific steps of intracellular trafficking (17,18). Previous studies indicate that the carboxyl-terminal cytoplasmic domain of certain GPCRs contains sequences that promote receptor endocytic trafficking to lysosomes (19-21). In contrast, recycling of internalized membrane proteins back to the plasma membrane is generally thought to occur by

"default" without any requirement for cytoplasmic sorting signals (17,18). Support for this hypothesis includes previous studies establishing that major lipid constituents of the plasma membrane recycle rapidly by "bulk flow" (22) and that certain integral membrane proteins recycle rapidly in the absence of any exposed cytoplasmic residues (23).

Emerging evidence suggests that recycling of certain GPCRs may not occur by default but may require specific membrane sorting signals. Endocytosed V2 vasopressin receptors (V2Rs) recycle to the plasma membrane by a membrane pathway characterized by its remarkably slow kinetics (t1/2 > 2 hours) (12). Recycling of receptors via this "long pathway" requires a specific sequence present in the cytoplasmic tail of the V2R (12,24,25), and this sequence is sufficient to act as a sorting signal to cause a chimeric mutant V1 vasopressin (26) or β_2AR (25) to traverse this distinct recycling pathway. However, disruption of this sorting signal in the V2R causes internalized receptors to recycle with similarly rapid kinetics (t1/2 < 30min) as the wild type β_2AR (25). While these observations confirm that recycling of GPCRs by the specialized long pathway is mediated by a specific cytoplasmic sorting signal, they also support the hypothesis that more rapid recycling of GPCRs occurs by default.

A previous study of the β_2AR suggested that rapid recycling of certain GPCRs may require a specific sorting signal. Mutations of a sequence present in the distal portion of the carboxyl-terminal cytoplasmic domain of the β_2AR, which disrupt a specific interaction with the NHERF (Na^+/H^+ Exchanger Regulatory Factor) / EBP50 (Ezrin/Radixin/Moesin Binding Phosphoprotein of 50 kD) family of PDZ domain-containing proteins (27-29), strongly inhibited recycling of receptors after agonist-induced endocytosis (30). However, as NHERF / EBP50 proteins play multiple

important roles in cell physiology (including controlling ion transport across the plasma membrane (27,31), contributing to the structure of the cortical actin cytoskeleton (29,32) and cross-linking certain proteins in the plasma membrane (31)), impaired recycling of tail-mutant β_2ARs may not indicate the existence of a specific recycling signal but might instead reflect a secondary consequence of disrupting another aspect of receptor function or membrane organization. Furthermore, as the specific sequence required for high-affinity interaction of the β_2AR with NHERF / EBP50 –family proteins is not conserved in most other GPCRs (33), it was not established whether this PDZ-interacting sequence could play any role in controlling the membrane trafficking of a distinct GPCR.

We have addressed these questions by examining whether sequences derived from the carboxyl-terminal cytoplasmic domain of the β_2AR are sufficient to function as a transplantable sorting signal to promote rapid recycling of a heterologous GPCR. We have focused on studying effects on the endocytic trafficking of an epitope-tagged version of δOR expressed in HEK293 cells, where this GPCR is well established to endocytose via clathrin-coated pits but differs substantially in its postendocytic sorting from the β_2AR even when co-expressed at similar levels in the same cells (8). Our results indicate that the distal tail sequence from the β_2AR can indeed function as an autonomous sorting signal, which is fully sufficient to re-route endocytosed δOR into a rapid recycling pathway. This transplantable sorting activity is functionally significant because it also confers reduced proteolytic downregulation on mutant receptors, and it is possible to reduce the sorting signal sufficient to mediate both effects to a four residue sequence (DSLL) corresponding to the minimal structure required to mediate detectable binding of the mutant receptor tail to NHERF / EBP50 –family proteins. The autonomous activity of

this sorting signal is demonstrated by the ability of the four residue sequence to selectively re-route trafficking of a mutant δOR without causing any detectable effect on the endocytic trafficking of the co-expressed wild-type allele. Thus, at least in the case of certain GPCRs, rapid recycling does not occur by default but can instead be mediated by a specific signal-dependent sorting operation.

Experimental Procedures

cDNA Constructs and Mutagenesis

Several epitope-tagged versions of the cloned murine delta opioid receptor (δ OR (34)) and the human beta 2 adrenergic receptor (β_2AR (35)) were used in these studies: mutant receptors containing an HA or FLAG epitope in the amino-terminal extracellular domain (HAδ OR, HAβ_2AR or SFδ OR, SFβ_2AR, respectively) were described previously and demonstrated to be functional (8,36,37). Mutant delta opioid receptors containing a FLAG epitope in the amino-terminal extracellular domain and the last six carboxyl-terminal cytoplasmic residues (NH_2-GGGAAA-COOH) deleted, replaced with either the ten carboxyl-terminal residues from the β_2AR (NH_2-RNCSTNDSLL-COOH) or the ten residues plus an alanine (NH_2-RNCSTNDSLLA-COOH). This was accomplished by insertion of a synthetic linker-adapter (Operon Technologies) encoding the ten-residue or eleven-residue sequence followed by a stop codon into an Srf I site present near the 3' end of the sequence encoding the δ OR tail. FLAG-tagged δ OR-DSLL was made by adding a sequences encoding DSLL in frame at the 3' end of the full length receptor cDNA followed by a stop codon. This was constructed by oligonucleotide-directed mutagenesis using the polymerase chain reaction (Vent polymerase, New England Biolabs). Receptor cDNAs were cloned into pcDNA3 (Invitrogen) and all constructs were verified by dideoxynucleotide sequencing (UCSF Genetics Core Sequencing Facility.).

Cell Culture and Transfection

Human embryonic kidney 293 cells (ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (University of California

San Francisco Cell Culture Facility). Cells grown in 6-cm dishes were transfected with ~5 ug of plasmid DNA containing the indicated receptor by calcium phosphate precipitation (11,38). For studies of receptor trafficking in transiently transfected cells, cells were transfected as above, plated onto coverslips 24 h post transfection and experiments were conducted 48 h post transfection. Stably transfected cells expressing epitope tagged receptors were generated by transfecting 293 cells in 6-cm dishes as above. Cell clones expressing transfected receptors were selected in 500 ug/ml Geneticin (Life Technologies, Inc.) and colonies were isolated and selected to have similar levels of receptor expression, as estimated by radioligand binding assay conducted as described previously (11). Receptor levels in stably transfected cell lines ranged from 0.7 to 4.2 pmol/mg of total protein.

Examination of Receptor Endocytosis and Recycling by Fluorescence Microscopy

Endocytic trafficking of receptors labeled initially in the plasma membrane was visualized by fluorescence microscopy using a minor modification of a previously described method (30). Briefly, stably or transiently transfected 293 cells expressing the indicated receptor were grown on glass coverslips (Corning) treated with M1 anti-FLAG antibody (2.5 ug/ml, Sigma) at 37 °C for 25 min to label receptors. The cells were treated at the same time (37 °C for 25 min) in the presence of 10 mM isoproterenol (Research Biochemicals) or 10 mM DADLE (Research Biochemicals International). Following this incubation, cells were either fixed immediately, for determining internalization of FLAG-tagged receptors, or were subsequently washed twice in DMEM supplemented with 10% fetal bovine serum. After washing in DMEM, the cells were further incubated in DMEM

(an additional 45 min. at 37°C) to allow receptor recycling to occur before the cells were fixed. The cells were fixed with 3.7% formaldehyde in PBS, pH 7.4, for 10 min and then quenched with three washes of TBS with 1 mM CaCl2. Specimens were permeabilized with 0.1% Triton X-100 (Sigma) in Blotto (3% dry milk in TBS with 1 mM $CaCl_2$) and incubated with fluorescein isothiocyanate-conjugated donkey anti-mouse secondary antibody (1:500 dilution; Jackson ImmunoResearch) for 30 min to detect FLAG-tagged receptors. Conventional fluorescence microscopy was performed using inverted Nikon Diaphot microscope equipped with a Nikon 60X NA1.4 objective and epifluorescence optics; confocal fluorescence microscopy was carried out using a Bio-Rad MRC 1000 and a Zeiss 100X NA1.3 objective. Images were collected using a 12-bit cooled charge-coupled device camera (Princeton Instruments) interfaced to a Macintosh computer.

Quantitation of Receptor Recycling by Fluorescence Flow Cytometry

Recycling of epitope-tagged receptors back to the plasma membrane was estimated by assaying the recovery of immunoreactive receptors accessible at the cell surface to monoclonal antibody recognizing the extracellular epitope tag (FLAG). This assay is a variant of a previously described flow cytometric assay for estimating receptor internalization and recycling (8). Briefly, monolayers of cells stably expressing the indicated FLAG tagged receptor were incubated in the presence of 10 uM of the appropriate agonist (isoproterenol or DADLE) for 25 min at 37 °C to drive agonist-induced internalization, then rinsed twice with DMEM, and subsequently incubated at 37°C in the presence of the appropriate antagonist (10 uM alprenolol or naloxone (Research Biochemicals) to block additional endocytosis of receptors. At the indicated

time points, cell monolayers were chilled on ice to stop membrane trafficking, and cells were lifted with PBS containing 0.04% EDTA and lacking Ca++ or Mg++ (PBS/EDTA University of California San Francisco Cell Culture Facility). Cells were washed twice in 1 ml PBS and incubated at 4 °C for 45-60 min in 0.5 ml PBS with 2.5 ug/ml M1 anti-FLAG antibody that had been conjugated with fluorescein isothiocyanate (Molecular Probes) using standard methods. Receptor immunoreactivity was quantitated by fluorescence flow cytometry (FACScan, Becton Dickinson, Palo Alto, CA). Fluorescence intensity of 20,000 cells was collected for each sample. Cellquest software (Becton Dickinson) was used to calculate the mean fluorescence intensity of single cells in each population. All experiments were conducted ≥3 times with similar results. The mean values for each experiment were averaged to obtain the overall mean fluorescence intensity and standard error of the mean reported in the figure.

Biochemical analysis of receptor degradation

Western blotting to detect proteolysis of total cellular receptors- To determine the effect of agonist treatment on steady state levels of total receptor protein, immunoblotting was performed as described previously (8). Briefly, cells stably transfected with the indicated FLAG tagged receptors were grown in 10-cm dishes and treated for 0, 1, or 4 hours with the appropriate agonist (10 uM isoproterenol or DADLE) at 37 °C. Dishes of cells containing stably transfected cells were washed with 2.5 ml PBS and the cells were dissociates and harvested in 1.5 ml PBS/EDTA for 30 min at 4 °C. After pelleting the cells by centrifugation (1000 rpm for 5 min on benchtop microcentrifuge), the cells were lysed by placing them in 1 ml of hypotonic lysis solution (25 mM Tris-HCl or 25 uM

Hepes buffer, pH 7.4, 1 ug/ml leupeptin, 1 ug/ml, pepstatin, and 2 ug/ml aprotinin) while vortexing for 2 min. The crude membrane fraction was separated from the cytoplasmic fraction by centrifugation at 14000 rpm for 15 min on a microcentrifuge. The supernatant was discarded and the pellet resuspended in 0.5 ml of resuspension buffer (25 mM Tris-HCl or 50 mM Hepes buffer, pH 7.4, 1 ug/ml leupeptin, 1 ug/ml, pepstatin, 2 ug/ml aprotinin, and 0.25% v/v Triton-X100). The non-soluble fraction was removed by centrifugation as above at 14000 rpm and the supernatant was decanted and analyzed for protein content by the Bradford method (39) using bovine serum albumin as standard. Lysate from the samples corresponding to ~40 ug of total protein were loaded and separated by SDS-PAGE under denaturing conditions. Resolved proteins were transferred to nitrocellulose membranes (Micron Separations, Inc.) and placed in TBSTM (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.1% v/v Tween-20, and 5% dry nonfat milk) for 60 min. Detection of receptors containing FLAG epitope was carried out by incubation of the blots with M1 anti-FLAG antibody (15 ug/ml in TBSTM) for 60 min, washing in TBST (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, and 0.1% v/v Tween-20), and incubation for 60 min in TBSTM containing 400 ng/ml goat anti-mouse conjugated to horseradish peroxidase (Jackson ImmunoResearch). After washing in TBST, proteins bands were detected using Super Signal (Pierce). Band intensities were quantitated by densitometry of films exposed in the linear range, imaged using a charge-coupled device camera, and analyzed using National Institutes of Health Image software or FluorChem 2.0 (AlphaInnotech Corp). Alternatively, some blots were directly imaged via chemiluminescence detection on a

FluorChem 8000 instrument (AlphaInnotech Corp.) using a 16-bit cooled charge-coupled device camera and analyzed using FluorChem 2.0 software (AlphaInnotech Corp.).

Surface biotinylation to specifically detect proteolysis of receptors present in the plasma membrane- To examine the effect of prolonged agonist exposure on the levels of receptor initially present on the surface of stably transfected cells containing the indicated receptors, a modification of an established assay using cell surface biotinylation was applied (30). Stably transfected 293 cells expressing FLAG tagged receptors were grown in 10-cm dishes, washed twice with ice-cold PBS, and surface-biotinylated by incubating intact cells with 300 ug/ml sulfo-NHS-biotin (Pierce) in PBS for 30 min at 4 °C. Unreacted biotin was quenched and removed by three washes with ice-cold TBS at 4 °C. Biotinylated cells were then transferred to prewarmed medium (37 °C) & 10 uM isoproterenol or DADLE for 1, 2, or 4 hours then cells were again chilled on ice to stop membrane trafficking. Cells were then extracted with Triton X-100 extraction buffer (0.2% (v/v) Triton X-100, 10 mM Tris-HCl, pH 7.5, 120 mM NaCl, 25 mM KCl, 1 ug/ml leupeptin, 1 ug/ml, pepstatin, 2 ug/ml aprotinin, and 1 mg/ml iodoacetamide), and extracts were clarified by centrifugation in a microcentrifuge (12,000 X *g* for 10 min) prior to immunoprecipitation of receptors. Receptors were immunoprecipitated from cell extracts using 4 ug/ml anti-FLAG M2 monoclonal antibody (Sigma), 4 ug/ml rabbit anti-mouse linker antibody, and 25 ul of protein A-Sepharose beads (Amersham Pharmacia Biotech). Immunoprecipitations were washed 5X with PBS containing 0.2% Triton X-100. Washed beads were extracted with SDS sample buffer, and eluted proteins were resolved by SDS-polyacrylamide gel electrophoresis under nonreducing conditions.

Resolved proteins were transferred to nitrocellulose membranes (Micron Separations, Inc.) and placed for 60 min in blocking solution (5% dry milk, 0.5% Tween 20 in TBS). Biotinylated proteins were then complexed with horseradish peroxidase by incubating membranes with VectaStain ABC detection system (Vector Laboratories), and biotinylated proteins were detected by enzyme-linked chemiluminescence using Super Signal (Pierce). Identical results were obtained using an alternative Immunoprecipitation procedure. In this technique, clarified cell extracts from biotinylated cells were immunoprecipitated with streptavidin conjugated Sepharose beads, thus precipitating solubilized proteins conjugated to biotin. Immunoprecipitations were washed, extracted, run on SDS-PAGE, and transferred as above. Blots were placed for 60 min in blocking solution and receptors were detected by sequential incubation of blots with 15 ug/ml M2 anti-FLAG anti-body (Sigma) and 400 ng/ml goat anti-mouse antibody conjugated to horseradish peroxidase; both 60 min. incubations were in 5% dry milk, 0.1% Tween 20 in TBS. Proteins bands were detected using Super Signal (Pierce). Band intensities were quantitated by densitometry of films exposed in the linear range, imaged using a charge-coupled device camera, and analyzed using National Institutes of Health Image software or FluorChem 2.0 (AlphaInnotech Corp.).

Radioligand Binding Assays

Radioligand binding assays to estimate receptor expression level in transfected cells were performed as described previously (8). Agonist-induced down-regulation of receptors was assayed as described previously (8,40). Briefly, monolayers of cells expressing FLAG-tagged mutant receptors were incubated for 0, 1, or 4 h at 37 °C in the absence or

presence of 10 uM DADLE (Research Biochemicals). To ensure a saturating concentration of peptide agonist over the incubation period, monolayers incubated with DADLE were supplemented with fresh peptide every hour during the incubation. At the end of the incubation, cells were lifted with PBS/EDTA and washed four times by centrifugation with 10 ml of warm (37 °C) PBS. Then cells were washed once by centrifugation in 10 ml of Krebs-Ringer HEPES buffer (KHRB: 110 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, 25 mM glucose, 55 mM sucrose, 10 mM HEPES, pH 7.3). Radioligand binding was carried out at room temperature in 120 ul of KHRB containing 50–100 ug of cell protein and 10 nM [^{3}H]-diprenorphine (50 Ci/mmol, New England Nuclear). Incubations were terminated by vacuum filtration through glass fiber filters (Packard Instruments) and repeated washes with ice-cold Tris-buffered saline, pH 7.4. Bound radioactivity was determined by scintillation counting (Scintiverse, Fisher) using a Beckman LS 6500 instrument. Bound counts represented ≤10% of input radioligand. Nonspecific binding, defined by assays conducted in the presence of 10 uM naloxone, was ≤10% of total counts isolated on filters. All assays were conducted in triplicate with similar results.

Results

The distal portion of the carboxyl-terminal cytoplasmic domain of δ OR is not required for agonist-induced endocytosis of receptors or trafficking of receptors to non-recycling endocytic vesicles

Previous studies indicate that the distal portion of the cytoplasmic tail of the β_2AR is essential for rapid recycling of receptors to the plasma membrane after agonist-induced endocytosis (30) but that the corresponding portion of the δ OR tail is not required for agonist induced endocytosis (41) or subsequent post-endocytic trafficking to lysosomes (42). To establish this in our system, we constructed a mutant δ OR in which the terminal six residues were truncated (δ ORt mutant receptor) and examined the endocytic trafficking of this mutant receptor using fluorescence microscopy, as used previously to distinguish the endocytic trafficking of the wild type β_2AR from that of wild type δ OR. These studies indicated that the δ ORt truncated mutant receptor exhibited endocytic trafficking closely similar to that of the full length δ OR and readily distinguishable from that of the β_2AR. In the absence of agonist, δ ORt mutant receptors were visualized primarily in the plasma membrane (Fig 1A panel a). After incubation of cells with agonist (10 uM DADLE) for 25 min., antibody labeled receptors redistributed from the plasma membrane to numerous cytoplasmic puncta representing endocytic vesicles (Fig 1A panel b). After agonist incubation followed by subsequent incubation of cells for 45 minutes in the absence of agonist (and presence of 10 uM of the antagonist naloxone to prevent possible receptor activation by residual agonist), antibody-labeled receptors remained primarily in intracellular vesicles and little redistribution of receptors to the

plasma membrane was observed (Fig 1A panel c). These results suggest that the δ ORt mutant receptor, like the wild type δ OR characterized previously by this assay (8), undergoes rapid agonist-induced endocytosis but fails to recycle efficiently even when examined 45 minute after agonist removal, conditions under which essentially complete (>95%) recycling of the β_2AR is observed (8,30).

The distal portion of the β_2AR tail contains a sequence that is sufficient to re-route internalized δ OR into a rapid recycling pathway

To determine whether the carboxyl-terminal cytoplasmic domain of the β_2AR contains a sorting signal that is sufficient to promote recycling of a heterologous GPCR, we examined the effect of appending a sequence corresponding to the carboxyl-terminal ten residues of the β_2AR to the δ ORt sequence (δ/β_{10} mutant receptor). As with the δ ORt mutant receptor, the δ/β_{10} chimera was localized primarily to the PM in the absence of agonist and exhibited rapid agonist induced internalization (Fig 1A panel d,e). In contrast to the δ ORt receptor, however, the δ/β_{10} chimeric mutant receptors disappeared from intracellular vesicles after agonist washout and immunoreactive receptors appeared to redistribute to the plasma membrane (Fig 1A panel f). To determine whether this enhanced recycling was a specific effect of the added β_2AR tail sequence, or if it might be a nonspecific consequence of extending the length of the cytoplasmic tail of δ OR, we examined the effect of adding a single alanine residue to the β_2AR-derived tail sequence (δ/β10-Ala mutant receptor). The corresponding mutation disrupts rapid recycling of β_2AR by preventing PDZ domain-mediated protein interaction with NHERF / EBP50 –

family protein(s) present in the cytoplasm (30). δ/β10-Ala mutant receptors exhibited pronounced agonist-induced internalization but, in contrast to the δ/β10 mutant receptor, remained primarily in intracellular vesicles after agonist washout (Fig 1A panels g-i). Identical results were obtained in studies of stably transfected HEK293 cells (data not shown) and the specific effect of the β_2AR-derived tail sequence was observed using both FLAG and HA –tagged receptors (Fig 1B). Confocal optical sections imaged through the center of cells, which allow endocytic vesicles to be resolved more clearly from the limiting plasma membrane, further confirmed differences in the localization of the mutant receptors deduced from epifluorescence microscopy (Fig 1B). The reproducibility of these observations was assessed using a previously established method (41) of counting of receptor-containing endocytic vesicles visualized in multiple cells examined at random in coded specimens (Fig 1C).

To confirm that the recovery of receptor immunoreactivity to the plasma membrane resulted from recycling of previously internalized receptors, rather than a possible effect on new receptor synthesis or delivery of receptors from a distinct intracellular "storage" pool (43), FLAG-tagged receptors present in the plasma membrane were specifically pre-labeled with monoclonal antibody before agonist addition. The cells were next washed with EDTA-containing medium to remove residual antibody from the cell surface before beginning the agonist washout period in the presence of antagonist (8). Under these conditions the only receptors labeled with antibody are those that were initially endocytosed in the presence of agonist. Whereas endocytosed δ ORt and δ/β_{10}-Ala mutant receptors visualized by this assay remained predominantly in intracellular vesicles

after agonist withdrawal, antibody-labeled δ/β_{10} chimeric mutant receptors were observed to translocate from endocytic vesicles back to the plasma membrane (Fig 1D).

The ability of the β_2AR-derived tail sequence to promote recycling of mutant opioid receptors was quantitated in stably transfected cells using a previously established flow cytometric method that measures the amount of immunoreactive receptor protein present in the plasma membrane (8). Briefly, stably transfected cells expressing FLAG-tagged mutant opioid or adrenergic receptors were maintained in the absence of agonist ("control" in Fig 2A), incubated for 30 minutes in the presence of the appropriate agonist (10uM DADLE or isoproterenol, respectively; "+agonist" in Fig 2 A), or incubated for 30 minutes with agonist and then washed and incubated for an additional 45 minutes in the presence of an excess concentration of the appropriate antagonist (10uM naloxone or alprenolol, respectively; "+agonist → antagonist" in Fig 2 A) to prevent possible receptor activation by residual agonist. After incubating cells at 37°C cells under these conditions, cells were then chilled on ice to block subsequent membrane trafficking, receptors present in the plasma membrane were labeled specifically with fluorescein-conjugated antibody and fluorescence flow cytometry was used to quantitate the relative number of immunoreactive receptors present in the plasma membrane. All cell clones studied exhibited comparable amounts of surface receptor under control conditions, consistent with their initial selection based on similar levels of receptor expression estimated by radioligand binding (see Experimental Procedures). As expected, all cell clones studied exhibited a substantial reduction (~40%) in surface receptor immunoreactivity following 30 minute incubation with agonist. After agonist washout for 45 minutes, immunoreactive β_2AR present in the plasma membrane recovered to levels close to those

observed in control (untreated) cells, whereas δ ORt mutant receptors exhibited minimal recovery in the plasma membrane under similar conditions (Fig 2A, first and second set of bars, respectively). Consistent with the ability of the β_2AR-derived tail sequence to promote recycling of internalized receptors visualized by fluorescence microscopy, surface immunoreactivity of the δ/B_{10} mutant receptor recovered nearly to control levels after agonist washout, whereas minimal recycling of the δ/B_{10}-Ala mutant receptor was observed (Fig 2A, third and fourth sets of bars, respectively). This specific effect of the β_2AR-derived tail sequence on promoting recycling of mutant opioid receptors was evident both by examination of the raw surface fluorescence data (Fig 2A) and by calculation of the fractional recovery of surface receptors after agonist washout (Fig 2B).

Enhanced recycling mediated by the β_2AR-derived tail sequence is associated with inhibited proteolytic degradation of receptors

As an independent assay of the functional activity of the β_2AR-derived sorting signal, we examined the effects of this sequence on agonist-induced proteolysis of mutant receptors in stably transfected cells. We first used immunoblotting to estimate proteolysis of the total cellular pool of receptors after continuous incubation of cells with a saturating concentration of agonist. No detectable loss of immunoreactive β_2AR was observed in lysates prepared from cells incubated in the presence of 10uM isoproterenol for 4 hours, confirming that the β_2AR is relatively resistant to proteolytic degradation under these conditions (30). In contrast, the δ ORt mutant receptor (like wild type δ OR (8)) was extensively proteolyzed under similar conditions (incubation of cells with 10uM DADLE

for 4 hours; Fig 3 A and C). The δ/β_{10} mutant receptor exhibited substantially reduced proteolysis relative to the δ ORt mutant receptor, whereas the δ/β_{10}-Ala mutant receptor exhibited extensive proteolysis closely comparable to that observed for the δ ORt mutant receptor (Fig 3 A and C). Previous studies have established that agonist-induced proteolysis of δ OR observed at this time point occurs primarily in lysosomes (8,44). Therefore the present observations indicate that the β_2AR-derived tail sequence, in addition to promoting rapid recycling of δ/β_{10} mutant receptors, inhibits trafficking of internalized receptors to lysosomes.

To specifically examine the effect of the β_2AR-derived tail sequence on the fate of endocytosed receptors, we applied a previously established cell surface biotinylation method to examine proteolysis of receptors labeled initially in the plasma membrane (30). Surface-biotinylated β_2AR exhibited little detectable proteolysis after incubation of stably transfected cells with agonist for four hours, whereas the surface-biotinylated δ ORt mutant receptor (like wild type δ OR (8)) was extensively proteolyzed under similar conditions (Fig 3 B and D). The β_2AR-derived sorting signal specifically inhibited proteolysis of surface-labeled δ/β_{10} mutant receptors, and this effect was abrogated by the addition of a single alanine residue to the tail sequence (δ/β_{10}-Ala mutant receptor). Taken together these observations provide independent confirmation that the β_2AR-derived tail sequence contains a transplantable "sorting signal" and they suggest that this signal functions by re-routing internalized opioid receptors from a membrane pathway leading to lysosomes to a distinct membrane pathway mediating rapid recycling of receptors to the plasma membrane.

The transplantable recycling signal is encoded by the terminal four-residues derived from the β_2AR tail

Previous studies have established that rapid recycling of the β_2AR requires binding of the receptor tail to NHERF / EBP50 / E3KARP -family proteins via a specific PDZ domain interaction (27,30). The ability of a single alanine residue, which blocks PDZ domain-mediated interactions with the β_2AR tail (30), to abrogate recycling of the δ/β_{10} mutant receptor strongly suggests that a similar protein interaction is also required for the β_2AR-derived tail sequence to function as a transplantable sorting signal. To begin to address whether such a protein interaction might be sufficient by itself to mediate the transplantable sorting activity of the β_2AR-derived tail sequence, we examined a mutant receptor in which only these four resides (DSLL) derived from the β_2AR tail were appended to the tail of the full length δ OR (δ/β_4 mutant receptor). These residues were chosen because they have been established previously to comprise a minimal sequence sufficient to mediate PDZ domain-mediated protein interactions with the β_2AR tail (33,45) and are sufficient to mediate detectable interaction of the δ/β_4 tail with human with human NHERF / EBP50 (data not shown). Fluorescence flow cytometry confirmed that the δ/β_4 mutant receptor is able to recycle to the plasma membrane after agonist-induced endocytosis and subsequent agonist washout (Fig 4 A) and demonstrated that the extent of recycling mediated by this four residue sequence is closely similar to that mediated by the ten residue sequence examined in the δ/β_{10} mutant receptor (Fig. 2 A and B). To examine effects of the β_2AR-derived tetrapeptide sequence on trafficking of internalized δ OR to lysosomes, we assayed agonist-induced proteolysis using

immunoblotting and surface biotinylation assays (Figs 4 B and 4 C, respectively). Both assays indicated that the terminal four residue DSLL sequence is sufficient to significantly inhibit proteolysis of receptors after agonist-induced endocytosis. Thus the β2AR-derived tetrapeptide DSLL, when attached to the c-terminal tail of the full length δ OR, is sufficient both to promote recycling of internalized receptors to the plasma membrane and to inhibit receptor trafficking to lysosomes.

Functional effects of the β_2AR-derived sorting signal on agonist-induced down-regulation measured by radioligand binding

To examine the functional consequences of the β_2AR-derived sorting signal on downregulation of mutant opioid receptors, we assayed the effect of the β_2AR-derived sorting signal on DADLE-induced downregulation of opioid receptor binding sites estimated by a previously established radioligand binding assay using [^{3}H]-diprenorphine (8). The δ opioid receptor exhibited extensive downregulation after preincubation of cells for four hours in the presence of 10uM DADLE (FIG 5, bar 1), consistent with previous results (8,42). Addition of ten residues of β_2AR-derived tail sequence significantly reduced the amount of δ/β_{10} mutant receptor downregulation (Fig 5 bar 2), whereas this effect was abrogated by adding a terminal alanine residue (δ/β_{10}-Ala mutant receptor, FIG 5, bar 3). The four residues comprising the minimal rapid recycling signal also inhibited downregulation of the δ/β_4 mutant receptor (FIG 5, bar 4). These observations are consistent with the results from the immunocytochemical and biochemical assays of receptor trafficking, and they indicate that the β_2AR-derived sorting signal is functionally

sufficient to mediate a significant reduction in agonist-induced downregulation when transplanted into a heterologous GPCR.

The β_2AR-derived sorting signal is sufficient to mediate sorting of otherwise identical receptors when co-expressed in the same cells

To determine whether the DSLL sequence functions as a sorting signal specifically for a mutant GPCR containing this sequence, or if the presence in cells of receptors containing this signal might cause a more general effect on endocytic trafficking of other receptors, we co-expressed in stably transfected HEK293 cells a FLAG-tagged version of the δ/β_4 mutant receptor together with an HA-tagged version of the "wild type" δ OR. These distinct epitope tag sequences themselves do not confer detectable differences on endocytic trafficking (FIG 1) or proteolysis of receptors (8,42). Surface biotinylation was used to label receptors initially present in the plasma membrane, and the relative amount of each receptor recovered from cells after various periods of agonist incubation were determined using immunoprecipitation with the respective epitope tag antibody followed by detection of biotinylated receptor using streptavidin overlay. Using this technique we observed that the β_2AR-derived sorting signal specifically inhibits proteolytic degradation of the δ/β_4 mutant receptor relative to the co-expressed δ OR (FIG 6A). Furthermore we confirmed that differences in the endocytic trafficking of δ OR and δ/β_4 mutant receptors could also be observed following receptor activation with the potent alkaloid agonist etorphine (which is difficult to use for studies of receptor recycling because it washes out less efficiently than the peptide agonist DADLE). Quantitation of these observations

Discussion

In this study we examined the effect of transplanting sequences derived from the carboxyl-terminal cytoplasmic domain of the β_2AR on the endocytic trafficking of the δ OR. These experiments were motivated by the fact that, although both the β_2AR and δ OR are co-endocytosed in HEK293 by clathrin-coated pits, these distinct GPCRs differ substantially in their postendocytic trafficking between recycling and degradative membrane pathways, respectively. Furthermore, as these GPCRs signal via coupling to distinct heterotrimeric G proteins, the sorting activity of the β_2AR-derived sorting signal does not appear to be limited to G_s-coupled GPCRs. A previous study indicated that a distal portion of the carboxyl-terminal cytoplasmic domain is necessary for rapid recycling of the β_2AR. However, as the NHERF / EBP50-family proteins that interact with this sequence serve multiple functions in cell physiology and the sequence required for this protein interaction is not conserved in most other GPCRs (including δOR), it was not determined whether the requirement of this sequence for recycling of receptors reflects its activity as an "autonomous" sorting signal or an indirect consequence perturbing other aspects of cellular function or β_2AR signaling activity. The present results demonstrate that this PDZ domain-binding sequence is sufficient to re-route the endocytic trafficking of δOR from a lysosomal degradative pathway into a rapid recycling pathway and to mediate autonomous sorting of a mutant δOR from the wild type δOR when co-expressed in the same cells. Thus we conclude that the β_2AR does indeed contain an autonomous, transplantable endocytic sorting signal, which is sufficient to re-route a heterologous GPCR into a rapid recycling pathway and to cause functionally

significant changes in agonist-induced downregulation of receptors. To our knowledge, this is the first direct demonstration of the existence of a modular sorting signal mediating rapid recycling of any GPCR.

It has been well documented that GPCRs contain cytoplasmic sequences which influence specific membrane trafficking steps. For example, the D1 dopamine receptor contains a sequence in the carboxyl-terminal cytoplasmic domain that influences trafficking of newly synthesized receptors in the biosynthetic pathway (46). Studies of the PAR1 thrombin receptor and thromboxane A2 receptor have identified distinct portions of the carboxyl-terminal cytoplasmic domain that mediate regulated and constitutive endocytosis (47,48). Studies of PAR1 have also identified a distinct function of the carboxyl-terminal cytoplasmic domain in promoting receptor trafficking to lysosomes (21). Recent studies of the V2 vasopressin receptor identified a cytoplasmic sequence that mediates trafficking of receptors via a specialized recycling pathway characterized by its remarkably slow kinetics (12,25,26). However, as disruption of this sequence causes rapid recycling of receptors (26), these studies suggest that only slow pathway(s) of GPCR recycling are mediated by specific sorting signals and more rapid recycling can occur by default. To our knowledge, the present results provide the first direct demonstration of the existence of a modular sorting signal that specifically mediates rapid recycling of any GPCR.

The idea that rapid recycling can occur by default is supported by a large number of previous studies of various membrane proteins (49). For example, elegant studies of endocytic trafficking of the transferrin receptor indicate that rapid recycling of this transmembrane protein can occur after removal of all exposed cytoplasmic residues (23).

Similar experiments suggest that the Epidermal Growth Factor Receptor (EGFR) can recycle to the plasma membrane in the absence of any specific sequence present in the cytoplasmic domain of the receptor (17). Moreover, as discussed above, truncation and point mutations of the portion of the V2R tail that abolish slow recycling via the "long pathway" cause receptors to recycle to the plasma membrane with rapid kinetics (26). These considerations suggest that the rapid recycling pathway mediated by the β_2AR-derived tail sequence represents a specialized mechanism of receptor regulation, consistent with previous observations suggesting that the mechanisms mediating rapid recycling of the β_2AR and transferrin receptors are distinguishable (30). In addition, they expand on previous evidence suggesting that specialized membrane trafficking mechanisms may play an important role in distinguishing the functional regulation of specific GPCRs (5,14,16,25,50). Although our studies have focused exclusively on the functional role of endocytic sorting in controlling proteolytic downregulation mediated by receptor trafficking to lysosomes, we note that multiple mechanisms can contribute to downregulation of GPCRs under physiological conditions (51). In particular, recent studies provide strong evidence for an important role of proteasomes in mediating proteolysis of opioid receptors (52,53) and additional mechanism(s) contributing to proteolysis of other GPCRs (54,55). In future studies it will be interesting to determine what physiological significance this remarkable diversity of mechanisms mediating both recycling and proteolysis of GPCRs might have.

References

1. von Zastrow, M., and Kobilka, B. K. (1994) *J. Biol. Chem.* **269,** 18448-52

2. Goodman, O. B., Jr., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1998) *Advances in Pharmacology* **42,** 429-33

3. Ferguson, S. S., Zhang, J., Barak, L. S., and Caron, M. G. (1998) *Life Sci* **62,** 1561-5

4. Lefkowitz, R. J., Pitcher, J., Krueger, K., and Daaka, Y. (1998) *Advances in Pharmacology* **42,** 416-20

5. Gagnon, A. W., Kallal, L., and Benovic, J. L. (1998) *Journal of Biological Chemistry* **273,** 6976-81

6. Zhang, J., Ferguson, S., Barak, L. S., Menard, L., and Caron, M. G. (1996) *J Biol Chem* **271,** 18302-5

7. Chu, P., Murray, S., Lissin, D., and von Zastrow, M. (1997) *J Biol Chem* **272,** 27124-30

8. Tsao, P. I., and von Zastrow, M. (2000) *Journal of Biological Chemistry* **275,** 11130-11140

9. Law, P.-Y., Hom, D. S., and Loh, H. H. (1984) *J. Biol. Chem.* **259,** 4096-4104

10. Law, P. Y., and Loh, H. H. (1999) *Journal of Pharmacology and Experimental Therapeutics* **289,** 607-24

11. Tsao, P., Cao, T., and von Zastrow, M. (2001) *Trends Pharmacol Sci* **22,** 91-96.

12. Innamorati, G., Sadeghi, H. M., Tran, N. T., and Birnbaumer, M. (1998) *Proceedings of the National Academy of Sciences of the United States of America* **95,** 2222-6

13. Zhang, J., Barak, L. S., Anborgh, P. H., Laporte, S. A., Caron, M. G., and Ferguson, S. S. (1999) *Journal of Biological Chemistry* **274,** 10999-1006

14. DeFea, K. A., Zalevsky, J., Thoma, M. S., Dery, O., Mullins, R. D., and Bunnett, N. W. (2000) *Journal of Cell Biology* **148,** 1267-1281

15. Claing, A., Perry, S. J., Achiriloaie, M., Walker, J. K., Albanesi, J. P., Lefkowitz, R. J., and Premont, R. T. (2000) *Proc Natl Acad Sci U S A* **97,** 1119-24

16. Luttrell, L. M., Roudabush, F. L., Choy, E. W., Miller, W. E., Field, M. E., Pierce, K. L., and Lefkowitz, R. J. (2001) *Proc Natl Acad Sci U S A* **98,** 2449-2454.

17. Trowbridge, I. S., Collawn, J. F., and Hopkins, C. R. (1993) *Annual Review of Cell Biology* **9,** 129-61

18. Gruenberg, J., and Maxfield, F. R. (1995) *Current Opinion in Cell Biology* **7,** 552-63

19. Trapaidze, N., Keith, D. E., Cvejic, S., Evans, C. J., and Devi, L. A. (1996) *J Biol Chem* **271,** 29279-85

20. Cvejic, S., Trapaidze, N., Cyr, C., and Devi, L. A. (1996) *J Biol Chem* **271,** 4073-6

21. Trejo, J., and Coughlin, S. R. (1999) *Journal of Biological Chemistry* **274,** 2216-24

22. Hao, M., and Maxfield, F. R. (2000) *Journal of Biological Chemistry* **275,** 15279-86

23. Mayor, S., Presley, J. F., and Maxfield, F. R. (1993) *Journal of Cell Biology* **121,** 1257-69

24. Innamorati, G., Sadeghi, H., and Birnbaumer, M. (1999) *Journal of Receptor and Signal Transduction Research* **19,** 315-26

25. Oakley, R. H., Laporte, S. A., Holt, J. A., Barak, L. S., and Caron, M. G. (1999) *J Biol Chem* **274,** 32248-57

26. Innamorati, G., Le Gouill, C., Balamotis, M., and Birnbaumer, M. (2001) *Journal of Biological Chemistry* **276,** 13096-103

27. Hall, R. A., Premont, R. T., Chow, C. W., Blitzer, J. T., Pitcher, J. A., Claing, A., Stoffel, R. H., Barak, L. S., Shenolikar, S., Weinman, E. J., Grinstein, S., and Lefkowitz, R. J. (1998) *Nature* **392,** 626-30

28. Weinman, E. J., Steplock, D., Wang, Y., and Shenolikar, S. (1995) *Journal of Clinical Investigation* **95,** 2143-9

29. Reczek, D., Berryman, M., and Bretscher, A. (1997) *Journal of Cell Biology* **139,** 169-79

30. Cao, T. T., Deacon, H. W., Reczek, D., Bretscher, A., and von Zastrow, M. (1999) *Nature* **401,** 286-90

31. Raghuram, V., Mak, D. D., and Foskett, J. K. (2001) *Proceedings of the National Academy of Sciences of the United States of America* **98,** 1300-5

32. Pearson, M. A., Reczek, D., Bretscher, A., and Karplus, P. A. (2000) *Cell* **101,** 259-70

33. Hall, R. A., Ostedgaard, L. S., Premont, R. T., Blitzer, J. T., Rahman, N., Welsh, M. J., and Lefkowitz, R. J. (1998) *Proceedings of the National Academy of Sciences of the United States of America* **95,** 8496-501

34. Evans, C. J., Keith, D. J., Morrison, H., Magendzo, K., and Edwards, R. H. (1992) *Science* **258,** 1952-5

35. Kobilka, B. K., Dixon, R. A., Frielle, T., Dohlman, H. G., Bolanowski, M. A., Sigal, I. S., Yang-Feng, T. L., Francke, U., Caron, M. G., and Lefkowitz, R. J. (1987) *Proceedings of the National Academy of Sciences of the United States of America* **84,** 46-50

36. von Zastrow, M., and Kobilka, B. K. (1992) *J Biol Chem* **267,** 3530-8

37. Keith, D. E., Murray, S. R., Zaki, P. A., Chu, P. C., Lissin, D. V., Kang, L., Evans, C. J., and von Zastrow, M. (1996) *J Biol Chem* **271,** 19021-4

38. Okayama, H., and Berg, P. (1983) *Molecular and Cellular Biology* **3,** 280-9

39. Bradford, M. M. (1976) *Analytical Biochemistry* **72,** 248-54

40. Law, P.-Y., Hom, D. S., and Loh, H. H. (1982) *Mol. Pharmacol.* **72,** 1-4

41. Murray, S. R., Evans, C. J., and von Zastrow, M. (1998) *J. Biol. Chem.* **273,** 24987-24991

42. Whistler, J. L., Tsao, P., and von Zastrow, M. (2001) *J. Biol. Chem.* , M104627200

43. Hein, L., Ishii, K., Coughlin, S. R., and Kobilka, B. K. (1994) *J Biol Chem* **269,** 27719-26

44. Ko, J. L., Arvidsson, U., Williams, F. G., Law, P. Y., Elde, R., and Loh, H. H. (1999) *Brain Research. Molecular Brain Research* **69,** 171-85

45. Weinman, E. J., Steplock, D., Tate, K., Hall, R. A., Spurney, R. F., and Shenolikar, S. (1998) *Journal of Clinical Investigation* **101,** 2199-206

46. Bermak, J. C., Li, M., Bullock, C., and Zhou, Q. Y. (2001) *Nature Cell Biology* **3,** 492-8

47. Shapiro, M. J., Trejo, J., Zeng, D., and Coughlin, S. R. (1996) *J Biol Chem* **271,** 32874-80

48. Parent, J. L., Labrecque, P., Orsini, M. J., and Benovic, J. L. (1999) *Journal of Biological Chemistry* **274,** 8941-8

49. Mukherjee, S., Ghosh, R. N., and Maxfield, F. R. (1997) *Physiological Reviews* **77,** 759-803

50. Trejo, J., Hammes, S. R., and Coughlin, S. R. (1998) *Proceedings of the National Academy of Sciences of the United States of America* **95,** 13698-702

51. Tsao, P., and von Zastrow, M. (2000) *Curr Opin Neurobiol* **10,** 365-9.

52. Petaja-Repo, U. E., Hogue, M., Laperriere, A., Bhalla, S., Walker, P., and Bouvier, M. (2000) *J Biol Chem*

53. Chaturvedi, K., Bandari, P., Chinen, N., and Howells, R. D. (2001) *J Biol Chem* **6,** 4

54. Kojro, E., and Fahrenholz, F. (1995) *Journal of Biological Chemistry* **270,** 6476-81

55. Jockers, R., Angers, S., Da Silva, A., Benaroch, P., Strosberg, A. D., Bouvier, M., and Marullo, S. (1999) *Journal of Biological Chemistry* **274,** 28900-8

Acknowledgements

We thank Heather Deacon, Denise Kenski, Aaron Marley and Michael Tanowitz for valuable advice and assistance. These studies were supported by the National Institutes of Health and the National Alliance for Research on Schizophrenia and Depression (NARSAD).

Figure Legends

Figure 1: Endocytic trafficking of mutant opioid receptors visualized by fluorescence microscopy. Transiently transfected cells expressing δ ORt, δ/β_{10}, or δ/β_{10}-Ala were surface-labeled with M1 anti-FLAG antibody, treated as indicated, then the localization of labeled receptors was visualized in fixed cells using fluorescence microscopy. **A.** δ ORt, similar to full length δ OR, endocytosed efficiently following incubation with the peptide agonist DADLE for 25 minutes, and remained primarily in endocytic vesicles after incubation in the absence of agonist for an additional 45 minutes (**a-c**). The δ/β_{10} chimera also exhibited robust agonist-induced endocytosis but returned to the plasma membrane following agonist washout (**d-f**). The δ/β_{10}-Ala mutant receptor had a similar trafficking phenotype as the δ OR and δ ORt receptors, indicating that the β_2AR-derived recycling signal could be abrogated by addition of a single carboxyl-terminal alanine residue; **g-i**). **B.** Differences in the localization of receptors after agonist washout were examined using an amino-terminal HA (rather than FLAG) epitope tag, and the differences suggested using standard epifluorescence microscopy (panel **A**) were confirmed using confocal microscopy to image optical sections (~0.7 um thick) imaged through the center of cells. **C.** The fluorescence microscopy data shown in panel **A** were quantitated by counting the number of receptor-containing endocytic vesicles in cells at random in coded specimens representing each experimental condition. Error bars represent the S.D. of individual data points ($n \geq 20$ cells / condition). **D.** Receptors present in the plasma membrane were specifically labeled with anti-FLAG antibody (as in panel **A**), but antibodies attached to receptors remaining in the plasma membrane after 25 minute DADLE exposure were dissociated using a brief wash at 4°C with EDTA-

containing PBS, as described in *Experimental Procedures*, in order to selectively label only those receptors endocytosed after agonist exposure. The ability of endocytosed receptors to return to the plasma membrane after agonist washout was then assessed using confocal fluorescence microscopy. Each panel shows a representative example of a cell from each condition ($n \geq 20$ cells per condition per experiment, experiment replicated twice with similar results).

Figure 2: Quantitative assessment of receptor recycling using fluorescence flow cytometry. Stably transfected cells expressing the indicated FLAG-tagged mutant receptors were left untreated ("Control"), treated with agonist for 25 min. ("Agonist"), or treated with agonist, washed, and treated with antagonist for 45 min. ("Agonist → Antagonist") . Then cells were chilled to 4°C, dissociated from tissue culture dishes, receptors present in the plasma membrane were specifically labeled with FITC-conjugated M1 antibody, and the relative number of surface receptors was quantitated by fluorescence flow cytometry as described in *Experimental Procedures*. **A.** A similar amount of internalization (approximately 40%) was observed for all receptor constructs. Efficient recycling of the β_2AR was observed after 45 min. agonist washout, as indicated by recovery of surface immunoreactivity to nearly control levels (first set of bars). The δ ORt receptor exhibited little recovery to the plasma membrane under similar conditions (second set of bars). Addition of the last ten residues of the β_2AR to the carboxyl terminus of the δ OR was sufficient to confer a rapid recycling phenotype on this chimeric receptor (third set of bars). This effect was negated by the addition of an alanine to the sequence (fourth set of bars). Results represent the mean fluorescence intensities

normalized to untreated cells under each experimental condition. Each condition was assayed in triplicate for each experiment and all conditions were assayed in ≥4 independent experiments with similar results. Bars represent means for data across all experiments; error bars represent the standard deviation of the mean among experiments. **B.** The fluorescence intensity data displayed in **A** were used to calculate the fractional recycling relative to the amount of internalization obtained following 25 minute incubation with agonist. This method corrects for small differences in the extent of internalization of each mutant receptor observed before agonist washout. The following formula used to calculate fractional recycling and results (± standard error between individual experiments, n≥4) was expressed as a percentage.

$$\frac{(+Antagonist)-(+Agonist)}{(Control)-(+Agonist)}$$

Figure 3: Effects of the β_2AR-derived recycling signal on agonist-induced proteolysis of mutant opioid receptors. 10 cm dishes of HEK-293 cells stably expressing the indicated constructs were grown to confluency and treated for various times in the continuous presence of agonist. The relative levels of total or surface receptor present in lysates after the indicated times were determined by western blotting or biotinylation/immuno-precipitation, respectively, as described in *Experimental Procedures*. **A.** For the δ ORt and δ/β_{10}-Ala receptors, a pronounced decrease in amount of total cellular receptor was observed after 4 hours of agonist treatment, consistent with relatively rapid agonist-induced proteolysis, whereas the β_2AR and δ/β_{10} receptors exhibited markedly less agonist-induced proteolysis at this time point. Immunoblots of equal amounts (20 ug) of

cell extract are shown from a typical experiment are shown, and experiments were conducted in quadruplicate with similar results. **B.** Surface-biotinylated δ ORt and δ/β_{10}-Ala exhibited pronounced agonist-induced degradation, whereas the δ/β_{10} mutant receptor was proteolyzed to a much smaller extent, similar to that of the β_2AR, under similar conditions. The results of a typical experiment are shown from a set of 4 experiments with similar results. **C.** and **D.** The western blots or surface biotinylation results shown in **A** and **B**, respectively, were analyzed by scanning densitometry and plotted relative to the amount of receptor detected in untreated cells.

Figure 4: Effects of adding the carboxyl-terminal four residues derived from the β_2AR on recycling and agonist-induced proteolysis of the full length δ OR. **A.** Fluorescence flow cytometry was used to quantitate fractional recycling of δ/β_4 mutant receptors relative to β_2AR and full length δ OR as described in Figure **2.** **B.** Immunoblotting analysis (as in Figure 3 A) was used to determine total amounts of FLAG-tagged receptors present in extracts prepared from stably transfected cells after the indicated times of incubation with 10 uM isoproterenol (β_2AR) or DADLE (δ OR and δ/β_4 mutant receptors). The δ/β_4 mutant receptor exhibited a marked reduction in the amount of agonist-induced proteolysis compared to the full length δ OR. A representative experiment is shown from a set of four experiments with similar results. in all experiments, >65% recovery (< 35% proteolysis) of δ/β_4 mutant receptors was observed after 4 hours of continuous exposure of cells to a saturating concentration of agonist (10 uM DADLE), whereas <30% recovery (>70% proteolysis) of the δ OR was measured

under similar conditions. C. Reduced agonist-induced proteolysis of surface-biotinylated δ/β_4 mutant receptor relative to δ OR levels. Experiments were conducted as in Figure 3 B and results from a representative experiment (n = 4) are shown. Scanning densitometry estimated recovery of surface-labeled δ/β_4 mutant receptors of >90% (<10% proteolysis) after continuous exposure to agonist for 4 hours, whereas recovery of δ OR observed under the same conditions was < 50% (>50% proteolysis).

Figure 5: Effects of the β_2AR-derived recycling signal on agonist-induced downregulation measured by radioligand binding. 10 cm dishes of HEK-293 cells stably expressing the indicated constructs were grown to confluency and incubated in the continuous presence of 10 uM DADLE for 4 hours. Receptor downregulation was measured using [^{3}H]-diprenorphine, as described in *Experimental Procedures*. The results shown represent ≥ 3 experiments conducted in triplicate with binding specificity controls performed in duplicate. In each experiment the standard deviation of individual data points was <10% of the mean. Bars represent the overall means from the three experiments combined. Error bars represent the standard error of the mean between the results calculated from individual experiments.

Figure 6: δ OR and δ/β_4 mutant receptors differ in their endocytic sorting when co-expressed in the same cells. Stably transfected cells co-expressing HA-tagged δ OR and FLAG-tagged δ/β_4 mutant receptors were surface biotinylated and incubated at 37°C with a saturating concentration of alkaloid agonist (10 uM etorphine) for the indicated times. Receptors were purified from cell extracts by immunoprecipitation using anti-FLAG

antibody and recovery of biotinylated receptors was determined by streptavidin overlay. **A.** Representative results of the streptavidin overlay, from a series of fours experiments conducted with similar results. **B.** Quantitation of recovery of surface-biotinylated receptors by scanning densitometry. Each data point represents the mean calculated from the results of three independent experiments. Error bars represent the standard deviation of these determinations.

Figure 1

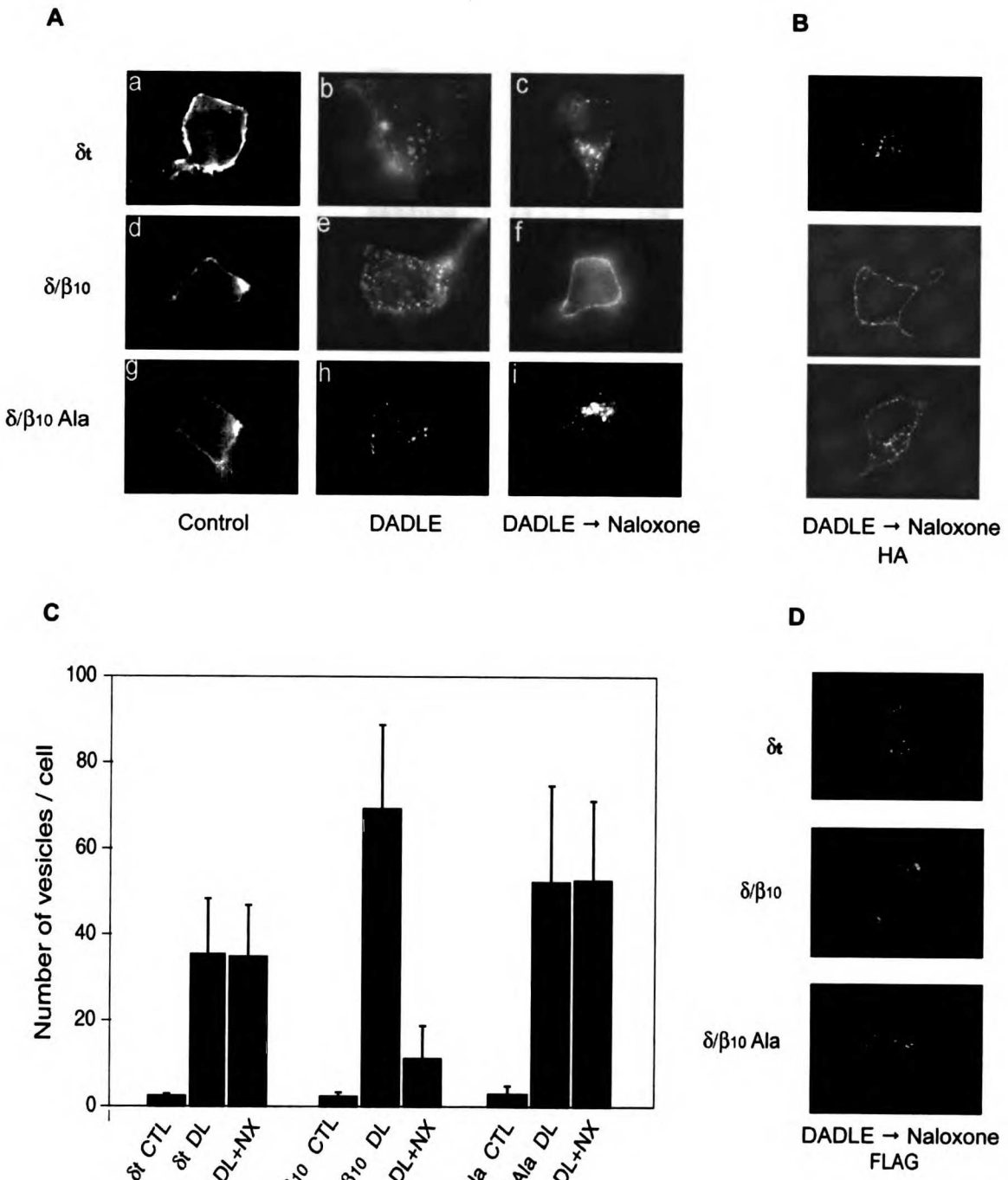
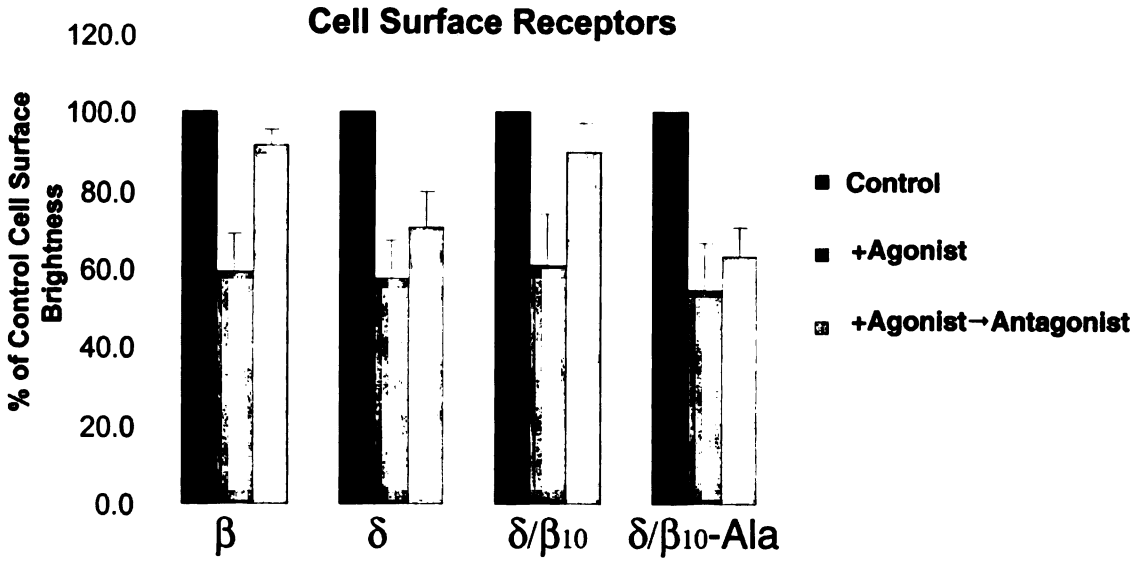


Figure 2

A


Cell Surface Receptors
% of Control Cell Surface Brightness
120.0
100.0
80.0
60.0
40.0
20.0
0.0
β
δ
δ/β10
δ/β10-Ala
Control
+Agonist
+Agonist→Antagonist

B

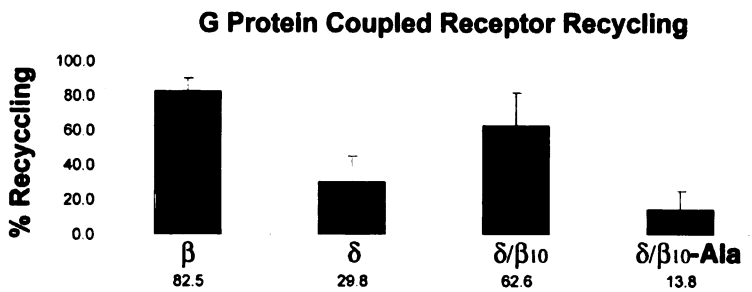

G Protein Coupled Receptor Recycling
% Recyccling
100.0
80.0
60.0
40.0
20.0
0.0
β
82.5
δ
29.8
δ/β10
62.6
δ/β10-Ala
13.8

Figure 3

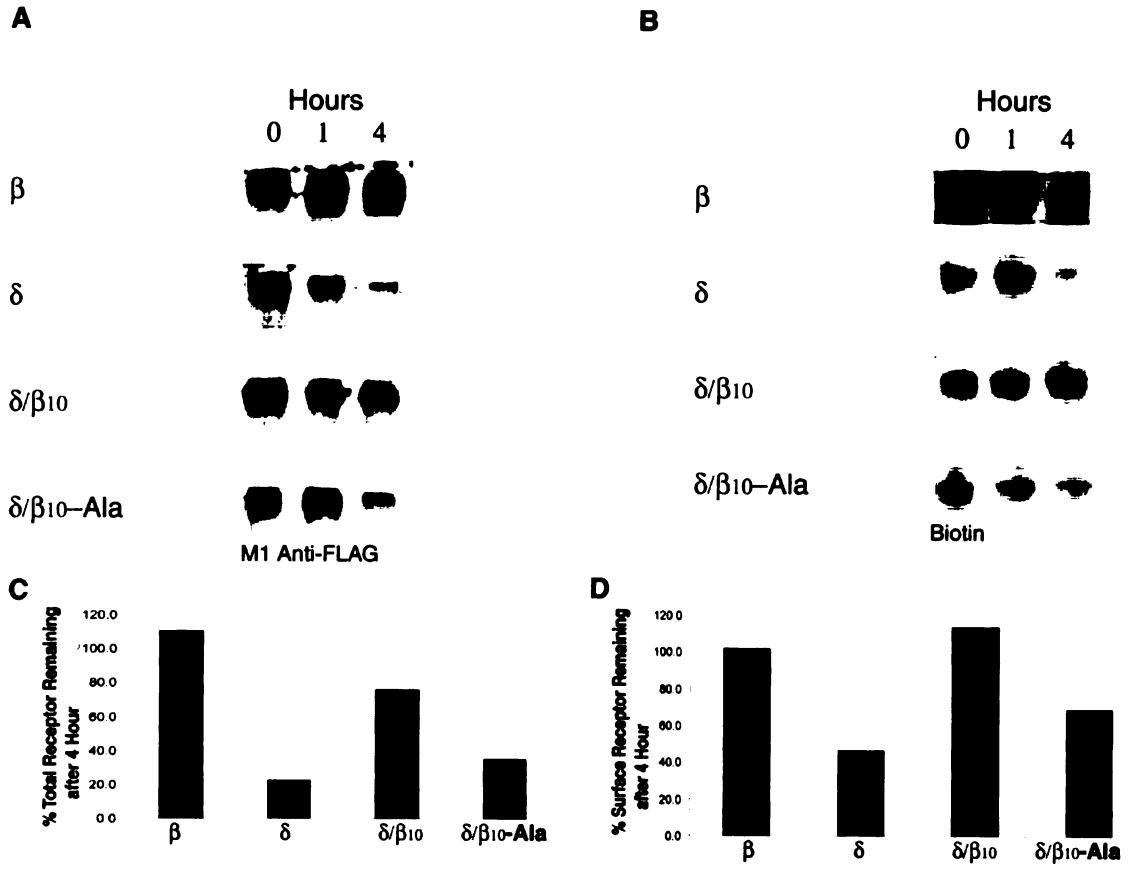
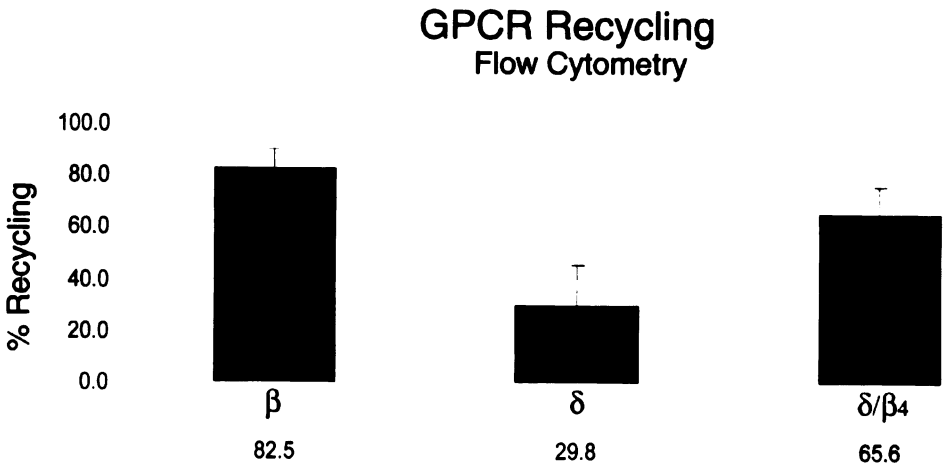
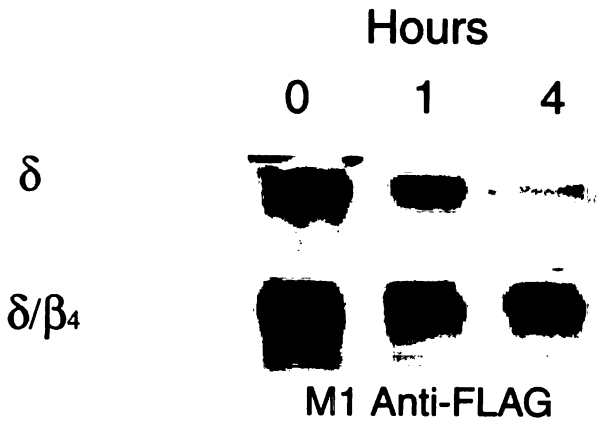

A
Hours
0 1 4
β
δ
δ/β10
δ/β10–Ala
M1 Anti-FLAG
B
Hours
0 1 4
β
δ
δ/β10
δ/β10–Ala
Biotin
C
% Total Receptor Remaining after 4 Hour
120.0
100.0
80.0
60.0
40.0
20.0
0.0
β
δ
δ/β10
δ/β10-Ala
D
% Surface Receptor Remaining after 4 Hour
120.0
100.0
80.0
60.0
40.0
20.0
0.0
β
δ
δ/β10
δ/β10-Ala

Figure 4

A


GPCR Recycling
Flow Cytometry
% Recycling
100.0
80.0
60.0
40.0
20.0
0.0
β
82.5
δ
29.8
δ/β4
65.6

B


Hours
0
1
4
δ
δ/β4
M1 Anti-FLAG

C

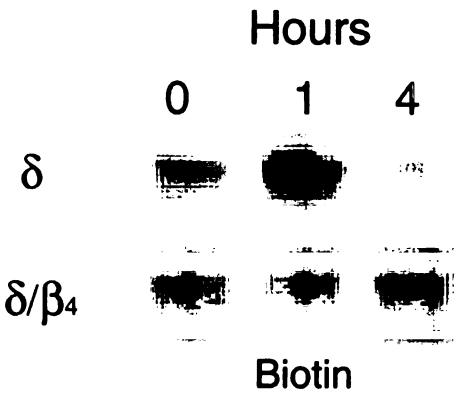

Hours
0
1
4
δ
δ/β4
Biotin

Figure 5

Agonist Induced G Protein Coupled Receptor Down-regulation

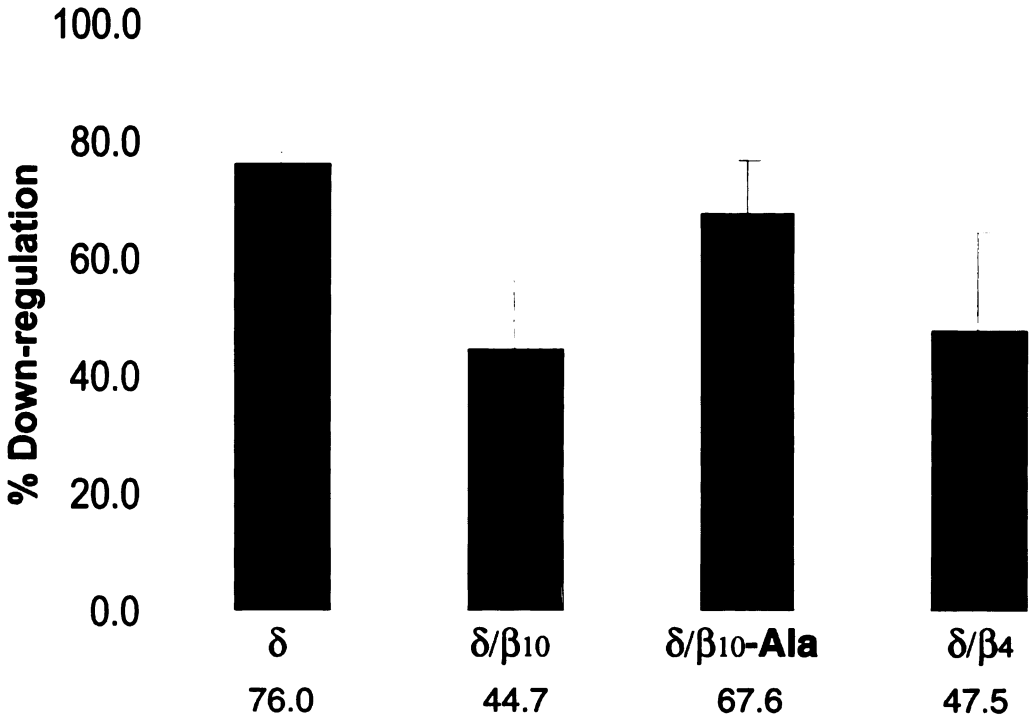
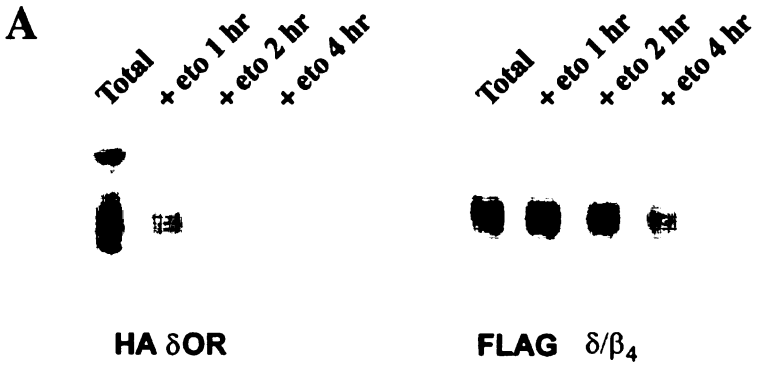
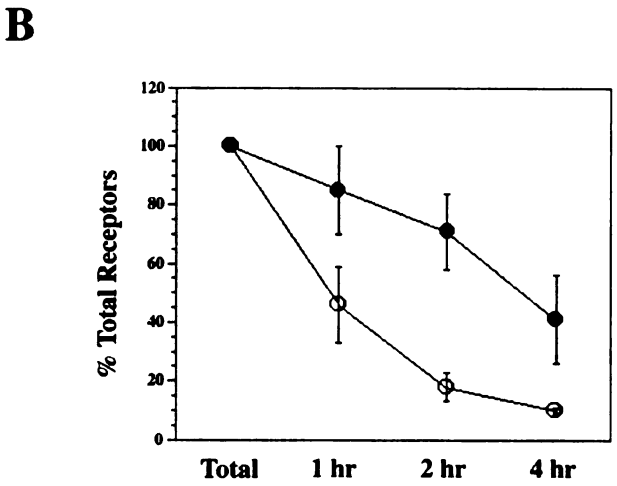


Figure 6


A
Total
+ eto 1 hr
+ eto 2 hr
+ eto 4 hr
Total
+ eto 1 hr
+ eto 2 hr
+ eto 4 hr
HA δOR
FLAG δ/β4


B
% Total Receptors
120
100
80
60
40
20
0
Total
1 hr
2 hr
4 hr

Chapter 3: Type I PDZ ligands are sufficient to promote rapid recycling of G protein-coupled receptors independent of binding to NSF

All work in this chapter was performed by Robert M. Gage with the following exception:

(1) Work presented in Figure 1 (panels A and B) showing NSF binding to the β_2AR tail via GST pull down and effects of α-SNAP were performed by Elena A. Matveeva in the laboratory of Sidney W. Whiteheart (Department of Molecular and Cellular Biochemistry, University of Kentucky, College of Medicine, Lexington KY 40536)

Summary

Molecular sorting of G protein-coupled receptors (GPCRs) between divergent recycling and lysosomal membrane pathways plays a fundamental role in determining the functional consequences of agonist-induced endocytosis. A sequence present in the carboxyl-terminal cytoplasmic domain of the beta-2 adrenergic receptor (β_2AR), which is necessary for rapid recycling of receptors, mediates both PDZ domain-dependent binding to NHERF / EBP50 -family proteins and non-PDZ binding to the NEM-sensitive factor (NSF). These considerations raise the questions of whether PDZ interaction(s) are actually sufficient to promote rapid recycling of endocytosed receptors and, if so, whether PDZ-mediated sorting is restricted to the β_2AR tail or to sequences that bind NHERF / EBP50. We have addressed these questions by examining the ability of short (10 residue) sequences differing in PDZ and NSF binding properties to promote rapid recycling, and inhibit lysosomal proteolysis, when fused to a distinct GPCR (the delta opioid receptor) that normally traffics to lysosomes after agonist-induced endocytosis. The recycling activity of the β_2AR –derived tail sequence was not blocked by a point mutation that selectively disrupts binding to NSF, and naturally occurring PDZ ligand sequences were identified that do not bind detectably to NSF yet function as strong recycling signals. The carboxyl-terminal cytoplasmic domain of the beta-1 adrenergic receptor, which does not bind either to NSF or NHERF / EBP50 and interacts selectively with a distinct group of PDZ proteins, promoted rapid recycling of chimeric mutant receptors with similarly high efficiency as the β_2AR tail. These results indicate that PDZ domain-mediated protein interactions are indeed fully sufficient to promote rapid

recycling of GPCRs, independent of binding to NSF. They also suggest that PDZ-directed recycling is a rather general mechanism of GPCR regulation, which is not restricted to a single GPCR, and may involve additional PDZ domain containing protein(s) besides NHERF / EBP50.

Introduction

Many G protein-coupled receptors (GPCRs)[1] undergo rapid endocytosis after agonist-induced activation(1-4). The functional consequences of this process depend, in large part, on molecular sorting of endocytosed receptors between divergent downstream membrane pathways(5,6). When expressed in human embryonic kidney (HEK-293) cells, both the beta-2 adrenergic receptor (β_2AR) and delta opioid receptor (δOR) undergo rapid, agonist-induced endocytosis via clathrin-coated pits(1,7,8). At early times after endocytosis, these distinct GPCRs are extensively colocalized(9). At later times after endocytosis, the membrane trafficking properties of these GPCRs differ greatly(9). Endocytosed β_2ARs can recycle to the plasma membrane rapidly and efficiently after agonist removal and receptors are capable of undergoing multiple rounds of continuous endocytosis / recycling without detectable proteolysis(9-11). In contrast, endocytosed δORs do not recycle efficiently and, instead, traffic preferentially to lysosomes(9,12,13). Rapid recycling of the β_2AR is well established to promote functional resensitization of signal transduction(3,4). Lysosomal trafficking of δOR to lysosomes contributes to the essentially opposite process of agonist-induced proteolytic downregulation(13-15). Despite the physiological importance of these opposite regulatory processes, the

[1] The abbreviations used are: GPCR, G protein-coupled receptor; β_2AR, β_2-adrenergic receptor; β_1AR, β_1-adrenergic receptor; δOR, δ opioid receptor; DMEM, Dulbecco's modified Eagle's medium; DADLE, [$_D$-Ala^2, $_D$-Leu^5 enkephalin]; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; NSF, N-ethyl maleimide sensitive factor; SNARE, soluble NSF attachment protein receptor; α-SNAP, α soluble NSF attachment protein; PDZ, PSD-95/Discs-Large/ZO-1; NHERF / EBP50, Na^+ / H^+ exchanger regulatory factor / ezrin/radixin/moesin-binding phosphoprotein of 50 kDa; CFTR, cystic fibrosis transmembrane conductance regulator.

molecular mechanisms that determine the sorting of distinct GPCRs remain poorly understood.

Plasma membrane recycling of many integral membrane proteins is thought to occur via bulk membrane flow, without requiring any specific sorting information(16-19). However, efficient recycling of the β_2AR requires a specific structural determinant present in a distal portion of the carboxyl-terminal cytoplasmic domain(20). A tetrapeptide sequence derived from the β_2AR carboxyl-terminus forms a minimal structural determinant that is both necessary for efficient recycling of the β_2AR and sufficient to promote rapid recycling when fused to a distinct GPCR, thereby fulfilling functional criteria of a modular endocytic sorting signal(21). This sequence conforms to a classical type I PDZ ligand and interacts with a PDZ domain present in NHERF / EBP50 –family proteins (22-24) both in vitro and in vivo(20,22,25), leading to the hypothesis that PDZ domain-mediated protein interaction(s) with the β_2AR –derived recycling sequence are sufficient to mediate its post-endocytic sorting activity(21).

The β_2AR tail can also interact with other cytoplasmic proteins, in addition to PDZ proteins such as NHERF / EBP50(26). Of particular interest is the N-ethyl maleimide sensitive factor (NSF), a cytoplasmic protein that does not contain recognizable PDZ domains but also requires a distal portion of the β_2AR tail for detectable binding(27). A number of mutations of the β_2AR tail that alter the endocytic membrane trafficking of receptors also disrupt receptor interaction with NSF, and in vitro studies indicate that NSF and PDZ proteins bind to the β_2AR tail competitively. This has led to the alternative hypothesis that NSF, and not PDZ, interactions with the β_2AR tail are important for its endocytic sorting activity (27).

A potential limitation of the previous studies is that they rely on a loss-of-function approach. In the present study the ability of the β_2AR –derived recycling signal to function as an autonomous membrane trafficking signal was used, in a gain-of-function design, to define sequences that are actually *sufficient* to promote plasma membrane recycling of endocytosed receptors. These results verify that PDZ domain-mediated protein interaction(s) with the cytoplasmic tail are indeed sufficient to promote rapid and efficient recycling of endocytosed GPCRs, and can do so in the absence of detectable NSF binding. Interestingly, these results demonstrate further that PDZ-mediated endocytic sorting activity is not limited to the β_2AR –derived cytoplasmic tail or to sequences that interact with NHERF / EBP50. Instead it appears that PDZ domain-mediated protein interaction(s) play a more general role in controlling post-endocytic sorting of GPCRs than previously anticipated.

Experimental Procedures

cDNA Constructs and Mutagenesis

Several epitope-tagged versions of the cloned murine delta opioid receptor (δOR(28)) and the human beta 2 adrenergic receptor (β_2AR (29)) were used in these studies: mutant receptors containing a FLAG epitope in the amino-terminal extracellular domain (SFδOR, SFβ_2AR, respectively) were described previously and demonstrated to be functional(9,11,30). Mutant delta opioid receptors containing a FLAG epitope in the amino-terminal extracellular domain and the last six carboxyl-terminal cytoplasmic residues (NH_2-GGGAAA-COOH) replaced with ten or eleven amino acids were generated (see table below). This was accomplished by insertion of a synthetic linker-adapter (Operon Technologies) encoding the ten-residue or eleven-residue sequence followed by a stop codon into an SrfI site present near the 3' end of the sequence encoding the δOR tail. Receptor cDNAs were cloned into pcDNA3 (Invitrogen) or pIRES (Clontech) and all constructs were verified by dideoxynucleotide sequencing (UCSF Genetics Core Sequencing Facility and UCSF Biomolecular Resource Center).

GST fusion proteins were constructed appending the carboxyl terminal tail from either the δOR or the β_2AR to GST (pGEX vector, Amersham Biosciences). Chimeric GST constructs were generated using the same SrfI restriction site present in the δOR tail and synthetic linker-adapters.

Chimeric Receptor	Linker Amino Acid sequence
β_2[10]	NH_2-RNCSTNDSLL-COOH
β_2[10]-Ala	NH_2-RNCSTNDSLLA-COOH
β_2[10] DSAL	NH_2-RNCSTNDSAL-COOH
β_2[10] ASLL	NH_2-RNCSTNASLL-COOH
β_1[10]	NH_2-RPGFASESKV-COOH
β_1[10]-Ala	NH_2-RPGFASESKVA-COOH
CFTR[10]	NH_2-TEEEVQDTRL-COOH
CFTR[10]-Ala	NH_2-TEEEVQDTRLA-COOH
PDZ II[10] (GLP C[10])	NH_2-GDSSRKEYFI-COOH
GluR2[10]	NH_2-KRMKVAKNPQ-COOH

Note: The same linker-adapter was used for both the full length receptor and GST constructs.

Cell Culture and Transfection

Human embryonic kidney 293 cells (ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (University of California San Francisco Cell Culture Facility). Cells grown in 6-cm dishes were transfected with ~5 μg of plasmid DNA containing the indicated receptor by calcium phosphate precipitation(13,31). For studies of receptor trafficking in transiently transfected cells, cells were transfected as above, plated onto coverslips 24 hr post transfection and experiments were conducted 48 hr post transfection. Stably transfected cells expressing epitope tagged receptors were generated by transfecting 293 cells in 6-cm dishes as above. Cell clones expressing transfected receptors were selected in 500 μg/mL Geneticin (Life Technologies, Inc.) and colonies were isolated and selected to have similar levels of receptor expression.

NSF Binding via GST pull-down

Wild type His_6-NSF and His_6-α-SNAP were produced as recombinant proteins in *Escherichia coli* and purified as described(32). The binding procedure was modified from previously described methods(33). Briefly, GST-□$_2$AR was incubated with preswollen, glutathione-agarose beads (100 μg of protein/100 μL of beads) at 4°C in phosphate-buffered saline with 0.01% (v/v) Tween 20, 0.1% (v/v) □-mercaptoethanol, and 2 mM EDTA. After 1 hr, the beads were washed four times (0.5 mL each) in the same buffer, and then equal volumes of the beads were aliquotted into the reaction tubes. NSF-binding reactions were performed in a final volume of 500 μL containing 15 μL of beads with GST-□$_2$AR in binding buffer: 20 mM HEPES/KOH (pH7.4), 250 mM imidazole, 150 mM potassium acetate, 5 mM EGTA, 5 mM $MgCl_2$, 1% (v/v) glycerol, 1% (v/v) Triton X-100, 10% (w/v) ovalbumin and 2.5 mM AMP-PNP. To check nucleotide requirement ATP and ADP were added instead AMP-PNP at same concentration. After 3 hr at 4°C, the beads were washed five times (0.5 mL each) with binding buffer without ovalbumin. The bound NSF was eluted with SDS-PAGE sample buffer and detected by western blotting using the INDIA^{TM}HRP probe for hexahistidine tags (Pierce) with enhanced chemiluminescence.

NSF binding via protein overlay

Protein overlay experiments were performed as previously described (27). Briefly, 15 μg of GST-fusion proteins were resolved on 10% SDS-PAGE gels and transferred to nitrocellulose filters. Filters were blocked 1 hr with 5% w/v fat-free milk powder in Tris-buffered saline with Tween 20 (TBST: 25 mM Tris, pH 7.4, 137 mM NaCl, 3 mM KCl,

1mM $CaCl_2$, 0.1% v/v Tween 20) and incubated overnight at 4°C in a solution containing 100 nM purified NSF. Blots were then washed three times with TBST buffer and incubated with anti-NSF monoclonal antibody (2E5) for 1 hr at room temperature. After three washes with TBST, filters were incubated for 1 hr with horseradish peroxidase-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch). The blots were washed again with TBST and proteins bands were detected using Super Signal (Pierce). Band intensities were quantified in the linear range by direct imaging via chemiluminescence detection on a FluorChem 8000 instrument (AlphaInnotech Corp.)

Assay of receptor recycling

Recycling of endocytosed GPCRs was measured by a ratiometric fluorescence assay, as described previously(34) and summarized briefly below. Transiently transfected cells grown on glass coverslips were incubated with Alexa488-conjugated M1 anti-FLAG antibody (prepared by standard methods using Alexa-fluor 488 *N*-hydroxysuccinimide ester, Molecular Probes) to selectively label FLAG-tagged receptors present in the plasma membrane at the beginning of the experiment. Then cells were incubated (at 37°C for 30 min) in the presence of 10 μM DADLE or 10 μM isoproterenol to drive internalization. At the end of this incubation cells were quickly washed three times in PBS lacking Ca^{2+} or Mg^{2+} and supplemented with 0.04% EDTA to dissociate FLAG antibody bound to residual surface receptors remaining in the plasma membrane, thereby leaving antibody bound only to the internalized pool of receptors. EDTA-stripped cells were then incubated (at 37 °C for 45 min) in the presence of 10 μM naloxone or 10 μM alprenolol to prevent subsequent receptor activation (from possible residual agonist not removed by washing).

Cells were fixed with 4% paraformaldehyde, PBS under non-permeabilizing conditions, quenched with Tris-buffered saline with 3% bovine serum albumin (but no Triton X-100), and incubated with Cy3-conjugated donkey anti-mouse secondary antibody to detect recycled, antibody-labeled receptors. In each experiment, and for each receptor construct examined, two parallel control coverslips were included, one in which cells were fixed after a 30-min incubation in the absence of agonist and without an EDTA stripping step (100% surface receptor control) and one in which cells were fixed immediately after the EDTA-mediated stripping step (0% recycled control). Cells were examined by epifluorescence microscopy using a Nikon inverted microscope with 60X NA 1.4 objective, appropriate filter sets to selectively detect Alexa488 or Cy3. Staining intensities of each fluor in individual cells were integrated using a cooled CCD camera (Princeton Instruments) and IPLab Image software (Scanalytics). This analysis indicated that the efficiency of the EDTA strip (reduction of Cy3 staining intensity in the 0% recycled control relative to the 100% surface receptor control) was >95%, consistent with previous measures using fluorescence flow cytometry. The percentage of receptors recycled in individual cells following agonist washout was then calculated from the red/green ratios determined from the control conditions according to the following formula: $(E - Z)/(C - Z) \times 100$, where E = the mean ratio for the experimental coverslip, Z = the mean ratio for the zero surface control, and C = the mean ratio for the 100% surface control. 20-30 cells/construct/condition were analyzed at random in this manner for each experiment, and average values reported under "Results" represent mean recycling percentages derived from five to eight independent experiments.

Assay of receptor degradation

To determine the effect of agonist treatment on steady state levels of total receptor protein, immunoblotting was performed as described previously(9). Briefly, cells stably transfected with the indicated FLAG tagged receptors were grown in 10-cm dishes and treated for 0, 1, or 4 hr with the appropriate agonist (10 μM isoproterenol or DADLE) at 37°C. Dishes of cells containing stably transfected cells were washed with 2.5 mL PBS and the cells were dissociated and harvested in 1.5 mL PBS/EDTA for 3 min at 4°C. After pelleting the cells by centrifugation (1000 rpm for 5 min on benchtop microcentrifuge), the cells were lysed by placing them in 1 mL of hypotonic lysis solution (25 mM Tris-HCl or 25 mM HEPES buffer, pH 7.4, 1 μg/mL leupeptin, 1 μg/mL, pepstatin, and 2 μg/mL aprotinin) while vortexing for 2 min. The crude membrane fraction was separated from the cytoplasmic fraction by centrifugation at 14000 rpm for 15 min on a microcentrifuge. The supernatant was discarded and the pellet resuspended in 0.5 mL of resuspension buffer (25 mM Tris-HCl or 50 mM HEPES buffer, pH 7.4, 1 μg/mL leupeptin, 1 μg/mL, pepstatin, 2 μg/mL aprotinin, and 0.25% v/v Triton-X100). The non-soluble fraction was removed by centrifugation as above at 14000 rpm and the supernatant was decanted and analyzed for protein content by the Bradford method (35) using bovine serum albumin as standard. Lysate from the samples corresponding to ~40 μg of total protein were loaded and separated by SDS-PAGE under denaturing conditions. Resolved proteins were transferred to nitrocellulose membranes (Micron Separations, Inc.) and placed in TBSTM (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.1% v/v Tween-20, and 5% dry nonfat milk) for 60 min. Detection of receptors containing FLAG epitope was carried out by incubation of the

blots with M1 anti-FLAG antibody (15 μg/mL in TBSTM) for 60 min, washing in TBST (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, and 0.1% v/v Tween-20), and incubation for 60 min in TBSTM containing 400 ng/mL goat anti-mouse conjugated to horseradish peroxidase (Jackson ImmunoResearch). After washing in TBST, proteins bands were detected using Super Signal (Pierce). Band intensities were quantitated by direct imaging via chemiluminescence detection on a FluorChem 8000 instrument (AlphaInnotech Corp.) using a 16-bit cooled charge-coupled device camera and analyzed using FluorChem 2.0 software (AlphaInnotech Corp.).

Quantification of Receptor Recycling by Fluorescence Flow Cytometry

Recycling of epitope-tagged receptors back to the plasma membrane was estimated by assaying the recovery of immunoreactive receptors accessible at the cell surface to monoclonal antibody recognizing the extracellular epitope tag (FLAG). This assay is a variant of a previously described flow cytometric assay for estimating receptor internalization and recycling (9). Briefly, monolayers of cells stably expressing the indicated FLAG tagged receptor were incubated in the presence of 10 uM of the appropriate agonist (isoproterenol or DADLE) for 25 min at 37 °C to drive agonist-induced internalization, then rinsed twice with DMEM, and subsequently incubated at 37°C in the presence of the appropriate antagonist (10 uM alprenolol or naloxone (Research Biochemicals) to block additional endocytosis of receptors. At the indicated time points, cell monolayers were chilled on ice to stop membrane trafficking, and cells were lifted with PBS containing 0.04% EDTA and lacking Ca++ or Mg++ (PBS/EDTA University of California San Francisco Cell Culture Facility). Cells were washed twice in

1 ml PBS and incubated at 4 °C for 45-60 min in 0.5 ml PBS with 2.5 ug/ml M1 anti-FLAG antibody that had been conjugated with Alexa 488 dye (Molecular Probes) using standard methods. Cells were washed in PBS and then fixed in PBS with 1% formaldehyde. Receptor immunoreactivity was quantitated by fluorescence flow cytometry (FACScan, Becton Dickinson, Palo Alto, CA). Fluorescence intensity of 10,000 cells was collected for each sample. Cellquest software (Becton Dickinson) was used to calculate the mean fluorescence intensity of single cells in each population. All experiments were conducted ≥3 times with similar results. The mean values for each experiment were averaged to obtain the overall mean fluorescence intensity and standard error of the mean reported in the figure.

Immunoprecipitations

To detect *in vivo* binding between NSF and FLAG-tagged receptors, co-immunoprecipitation experiments were performed. Briefly, cells stably transfected with the indicated FLAG tagged receptors were grown in 10-cm dishes and treated for 30 min with the appropriate agonist (10 μM isoproterenol or DADLE) at 37°C. Cells were washed three times in PBS / Hepes (PBS, 10 mM Hepes pH 7.5). Next cells were incubated for 30 min at room temperature in crosslinking buffer (PBS / Hepes, 123 μg/mL dithio-bis(succinimidyl propionate)) before quenching with PBS / Hepes / glycine (PBS / Hepes, 19 mM glycine). Cells were lysed in radioimmune precipitation buffer (RIPA buffer: 150 mM NaCl, 50 mM Tris pH 8.0, 5mM EDTA, 1% v/v Nonidet P-40, 0.5 % w/v Na-deoxycholate, 10 mM NaF, 10 mM Na_2-pyrophosphate, 0.1% w/v SDS, 1X complete Mini, EDTA-free protease inhibitor cocktail (Roche)) and insoluble cellular

debris was removed by centrifugation at 14,000 rpm for 15 minutes. After equalizing protein concentrations across all samples, lysates were added to M2 anti-FLAG agarose beads (Sigma) and allowed to rotate for 16 hr. After washing three times in RIPA buffer, FLAG-tag containing proteins were specifically eluted off the beads by 40 μL of 1X FLAG peptide (600 μg/mL peptide in PBS, Sigma). Proteins were incubated for 30 min in SDS sample buffer containing 100 μM Di-thio threitol (DTT) and 10 % 2-mercapto ethanol (Fisher) and separated by SDS-PAGE under denaturing conditions. Resolved proteins and lysate representing 1% of co-immunoprecipitation input were transferred to nitrocellulose membranes (Micron Separations, Inc.) and placed in TBSTM (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.1% v/v Tween-20, and 5% dry nonfat milk) for 60 min. Identical gels were run and transferred for separate detection of receptor and NSF. Detection of receptors containing FLAG epitope was carried out by incubation of the blots with M1 anti-FLAG antibody (15 μg/mL in TBSTM) for 60 min, washing in TBST (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, and 0.1% v/v Tween-20), and incubation for 60 min in TBSTM containing 400 ng/mL goat anti-mouse conjugated to horseradish peroxidase (Jackson ImmunoResearch). After washing in TBST, proteins bands were detected using Super Signal (Pierce). Detection of co-immunoprecipitated NSF was carried out as above using 1 μg/mL 2E5 mouse anti-NSF antibody in TBSTM and TBSTM containing 400 ng/mL goat anti-mouse conjugated to horseradish peroxidase (Jackson ImmunoResearch). Band intensities were recorded by direct imaging via chemiluminescence detection on a FluorChem 8000 instrument (AlphaInnotech Corp.) using a 16-bit cooled charge-coupled device camera and analyzed using FluorChem 2.0 software (AlphaInnotech Corp.).

Colocalization of Receptor and NSF Assay

To investigate the subcellular localization of endogenous NSF and FLAG-tagged receptors in response to agonist, confocal microscopy was conducted. HEK-293 cells stably transfected with the indicated receptor were grown on coverslips in DMEM with 10% fetal bovine serum at 37°C. Cells were treated with agonist (10 μM of isoproterenol or DADLE) for 0, 5, 10, or 30 minutes and then fixed and washed in phosphate buffered saline (PBS). Cells were permeabilized and blocked for 45 min at room temperature in Blotto (25 mM Tris, pH 7.4, 137 mM NaCl, 3 mM KCl, 1mM $CaCl_2$, 0.1% v/v Triton X-100, 3% w/v dry milk). Next, the coverslips were incubated for 45 minutes in Blotto containing 2E5 at 1 μg/mL and Rabbit anti-FLAG at 1 μg/mL (Sigma). After washing three times in tris buffered saline (TBS: 25 mM Tris, pH 7.4, 137 mM NaCl, 3 mM KCl, 1mM $CaCl_2$), the coverslips were incubated for 30 minutes in Blotto containing Cy3 conjugated donkey anti-mouse antibody at 1.4 μg/mL (Jackson ImmunoResearch) and Alexa 488 conjugated goat anti-rabbit antibody 2 μg/mL (Molecular Probes). The coverslips were mounted on slides using Vectashield mounting media following three washes in TBS. Endogenous NSF and FLAG-tagged receptors were visualized on an upright Zeiss LSM-5 Pascal confocal microscope and analyzed using Image Examiner software (Zeiss, version 3.2.0.70) and Photoshop (Adobe, version 6).

Results

PDZ domain-mediated protein binding of the full-length β_2AR tail sequence to NHERF / EBP50 binding can be blocked by addition of a single alanine residue to the carboxyl-terminus, and a distal 4 – 10 residue sequence derived from this cytoplasmic domain is sufficient to mediate PDZ binding(20,22,25). To determine if the NSF interaction with the tail of the β_2AR has similar requirements, GST fusion proteins, including various carboxyl-terminal tail sequences, were prepared. Initially binding was evaluated using a pull-down assay using GST-fusion proteins coupled to glutathione-agarose beads. NSF bound to the cytoplasmic tail of the β_2AR under these conditions. Binding was affected by nucleotide state, being optimal in the presence of the non-hydrolyzable AMP-PNP but also detectable in the presence of ATP or ADP (Figure 1A). Binding to the β_2AR however was not affected by the addition of the adapter protein soluble NSF attachment protein (α-SNAP) (Figure 1B). NSF binding did not require α-SNAP nor was it enhanced by α-SNAP addition. Given that NSF binding to the β_2AR tail did not require α-SNAP and was still detectable under various nucleotide conditions, we chose to use the previously reported overlay assay (27) to further probe the effect of β_2AR C-terminal mutations on NSF binding. This overlay assay allows the systematic evaluation of a number of binding interactions at the same time.

As previously reported (27) NSF bound strongly to the full-length β_2AR tail sequence (GST-β_2AR) using this protein overlay technique. No significant binding to the cytoplasmic tail of the delta opioid receptor (GST-δOR) was observed over nonspecific binding to GST (Figure 1, lanes 4 and 1). Addition of a single alanine residue to the

β_2AR-derived tail sequence (GST-β_2AR-Ala), shown previously to disrupt PDZ domain binding(20), also abrogated binding of NSF (lane 6). Appending only the distal 4 residues of the β_2AR (GST-δOR-DSLL), which is sufficient for PDZ-mediated binding to NHERF / EBP50, also conferred binding to NSF. Essentially identical results were obtained using a 10-residue sequence derived from the distal β_2AR tail. This short sequence (GST-δOR-β_2[10]) mediated NSF binding comparable to the full-length β_2AR tail which, like PDZ domain-mediated binding of this sequence to NHERF / EBP50, was disrupted by alanine addition (GST-δOR-β_2[10]-Ala). Alanine substitution of the –1 residue, shown previously to block NSF binding to the full-length β_2AR tail(27), also did so when introduced into the distal tail sequence (GST-δOR-β_2[10]DSAL). However mutation of the –3 residue (GST-δOR-β_2[10]ASLL), shown previously to selectively inhibit NHERF / EBP50 interaction(27), did not disrupt NSF binding. Together these results emphasize the close similarity between NSF and PDZ domain-interacting determinants in the β_2AR tail(27), and they establish that mutations established previously to affect protein binding to the full tail sequence also function similarly when transplanted into the distal 10-mer.

Co-immunoprecipitation of EBP50/NHERF with the β2AR in intact cells has been observed using both recombinant (27) and endogenous (20) protein. Co-immunoprecipitation of recombinant NSF was demonstrated previously(27). Despite these previous results, we were unable to detect significant co-immunoprecipitation of endogenous NSF with the FLAG-β2AR in stably transfected cells following DSP cross-linking (Supplemental Figure 1 A) or under various conditions in the absence of cross-linker (not shown). This prompted us to investigate the subcellular localization of

endogenous NSF relative to internalized receptors. Recombinant NSF is present diffusely in the cytoplasm of cells in the absence of agonist, and concentrates on receptor-containing endosomes following agonist activation(27). We observed endogenous NSF to be concentrated on the nuclear membrane and detectable also on various cytoplasmic membranes, consistent with previous reports of endogenous NSF localization using fluorescence and electron microscopy(36,37). However, we did not observe a detectable redistribution of endogenous NSF in response to agonist, and were unable to observe pronounced colocalization between endogenous NSF and receptor-containing endosomes in agonist-treated cells (Supplemental Figure 1 B).

To begin to investigate the functional effects of these protein interactions on endocytic membrane trafficking, the ability of additional mutations of the β_2AR-derived tail sequence to promote rapid recycling when fused to the lysosomally-targeted δOR was examined. For this purpose, an established ratiometric method (34) was used as a highly specific assay of mutant receptor recycling. This assay measures the ability of a previously internalized "pulse" of antibody-labeled receptors to return to the plasma membrane following agonist washout and "chase" incubation in the presence of excess antagonist, allowing specific detection of recycling without interference by other processes (such as new receptor biosynthesis or differences in amounts of initial receptor internalization) that could also influence the total number of receptors present in the plasma membrane. This assay is also advantageous because it allows recycling of receptors to be measured objectively in single cells, and then averaged over large numbers, providing an accurate overall assessment of recycling in transiently transfected cell populations varying widely in the expression level of recombinant receptors achieved

in individual cells. As expected, based on previous studies using a flow cytometric method in stably transfected cells(21), fusion of the distal 10 residues derived from the β_2AR tail (δ/β2[10] mutant receptor) strongly promoted recycling of antibody-labeled (internalized) receptors detected by the ratiometric assay as shown in Figure 2. Furthermore this recycling activity was specific because it was abrogated by the addition of a terminal alanine residue (δ/β2[10]-Ala mutant receptor), which disrupts both NSF and PDZ binding. Interestingly, mutation of the –1 residue in this sequence (δ/β2[10]DSAL mutant receptor), which selectively disrupts interaction with NSF, did not block the recycling activity of the fused β_2AR-derived tail sequence. However, mutation of the –3 residue (δ/β2[10]ASLL mutant receptor), which selectively reduces PDZ but not NSF binding, significantly reduced recycling of antibody-labeled receptors. Together these results confirm the ability of the β_2AR-derived tail sequence to function as a fully sufficient 'recycling signal' when fused to a heterologous GPCR, and they suggest that significant endocytic sorting activity may be observed in the absence of direct interaction of the cytoplasmic tail with NSF.

To further investigate whether tail interaction with PDZ domain-containing protein(s) is truly sufficient to mediate recycling, in the absence of NSF interaction, naturally occurring PDZ ligand sequences that fail to bind to NSF were tested. The cystic fibrosis transmembrane regulator (CFTR) contains a carboxyl-terminal PDZ ligand that binds to NHERF / EBP50 –family proteins with high affinity(25,38-41). This sequence contains an arginine residue at the –1 position, in contrast to the leucine residue present at the –1 position of the β_2AR tail. Considering the strong inhibitory effect on NSF binding of alanine substitution of the β_2AR-derived tail sequence at this position (Figure 1), this

PDZ ligand was anticipated not to interact with NSF. The beta-1 adrenergic receptor (β_1AR) contains a type I PDZ ligand sequence, which is biochemically distinct from that present in the β_2AR and has been shown previously not to interact with NSF (27). Consistent with these expectations, neither the CFTR-derived sequence (GST-δOR-CFTR) nor its alanine adduct (GST-δOR-CFTR-Ala) exhibited detectable interaction with NSF as demonstrated in Figure 3. Furthermore the β_1AR-derived tail sequences (GST-δOR-β_1[10] and GST-δOR-β_1[10]-Ala) also failed to bind NSF.

Despite its failure to bind NSF, the CFTR-derived PDZ ligand sequence strongly promoted recycling when fused to the cytoplasmic tail of the δOR (δ/CFTR[10] mutant receptor, Figure 4). Indeed the ratiometric recycling assay indicated that the ability of the CFTR-derived tail sequence to promote recycling of antibody-bound receptors was comparable to that of the β_2AR –derived tail sequence. Furthermore, as seen in Figure 4 the β_1AR-derived PDZ ligand sequence was also fully sufficient to strongly promote recycling (δ/β_1[10] mutant receptor). This latter result was particularly surprising because the β_1AR and β_2AR –derived tail sequences represents biochemically distinct type I PDZ ligands, which bind to distinct PDZ domains. In particular, the β_1AR tail is well known to interact with PDZ proteins such as PSD-95, but this sequence does not exhibit any detectable interaction with NHERF/EBP50 or related PDZ domains that bind strongly to the β_2AR tail(42,43). These data confirm that NSF binding is not required for the observed recycling activity of PDZ ligand sequences, and they suggest that PDZ-mediated recycling activity is not limited to tail interaction(s) with NHERF/EBP50 – family proteins.

To further explore the range of PDZ ligand sequences capable of controlling post-endocytic sorting, we next examined the effects of a more divergent PDZ ligand. While the cytoplasmic tails of the β_2AR, CFTR and β_1AR all correspond to conventional type I PDZ ligands, the cytoplasmic tail of glycophorin C is representative of a more divergent type II PDZ ligand. Fusion of this sequence to the δOR tail modestly increased recycling of internalized receptors after agonist washout, but the magnitude of this effect was significantly smaller than that of any of the type I PDZ ligands tested. These results further confirm the ability of various PDZ ligands to function as fully sufficient endocytic recycling signals, and they indicate that not all PDZ ligands are equally effective in controlling the endocytic sorting mechanism.

While the above results strongly indicate that PDZ domain-mediated protein interaction(s) are fully sufficient to mediate post-endocytic sorting of GPCRs, they do not exclude a possible additional effect on this process of NSF binding to the cytoplasmic tail. Indeed, mutation of the –3 residue in the β_2AR-derived tail sequence (δ/β_2[10]ASLL mutant receptor) inhibited, but did not completely block, its recycling activity. However, as it was not possible to fully exclude residual PDZ binding to this sequence, an alternative NSF-interacting sequence was sought. The cytoplasmic tail of the GluR2 ionotropic Glutamate receptor has been shown previously to bind specifically to NSF(44-46). The region of the GluR2 tail required for this binding was further mapped to a ten residue sequence that does not contain the carboxyl-terminal PDZ ligand(44,46). Despite previous evidence of specific interaction via yeast-2-hybrid assay, we were unable to detect NSF binding to this sequence biochemically (data not shown). However, this GluR2-derived sequence produced a modest, albeit detectable, enhancement of recycling

when fused to the δOR tail (δ/GluR2[10] mutant receptor). Thus it is not possible to rule out some functional role of NSF interaction with the cytoplasmic tail in controlling post-endocytic sorting of GPCRs. Nevertheless, a more pronounced effect on post-endocytic sorting is conferred by PDZ domain-interacting sequences that do not bind NSF.

When the results obtained from the ratiometric recycling assay were compared to those obtained by fluorescence flow cytometry, an interesting discepency was noted (see Figure 5). While the general pattern of recycling efficiencies was consistent with the previously determined order, the absolute magnitudes of recycling for three of the receptors was much reduced as compared to those determined by the ratiometric method. Specifically, the chimeric receptors δ/β_1[10], δ/β_2[10] DSAL, and δ/CFTR[10] have a lower recycling efficiency than expected considering the ratiometric recycling data. The derived recycling efficiencies of both the wild-type β_2 adrenergic receptor and δ opioid receptor are similar regardless of the experimental protocol. These results provide further evidence that class I PDZ ligands can act as recycling sequences independent of binding to NSF. Methodological differences between the ratiometric and fluorescence flow cytometry assays could contribute to the discrepancy in the absolute recycling efficiency numbers.

The β_2AR-derived cytoplasmic tail, in addition to promoting rapid recycling of receptors after short-term agonist exposure, also strongly inhibits lysosomal proteolysis of receptors in the prolonged presence of agonist(21). This effect is thought to reflect efficient recycling of receptors over repeated rounds of endocytosis(9,47), in contrast to agonist washout experiments that measure a single round of recycling. Thus the ability of the β_2AR-derived tail sequence to inhibit lysosomal proteolysis of receptors represents

a relatively stringent assay of its recycling activity. To determine whether PDZ ligand sequences defective in NSF binding are capable of mediating such efficient endocytic sorting effects, stably transfected HEK293 cells expressing mutant receptors at similar levels were generated and tested by immunoblotting for agonist-induced proteolysis. Consistent with its highly efficient recycling, negligible proteolysis of the wild type β_2AR (Figure 6, "β") was detected even after 4 hours in the continuous presence of a saturating concentration of agonist (10 μM isoproterenol). In contrast, the wild type δOR (δ) was extensively proteolyzed over a similar time course in the presence of its corresponding agonist (10 μM DADLE), consistent with the failure of δOR to recycle efficiently after endocytosis and to traffic preferentially to lysosomes(9,21). The ability of the β_2AR tail-derived 'recycling signal' to inhibit lysosomal proteolysis when fused to δOR tail was retained when its binding to NSF was disrupted by mutation at the -1 position (δ/β_2[10]DSAL). In contrast selective disruption of PDZ protein interaction by mutation of the –3 position (δ/β_2[10]ASLL) prevented the β_2AR 'recycling signal' from rescuing the δOR from its degradative fate. Furthermore, fusion of the GluR2-derived NSF-interacting sequence (δ/GluR2[10]) failed to inhibit proteolysis of chimeric receptors. Moreover, fusion of PDZ-selective ligands derived from the CFTR cytoplasmic tail (δ/CFTR[10]) or β_1AR tail (δ/β_1[10]) strongly inhibited agonist-induced proteolysis of receptors. Taken together, these results strongly support the ability of multiple type I PDZ ligands to function as highly efficient endocytic sorting signals, independent of detectable binding to NSF, and to inhibit receptor trafficking to lysosomes in the continuous presence of agonist.

Discussion

In the present study several approaches were used to test the hypothesis that PDZ domain-mediated protein interaction(s) with the carboxyl-terminal cytoplasmic tail of GPCRs are sufficient to promote sorting of endocytosed receptors into the rapid recycling pathway. This idea was proposed initially based on studies of the β_2AR, in which it was shown that a distal portion of the cytoplasmic tail is required for efficient plasma membrane recycling(20) and conforms to a classical type I PDZ ligand(20,22,25). A subsequent study confirmed the importance of this sequence for plasma membrane recycling but identified a distinct non-PDZ protein interaction of this sequence with NSF. Thus it was proposed that interaction the β_2AR-derived recycling sequence with PDZ proteins is not important to recycling and that this function is exclusively mediated by interaction with NSF(27). The present study utilized a gain-of-function approach based on the ability of the β_2AR-derived tail sequence to function as an autonomous, transplantable sorting signal that is fully sufficient to re-route the endocytic trafficking of a distinct GPCR from lysosomal to recycling membrane pathways(21). It was observed that several mutations of the β_2AR-derived tail sequence, including several interpreted previously as specific evidence for PDZ-mediated endocytic sorting activity, actually affect both PDZ domain-mediated interaction of the receptor tail with NHERF / EBP50 and non-PDZ interaction with NSF. However a point mutation that selectively disrupts NSF binding to the full-length β_2AR tail, and has a similar effect on a 10-residue sequence representing the tail-derived recycling signal, was still fully sufficient to promote rapid recycling when fused to δOR. This distinct GPCR normally traffics

preferentially to lysosomes after agonist-induced endocytosis, and fusion of the β_2AR-derived recycling signal inhibited this process in accord with its ability to promote efficient plasma membrane recycling.

In addition to confirming the importance of PDZ domain-mediated protein interaction with the β_2AR-derived tail sequence, the experimental approach was extended to search for other naturally occurring PDZ ligand sequences that possess post-endocytic sorting activity. A PDZ ligand sequence derived from CFTR, which binds to a closely similar spectrum of PDZ domain-containing proteins (including NHERF / EBP50) as the β_2AR tail, was observed to possess strong recycling activity despite its failure to interact with NSF. Interestingly, a similar observation was made for a distinct PDZ ligand sequence derived from the β_1AR tail. This sequence fails to bind NSF but is capable of binding to a subset of PDZ proteins distinct from NHERF / EBP50(43). Nevertheless, the ability of this distinct PDZ ligand sequence to promote rapid recycling, and to prevent lysosomal trafficking of receptors, was comparable to that of the β_2AR-derived tail sequence. Taken together, these results strongly support the hypothesis that PDZ domain-mediated protein interaction(s) with the carboxyl-terminal cytoplasmic domain of GPCRs are, indeed, fully sufficient to promote plasma membrane recycling. In addition, the present results suggest that this property of PDZ domain-mediated protein interaction is not limited to the β_2AR-derived tail sequence or to interaction with NHERF / EBP50 –family proteins, and suggest that such endocytic recycling activity is a more general property of type I PDZ ligand sequences. While only a few GPCRs are thought to bind specifically to NHERF / EBP50, a considerable number of receptors have carboxyl-terminal sequences that correspond to consensus type I PDZ ligands. Thus it seems likely that

PDZ domain-mediated endocytic post-endocytic sorting is not limited to the β_2AR and is, instead, a more general principle of GPCR regulation. To what degree this mechanism of endocytic sorting might apply to other classes of integral membrane protein remains to be determined. However, it is interesting to note that studies of CFTR membrane traffic suggest an important role of PDZ domain-mediated protein interaction in controlling post-endocytic sorting of this ion channel(48).

While the present results strongly support a critical role of PDZ domain-mediated protein interactions in controlling post-endocytic sorting mechanism, and establish that PDZ interactions are sufficient to mediate this function independently of any detectable interaction with NSF, a potential additional function of NSF binding to the cytoplasmic tail cannot be excluded at present. The reason for this is that a point mutation that selectively reduces interaction with PDZ proteins did not completely abrogate endocytic recycling activity of the β_2AR-derived tail sequence. Furthermore a distinct sequence from the GluR2 tail, previously reported to bind NSF(44,46), possessed modest recycling activity. A caveat is that, while the GluR2-derived sequence does not resemble any known PDZ ligand, there is precedent for non-consensus interactions of cytoplasmic sequences with PDZ domains. Of particular interest, a carboxyl-terminal sequence present in the rodent kappa opioid receptor does not correspond to a consensus PDZ ligand yet interacts in vivo with NHERF / EBP50 and promotes recycling of internalized receptors(49,50). Furthermore, the cytoplasmic tail of the mu opioid receptor contains a sequence that does not bind detectably either to known PDZ proteins or to NSF, yet strongly and specifically promotes plasma membrane recycling of endocytosed receptors to a similar degree as the β_2AR tail and other PDZ ligands tested in the present study(34).

Thus it is possible that there exist a considerable variety of cytoplasmic protein interactions (both PDZ –dependent and –independent), which are capable of controlling post-endocytic sorting of particular GPCRs in a highly specific manner. While NSF activity is fundamentally required for endocytic trafficking because of its essential role in SNARE-dependent membrane fusion, the present results argue clearly that a direct interaction of the receptor tail with NSF is not essential for efficient post-endocytic sorting.

It is curious that two different means of determining receptor recycling gave dissimilar results. The recycling efficiencies of three key chimeric receptors, δ/β_1[10], δ/β_2[10] DSAL, and δ/CFTR[10], are lower when measured by fluorescence flow cytometry than by the ratiometric recycling assay. One of the key differences between the two assays is that the ratiometric recycling assay specifically looks at the recycling efficiency of only those receptors that have endocytosed while disregarding any receptors which did not undergo internalization. The flow cytometry assay, since it is a measure of total surface receptor count under the various conditions, is influenced by the extent of receptor internalization. We have examined the extent of receptor internalization and in fact these same three receptors, δ/β_1[10], δ/β_2[10] DSAL, and δ/CFTR[10], have impaired internalization by both fluorescence microscopy and flow cytometric measures (see Chapter 4). The observation of a discrepancy between two distinct measures of receptor recycling led directly to the discovery of an "endocytic brake" mediated through PDZ domain containing proteins at the cell surface.

It will be interesting in future studies to define specific PDZ domain-mediated protein interactions controlling post-endocytic sorting of receptors and to elucidate their

biochemical function. The present results suggest that post-endocytic sorting activity is not limited to a single GPCR or to a single PDZ domain-containing protein, but the actual spectrum of PDZ proteins that mediate post-endocytic sorting of receptors remains to be defined. Considering the potential diversity of protein interactions that can promote the rapid recycling process, another important question is whether distinct protein interactions mediating post-endocytic sorting of distinct GPCRs function via a similar or different biochemical mechanism. Current studies suggest that there exists a highly conserved set of endosomal sorting proteins, which mediate the membrane trafficking of a wide variety of endocytosed proteins(51). Thus it seems likely that distinct PDZ domain-mediated protein interactions occurring with GPCR tails ultimately link to a shared core sorting mechanism. As multiple distinct PDZ domains are often linked in the same protein(52-54), and non-covalent interactions between distinct PDZ proteins are also known to occur(55-57), it is tempting to speculate further that there may exist a multivalent protein complex that can link distinct recycling signals to a similar (or identical) endocytic sorting mechanism. In principle such a "combinatorial" strategy could allow membrane trafficking itineraries of co-expressed GPCRs (including closely homologous subtypes such as β_1 and β_2ARs) to be specifically programmed in different cell types.

In conclusion, the present results indicate that PDZ domain-mediated protein interaction(s) with GPCR cytoplasmic tails are indeed sufficient to control post-endocytic sorting, demonstrate that this activity can occur in the absence of detectable interaction with NSF, and suggest that this mechanism of post-endocytic sorting is considerably more widespread than previously anticipated. Together with previous evidence that PDZ

proteins function in receptor signaling and regulation in the plasma membrane, the present results add to the growing appreciation of PDZ interactions as extremely important and versatile regulators of GPCR signaling and membrane trafficking in mammalian cells.

Acknowledgements: We thank Michael Tanowitz for valuable advice. We also thank Aylin Hanyaloglu for expert assistance. These studies were supported by research grants from the National Institutes of Health. RMG was supported during part of these studies by a National Defense Science and Engineering Fellowship.

References

1. von Zastrow, M., and Kobilka, B. K. (1994) *J Biol Chem* **269**, 18448-18452.
2. Goodman, O. B., Jr., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1998) *Advances in Pharmacology* **42**, 429-433
3. Ferguson, S. S., Zhang, J., Barak, L. S., and Caron, M. G. (1998) *Life Sci* **62**, 1561-1565
4. Lefkowitz RJ, P. J., Krueger K, Daaka Y. (1998) *Adv Pharmacol* **42**, 416-420
5. Gagnon, A. W., Kallal, L., and Benovic, J. L. (1998) *Journal of Biological Chemistry* **273**, 6976-6981
6. Tsao, P. I., and von Zastrow, M. (2001) *Pharmacol Ther* **89**, 139-147.
7. Zhang, J., Ferguson, S., Barak, L. S., Menard, L., and Caron, M. G. (1996) *J Biol Chem* **271**, 18302-18305
8. Chu, P., Murray, S., Lissin, D., and von Zastrow, M. (1997) *J Biol Chem* **272**, 27124-27130.
9. Tsao, P. I., and von Zastrow, M. (2000) *J Biol Chem* **275**, 11130-11140.
10. Kurz, J. B., and Perkins, J. P. (1992) *Mol Pharmacol* **41**, 375-381
11. von Zastrow, M., and Kobilka, B. K. (1992) *J Biol Chem* **267**, 3530-3538.
12. Chaturvedi, K., Bandari, P., Chinen, N., and Howells, R. D. (2001) *J Biol Chem* **276**, 12345-12355
13. Tsao, P., Cao, T., and von Zastrow, M. (2001) *Trends Pharmacol Sci* **22**, 91-96.
14. Law, P.-Y., Hom, D. S., and Loh, H. H. (1984) *J. Biol. Chem.* **259**, 4096-4104

15. Law, P. Y., and Loh, H. H. (1999) *Journal of Pharmacology and Experimental Therapeutics* **289**, 607-624

16. Trowbridge, I. S., Collawn, J. F., and Hopkins, C. R. (1993) *Annual Review of Cell Biology* **9**, 129-161

17. Hao, M., and Maxfield, F. R. (2000) *Journal of Biological Chemistry* **275**, 15279-15286

18. Mayor, S., Presley, J. F., and Maxfield, F. R. (1993) *Journal of Cell Biology* **121**, 1257-1269

19. Gruenberg, J. (2001) *Nat Rev Mol Cell Biol.* **2**, 721-730

20. Cao, T. T., Deacon, H. W., Reczek, D., Bretscher, A., and von Zastrow, M. (1999) *Nature* **401**, 286-290.

21. Gage, R. M., Kim, K. A., Cao, T. T., and von Zastrow, M. (2001) *J Biol Chem* **276**, 44712-44720.

22. Hall, R. A., Premont, R. T., Chow, C. W., Blitzer, J. T., Pitcher, J. A., Claing, A., Stoffel, R. H., Barak, L. S., Shenolikar, S., Weinman, E. J., Grinstein, S., and Lefkowitz, R. J. (1998) *Nature* **392**, 626-630

23. Weinman, E. J., Steplock, D., Wang, Y., and Shenolikar, S. (1995) *Journal of Clinical Investigation* **95**, 2143-2149

24. Reczek, D., Berryman, M., and Bretscher, A. (1997) *Journal of Cell Biology* **139**, 169-179

25. Hall, R. A., Ostedgaard, L. S., Premont, R. T., Blitzer, J. T., Rahman, N., Welsh, M. J., and Lefkowitz, R. J. (1998) *Proceedings of the National Academy of Sciences of the United States of America* **95**, 8496-8501

26. Hall, R. A., Premont, R. T., and Lefkowitz, R. J. (1999) *Journal of Cell Biology* **145**, 927-932

27. Cong, M., Perry, S. J., Hu, L. A., Hanson, P. I., Claing, A., and Lefkowitz, R. J. (2001) *J. Biol. Chem.* **276**, 45145-45152

28. Evans, C. J., Keith, D. J., Morrison, H., Magendzo, K., and Edwards, R. H. (1992) *Science* **258**, 1952-1955

29. Kobilka, B. K., Dixon, R. A., Frielle, T., Dohlman, H. G., Bolanowski, M. A., Sigal, I. S., Yang-Feng, T. L., Francke, U., Caron, M. G., and Lefkowitz, R. J. (1987) *Proceedings of the National Academy of Sciences of the United States of America* **84**, 46-50

30. Keith, D. E., Murray, S. R., Zaki, P. A., Chu, P. C., Lissin, D. V., Kang, L., Evans, C. J., and von Zastrow, M. (1996) *J Biol Chem* **271**, 19021-19024.

31. Okayama, H., and Berg, P. (1983) *Molecular and Cellular Biology* **3**, 280-289

32. Nagiec, E. E., Bernstein, A., and Whiteheart, S. W. (1995) *J. Biol. Chem.* **270**, 29182-29188

33. Matveeva, E. A., He, P., and Whiteheart, S. W. (1997) *J. Biol. Chem.* **272**, 26413-26418

34. Tanowitz, M., and von Zastrow, M. (2003) *J. Biol. Chem.* **278**, 45978-45986

35. Bradford, M. M. (1976) *Analytical Biochemistry* **72**, 248-254

36. Mashima, J., Nagahama, M., Hatsuzawa, K., Tani, K., Horigome, T., Yamamoto, A., and Tagaya, M. (2000) *Biochemical and Biophysical Research Communications* **274**, 559-564

37. Tagaya, M., Furuno, A., and Mizushima, S. (1996) *J. Biol. Chem.* **271**, 466-470

38. Wang S, R. R., Schatz PJ, Guggino WB, Li M. (1998) *FEBS Lett.* **427**, 103-108

39. Karthikeyan, S., Leung, T., and Ladias, J. A. A. (2001) *J Biol Chem* **276**, 19683-19686

40. Karthikeyan S, L. T., Birrane G, Webster G, Ladias JA. (2001) *J Mol Biol.* **308**, 963-973

41. Ladias, J. (2003) *J Membr. Biol.* **192**, 79-88

42. Hu, L. A., Tang, Y., Miller, W. E., Cong, M., Lau, A. G., Lefkowitz, R. J., and Hall, R. A. (2000) *J. Biol. Chem.* **275**, 38659-38666

43. Xu, J., Paquet, M., Lau, A. G., Wood, J. D., Ross, C. A., and Hall, R. A. (2001) *J. Biol. Chem.* **276**, 41310-41317

44. Nishimune, A., Isaac, J. T., Molnar, E., Noel, J., Nash, S. R., Tagaya, M., Collingridge, G. L., Nakanishi, S., and Henley, J. M. (1998) *Neuron* **21**, 87-97

45. Osten, P., Srivastava, S., Inman, G. J., Vilim, F. S., Khatri, L., Lee, L. M., States, B. A., Einheber, S., Milner, T. A., Hanson, P. I., and Ziff, E. B. (1998) *Neuron* **21**, 99-110

46. Song, I., Kamboj, S, Xia, J, Dong, H, Liao, D, Huganir, RL. (1998) *Neuron* **21**, 393-400

47. Koenig, J. A., and Edwardson, J. M. (1997) *Trends in Pharmacological Sciences* **18**, 276-287

48. Swiatecka-Urban, A., Duhaime, M., Coutermarsh, B., Karlson, K. H., Collawn, J., Milewski, M., Cutting, G. R., Guggino, W. B., Langford, G., and Stanton, B. A. (2002) *J. Biol. Chem.* **277**, 40099-40105

49. Li, J.-G., Chen, C., and Liu-Chen, L.-Y. (2002) *J. Biol. Chem.* **277**, 27545-27552

50. Liu-Chen, L. Y. (2004) *Life Sciences* **75**, 511-536

51. Katzmann, D. J., Odorizzi, G., and Emr, S. D. (2002) *Nat Rev Mol Cell Biol.* **3**, 893-905

52. Sheng, M., Sala, C. (2001) *Annu Rev Neurosci* **24**, 1-29

53. Fanning, A. S., Anderson, J M. (1999) *Curr Opin Cell Biol.* **11**, 432-439

54. Jelen, F., Oleksy, A, Smietana, K, Otlewski, J. (2003) *Acta Biochim Pol.* **50**, 985-1017

55. Hirao, K., Hata, Y., Yao, I., Deguchi, M., Kawabe, H., Mizoguchi, A., and Takai, Y. (2000) *J. Biol. Chem.* **275**, 2966-2972

56. Fouassier, L., Yun, C. C., Fitz, J. G., and Doctor, R. B. (2000) *J. Biol. Chem.* **275**, 25039-25045

57. Jaffrey, S., Snowman, AM, Eliasson, MJ, Cohen, NA, Snyder, SH. (1998) *Neuron* **20**, 115-124

Figure Legends

Figure 1: NSF binding to the □$_2$AR-derived tail sequence. (**A**) GST-□$_2$AR was immobilized on agarose beads as described in Experimental Procedures and incubated with NSF under different nucleotide conditions: 2.5 mM AMP-PNP, 2.5 mM ADP or 2.5 mM ATP. (**B**) NSF binding (assayed in the presence of 2.5 mM AMP-PNP) was not affected by the addition of excess His$_6$-α-SNAP (100 and 500 □g). (**C**) Approximately 15 μg of the indicated GST fusion proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane. Blots were incubated overnight with 100 nM NSF protein and binding detected by protein overlay. A representative result is shown. (**D**) NSF binding to the indicated fusion proteins was quantified by fluorometric imaging of anti-FLAG immunoblots, as described in Experimental Procedures, and expressed relative to binding to GST-□$_2$AR. Bars indicate mean relative band intensities determined from 2 independent experiments. Error bars represent the standard deviation.

Figure 2: Effects of β_2AR tail-derived sequences on recycling of chimeric opioid receptors. Receptors were transiently expressed in HEK-293 cells and recycling of antibody-labeled receptors was analyzed as described in Experimental Procedures. Bars indicate recycling measurements averaged over 6-8 independent experiments per construct. Error bars represent the standard error of the mean.

Figure 3: Failure of NSF to bind detectably to the β_1AR or CFTR –derived tail sequences. (**A**) Approximately 15 μg of the indicated GST fusion proteins were

separated by SDS-PAGE, transferred to nitrocellulose membrane, incubated overnight with 100 nM NSF protein and binding detected by protein overlay. A representative result is shown. (**B**) NSF binding to the indicated fusion proteins was quantified by fluorometric imaging and expressed relative to binding to GST-β_2AR. Bars indicate mean relative band intensities determined from 2 independent experiments. Error bars represent the standard deviation.

Figure 4: Recycling activity of the β_1AR or CFTR –derived tail sequences. Receptors were transiently expressed in HEK-293 cells and recycling of antibody-labeled receptors was analyzed as described in Experimental Procedures. Bars indicate recycling measurements averaged over 6-8 independent experiments per construct. Error bars represent the standard error of the mean.

Figure 5: Quantification of recycling efficiency by Flow cytometry differs from that reported by the ratiometric recycling assay. Stably transfected cells expressing the indicated FLAG-tagged mutant receptors were left untreated, treated with agonist for 25 min., or treated with agonist, washed, and treated with antagonist for 45 min. Then cells were chilled to 4°C, dissociated from tissue culture dishes, receptors present in the plasma membrane were specifically labeled with Alexa 488-conjugated M1 antibody, and the relative number of surface receptors was quantitated by fluorescence flow cytometry as described in *Experimental Procedures*. **A**. The β_2AR recycles efficiently to the plasma membrane following agonist treatment while the δ OR does not despite a similar extent of receptor internalization. The chimeric δ/β_1AR receptor (δ/β_1[10]) recycles

more efficiently than the δ OR, but not as efficiently as the β_2AR when measured by fluorescence flow cytometry. The extent of receptor return is also less than that measured for the same receptor using a different technique (see Figure 4). The addition of a single alanine to the β_1AR derived sequence disrupts receptor recycling ((δ/β_1[10]-Ala). **B.** Two point mutations of the β_2AR derived sequence discriminate NSF and hNHERF / EBP50 binding. δ/β_2[10] DSAL which selectively binds to EBP50 and δ/β_2[10] ASLL which binds to NSF both have a similar intermediate recycling efficiency when analyzed by flow cytometry. This result contrasts with the results from those obtained by ratiometric recycling (see Figure 2) in which δ/β_2[10] DSAL clearly recycles much more efficiently than δ/β_2[10] ASLL. A previously described NSF binding motif from the GluR2 metabotropic glutamate receptor (δ/GluR2[10]) does not increase the recycling efficiency of the δ OR. Another EBP50 binding motif from the CFTR ((δ/CFTR[10]) enhances the recycling of the δ OR to a similar extent as that accorded by sequences from the β_1AR or either point mutant of the β_2AR. The calculated recycling efficiency is based upon at least three experiments with each data point performed in triplicate. Error bars represent the standard error of the mean.

Figure 6: Effects of PDZ and NSF –interacting sequences on agonist-induced degradation of chimeric opioid receptors. (**A**) Stably transfected HEK-293 cells expressing the indicated FLAG-tagged receptors at similar levels were exposed to a saturating concentration of the appropriate agonist (10 μM isoproterenol or DADLE) for 0, 1 or 4 hours. Total cell extracts were separated on SDS-PAGE, transferred to nitrocellulose, and blotted with M1 anti-FLAG. Representative blots are shown. (**B**)

Quantification of anti-FLAG band intensities by fluorometric imaging. Bars represent mean receptor levels (relative to 0 hour agonist exposure) determined from 4-8 independent experiments per construct. Error bars represent the standard error of the mean.

FIGURE 1:

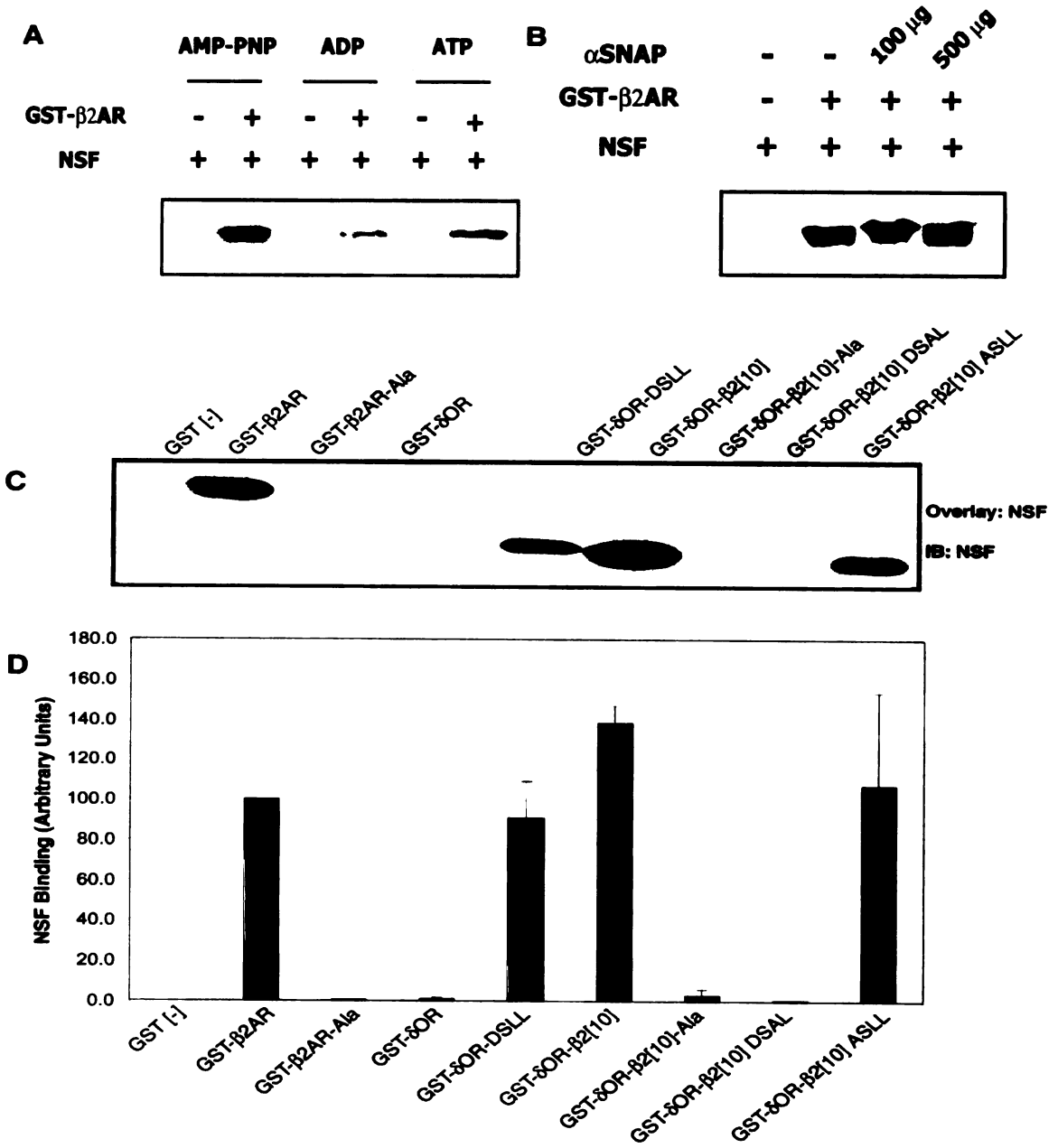

A
AMP-PNP
ADP
ATP
GST-β2AR
NSF
B
αSNAP
GST-β2AR
NSF
100 μg
500 μg
C
GST [-]
GST-β2AR
GST-β2AR-Ala
GST-δOR
GST-δOR-DSLL
GST-δOR-β2[10]
GST-δOR-β2[10]-Ala
GST-δOR-β2[10] DSAL
GST-δOR-β2[10] ASLL
Overlay: NSF
IB: NSF
D
NSF Binding (Arbitrary Units)
180.0
160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
GST [-]
GST-β2AR
GST-β2AR-Ala
GST-δOR
GST-δOR-DSLL
GST-δOR-β2[10]
GST-δOR-β2[10]-Ala
GST-δOR-β2[10] DSAL
GST-δOR-β2[10] ASLL

FIGURE 2:

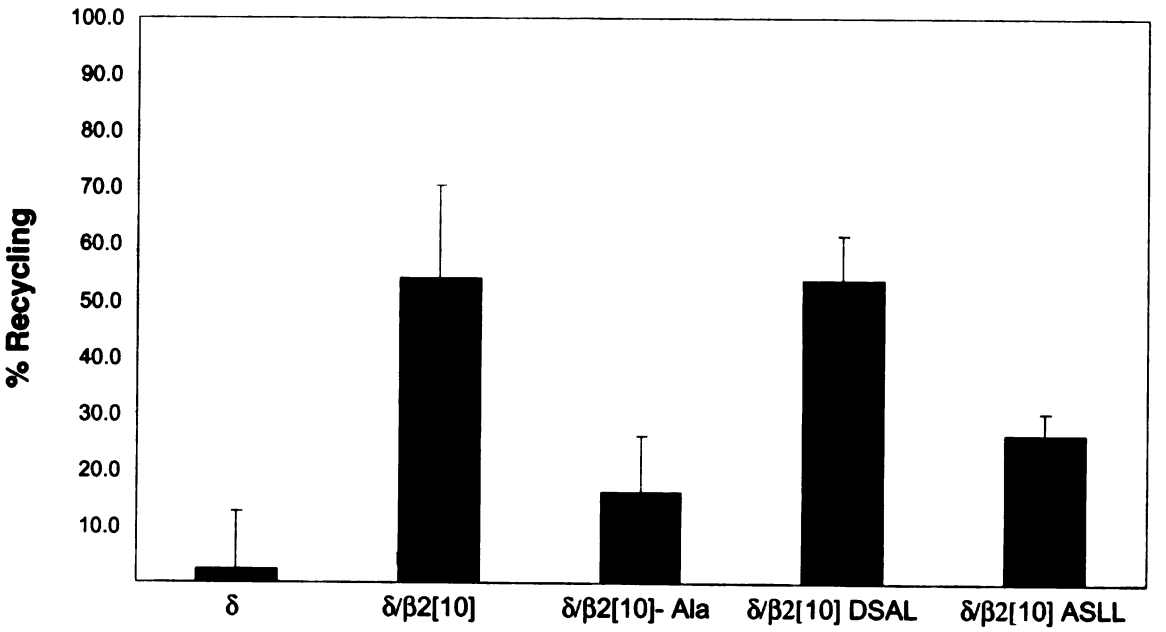

100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
% Recycling
δ
δ/β2[10]
δ/β2[10]- Ala
δ/β2[10] DSAL
δ/β2[10] ASLL

FIGURE 3:

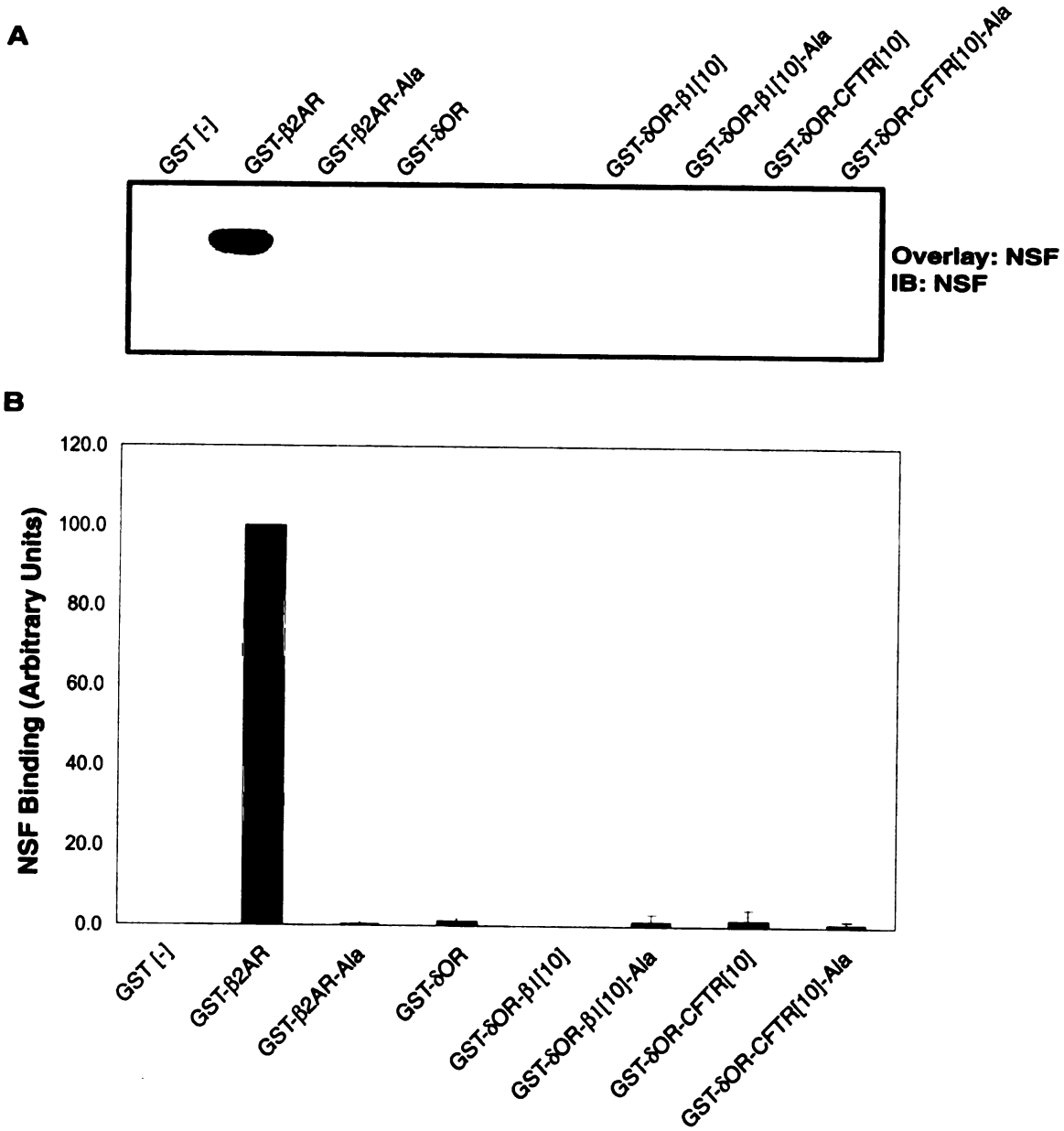

A
GST [-]
GST-β2AR
GST-β2AR-Ala
GST-δOR
GST-δOR-β1[10]
GST-δOR-β1[10]-Ala
GST-δOR-CFTR[10]
GST-δOR-CFTR[10]-Ala
Overlay: NSF
IB: NSF
B
NSF Binding (Arbitrary Units)
120.0
100.0
80.0
60.0
40.0
20.0
0.0
GST [-]
GST-β2AR
GST-β2AR-Ala
GST-δOR
GST-δOR-β1[10]
GST-δOR-β1[10]-Ala
GST-δOR-CFTR[10]
GST-δOR-CFTR[10]-Ala

FIGURE 4:

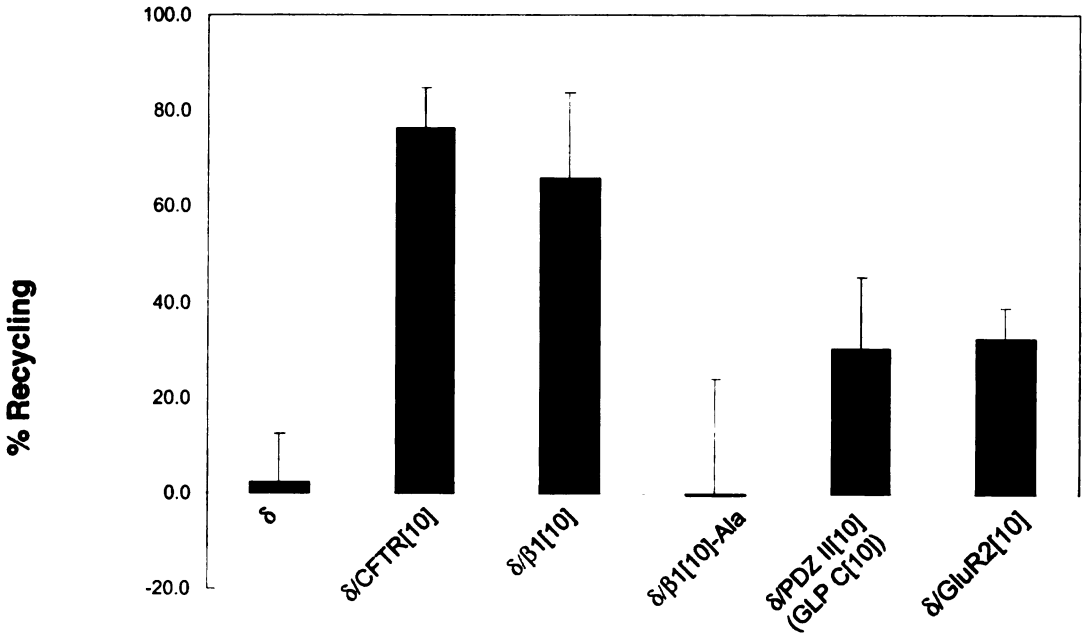
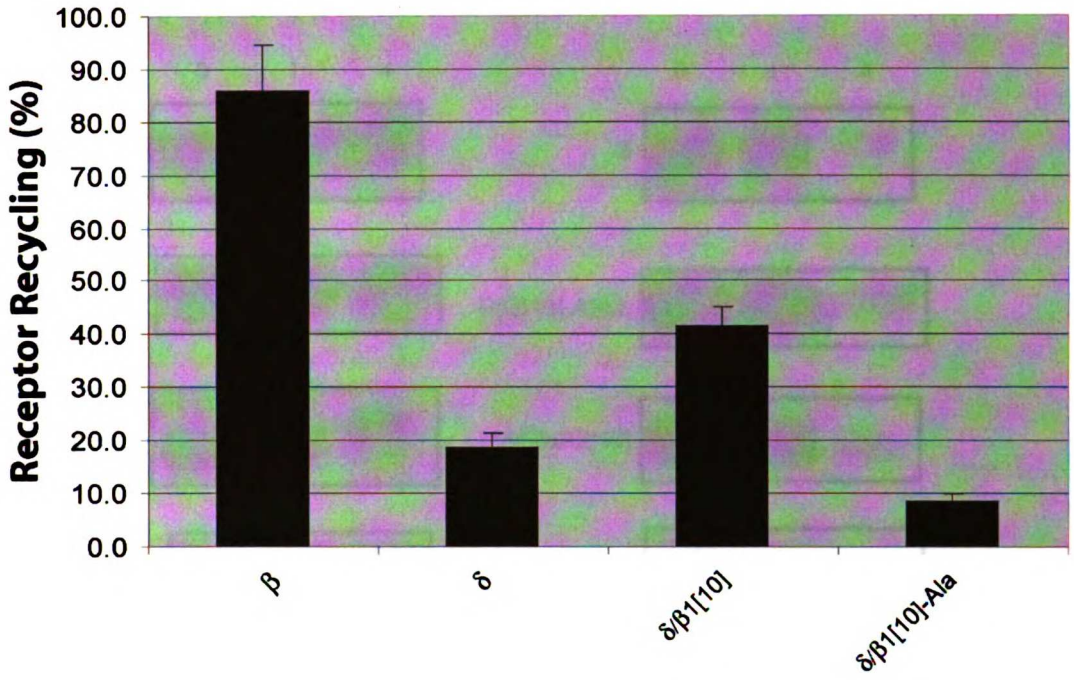

% Recycling
100.0
80.0
60.0
40.0
20.0
0.0
-20.0
δ
δ/CFTR[10]
δ/β1[10]
δ/β1[10]-Ala
δ/PDZ II[10] (GLP C[10])
δ/GluR2[10]

Figure 5:

A


Receptor Recycling (%)
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
β
δ
δ/β1[10]
δ/β1[10]-Ala

B

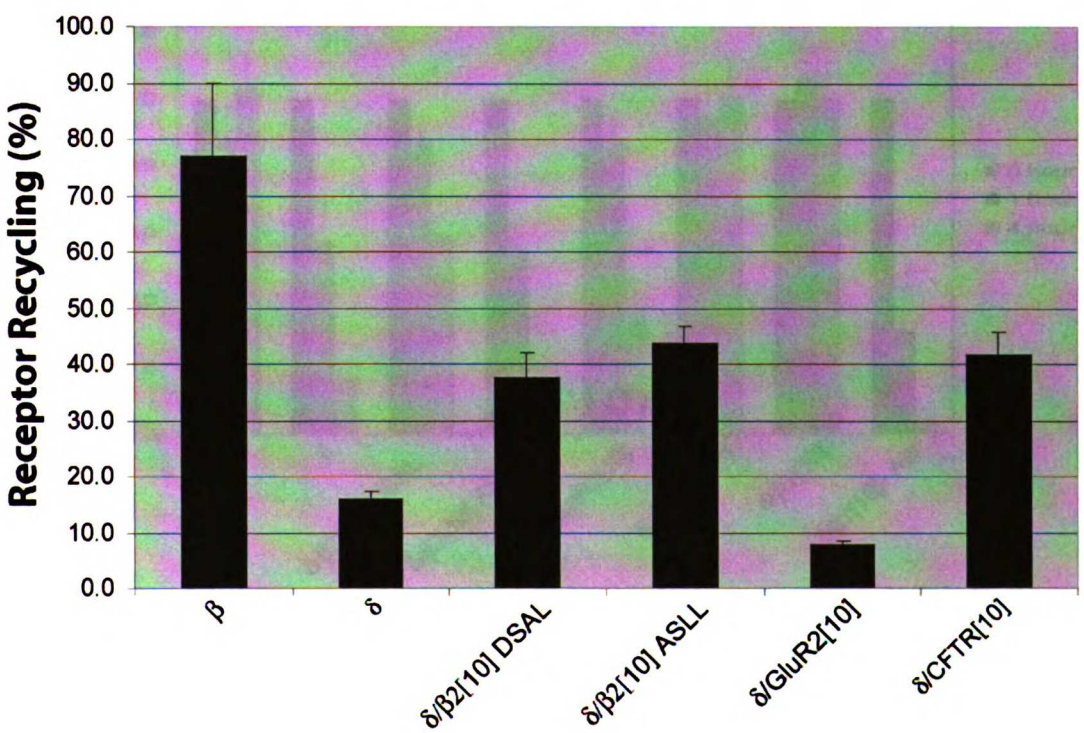
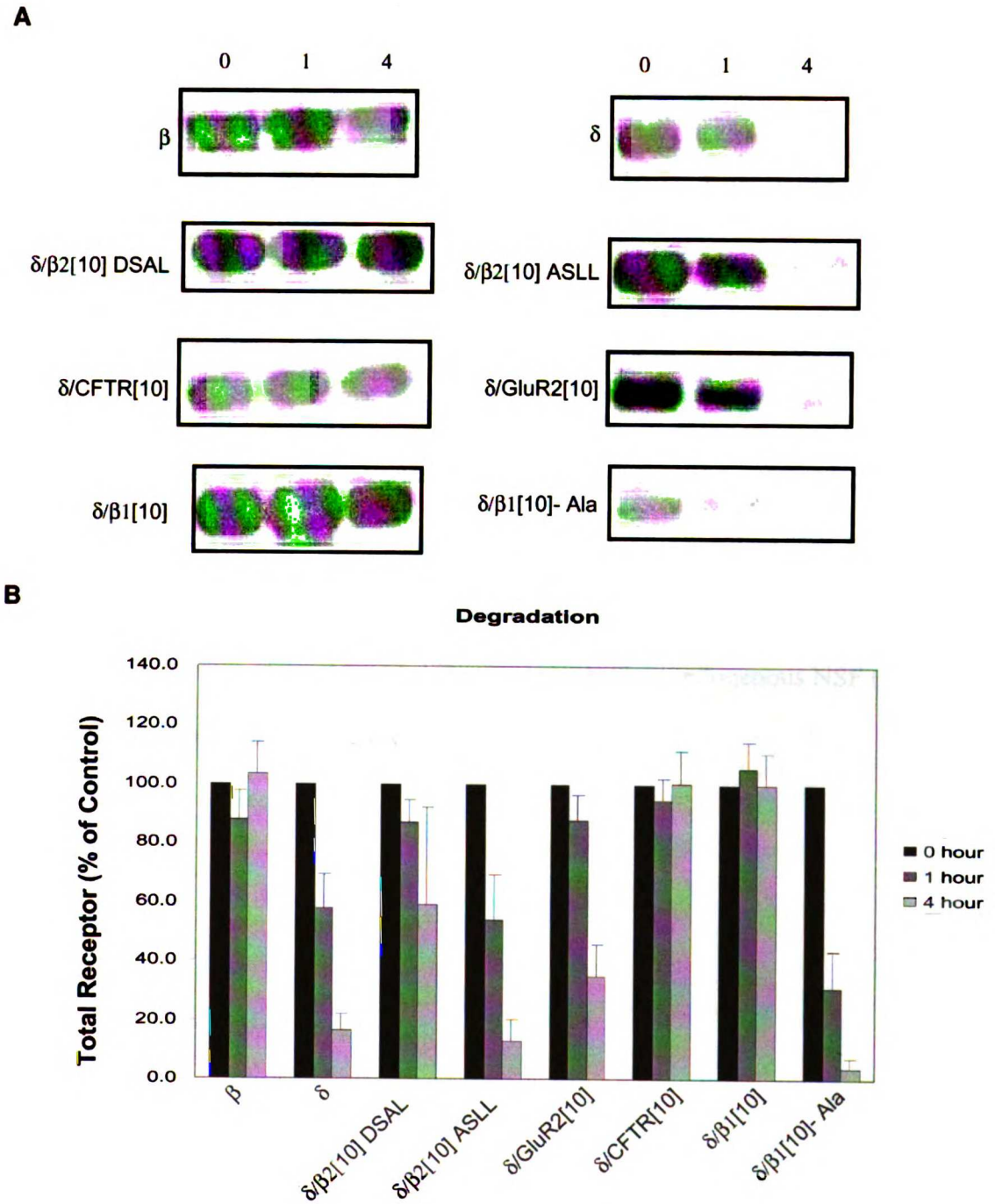

Receptor Recycling (%)
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
β
δ
δ/β2[10] DSAL
δ/β2[10] ASLL
δ/GluR2[10]
δ/CFTR[10]

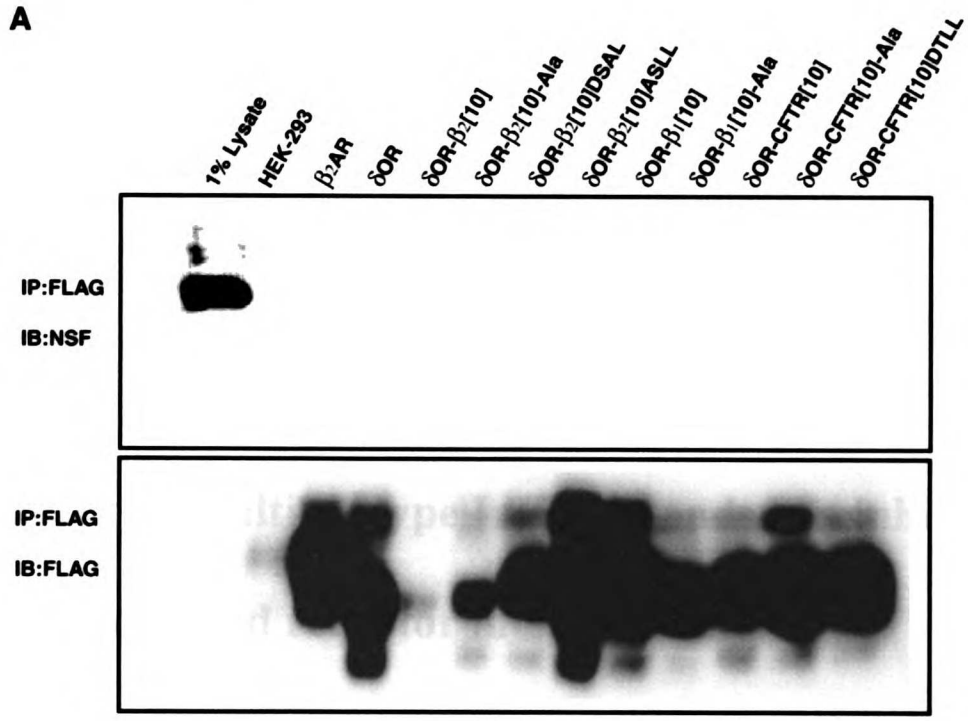
FIGURE 6:

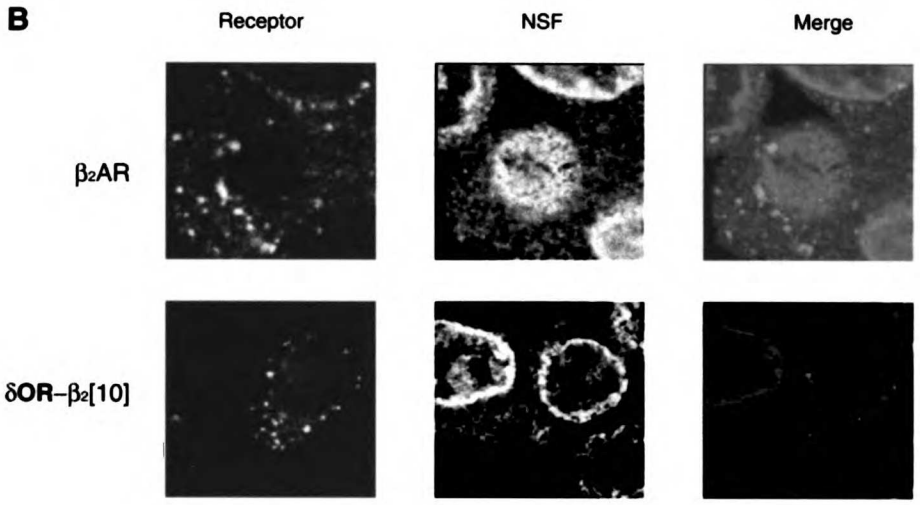


Supplemental Figure Legends

Supplemental Figure 1: *in vivo* NSF binding and co-localization to β_2AR, β_1AR, and CFTR tail derived sequences. (**A**) FLAG-tagged receptors were immunoprecipitated as described in Experimental Procedures and identical SDS-PAGE gels were transferred to nitrocellulose membranes. Detection of FLAG-tagged receptors or co-immunoprecipitated endogenous NSF was accomplished using M1 anti-FLAG or 2E5 antibodies respectively. A representative blot is shown from three independent experiments. (**B**) HEK-293 cells stably transfected with wt β_2AR or δOR-β_2[10] were treated with agonist (isoproterenol or DADLE respectively) for 30 minutes and then fixed. The cells were stained for FLAG epitope (green) or endogenous NSF (red) and visualized via confocal microscopy.

Supplemental Figure 1:





Chapter 4: Multiple type I PDZ ligands can inhibit G protein-coupled receptor internalization

All work presented in this chapter was performed by Robert Michael Gage and Mark von Zastrow.

Introduction

Endocytosis is a critical cellular process that allows cells to take up nutrients from the extracellular environment, macrophages to engulf and destroy invading microorganisms, and cells to bring old membrane proteins into the cell for degradation among other uses (1-12). Many cell types can endocytose relatively large areas of plasma membrane in order to incorporate fluid phase molecules in the extracellular milieu. For example, specialized cells like macrophages can engulf relatively large objects like bacteria through the process of phagocytosis (9-12). A related process, termed pinocytosis, also results in the uptake of a large volume of extracellular fluid (2-8). Cells also possess uptake mechanisms for smaller volumes and more specialized purposes.

The two major routes for the cellular uptake of membrane bound molecules are via clathrin coated pits and caveolae (13-18). These two methods of plasma membrane internalization derive their names from the principle protein constituents comprising the coat of endocytosed vesicles, clathrin and caveolin respectively (19-21). Both have been implicated in the uptake of signaling molecule receptors (13-17,22-27). Often, endocytosis of cell surface receptors is utilized as a mechanism to bring cargo into the cytoplasm of the cell. Such is the case for the transferrin and low density lipoprotein receptors (28-34). In other cases, such as the epidermal growth factor receptor (EGF R) internalization serves to decrease receptor signaling by removing the receptor from the source of agonist (the extracellular milieu) and/or directing the receptor to an intracellular compartment for degradation (14,35,36). Many G protein coupled receptors undergo similar processes.

Following agonist stimulation, many GPCRs are rapidly phosphorylated by G protein-coupled receptor kinases (GRKs) or other kinases on specific cytoplasmic residues (37-43). Phosphorylation of GPCRs can function to uncouple receptors from their cognate G proteins and diminish signaling (44-47). In most cases GRK phosphorylated receptors are substrates for arrestin binding. Arrrestin molecules serve to further decouple receptors from the signaling pathway and also act as an adapter to recruit receptors into clathrin coated pits where the receptors are concentrated prior to endocytosis (38,45,48-59). Scission of newly formed clathrin coated vesicles from the plasma membrane required the action of the GTPase dynamin (60-63). Dynamin molecules have been visualized via electron microscopy and seem to form a protein coat around the neck of the vesicle where it contacts the plasma membrane (62,64-72). Dynamin and the energy of GTP hydrolysis may directly act to pinch off vesicles from the membrane or they may act indirectly through other proteins (62,66,69,71-73). Internalization of signaling molecules away from the plasma membrane has been associated with many physiological processes.

Endocytosis of GPCRs has been implicated in receptor desensitization (38,45,48-59), the process by which greater amounts agonist are required to produce half-maximal biological effect. Endocytosis can also serve a number of other functions. It may be the first step in the endocytic pathway of a receptor targeted for degradation. Being membrane proteins, receptors must reach lysosomes for degradation. To reach the lysosomes for processing, however, the receptors must first be taken up from the cell surface and enter the cytoplasm in clathrin coated vesicles. Many rounds of vesicle fusion and fission with ever larger endocytic structures deposit the receptors first in early

endosomes then later in multi-vesicular bodies / late endosomes. Finally the receptors reach the lysosome, whose contents are kept at low pH and contains proteases which together act to degrade the receptor. From the early endosome, GPCRs can also recycle back to the plasma membrane in a process that dephosphorylates the receptor and returns it to a naïve state (74). In the process of "resensitization", the recycled receptors are ready to undergo a further round of agonist induced signaling. G protein coupled receptors can also exit the early endosome and traffick to recycling endosomes from which they return to the plasma membrane. A further possibility, exhibited by the V2 vasopressin receptor (V2R), is to remain within the cell for prolonged periods before returning to the cell surface (75). This may facilitate further signaling from the endocytosed V2R via signaling pathways distinct from G proteins (76). Evidence suggests that signaling may occur through the arrestin adapter molecule, which normally quickly dissociates from endosomes but remains bound to V2R containing endosomes for prolonged periods (76). Endocytosis of activated receptors can thus serve to dampen signaling, conversely to resensitize receptors, or to facilitate non-G protein based signaling.

Endocytosis of G protein coupled receptors has been reported to be influenced by protein : protein interactions (45,48,56,59,77-83). Specifically, the β_1AR interacts with PDZ domain containing proteins through its distal carboxyl terminus (81-83). Overexpression of the PDZ domain containing proteins which interact with the β_1AR, Magi-2 or PSD-95, can modulate the amount of receptor internalized after ten minutes of agonist treatment (81). We previously reported that certain PDZ ligand sequences were sufficient to mediate the recycling of the δ opioid receptor (84,85) independent of the

ATPase NSF. We sought in the current work to determine whether the PDZ ligand sequences found in the β_1AR and other membrane proteins could inhibit the internalization of a heterologous GPCR, the δ opioid receptor in an analogous manner. We also wanted to determine if PDZ ligand mediated recycling and inhibition of receptor internalization were biochemically separable events, occuring at different time points and/or different cellular locales during the membrane trafficking of a GPCR.

We found that the PDZ ligand from the β_1AR is capable of inhibiting the internalization of the δ OR when appended to the distal carboxyl terminus of the receptor. Other class I PDZ ligands, from the CFTR and a point mutation to a β_2AR derived sequence (DSAL), can also function as "endocytic brake" sequences. By modulating the ability of the ligand sequence to bind the ATPase protein NSF, we found that the ability of a PDZ ligand sequence to inhibit receptor internalization was independent of its ability to influence the efficiency of receptor recycling. We discovered that certain class I PDZ interactions can decrease the rate at which receptors cluster into clathrin coated pits following agonist stimulation. The recruitment and clustering of β-arrestin appears to proceed normally however. And it does so without any visible clustering of receptors into those clathrin coated structures occupied by the arrestin molecules. This surprising finding suggests that arrestin molecules, although initially recruited to the plasma membrane by activated G protein coupled receptors, do not need a tight association with the receptor to move laterally into clathrin coated pits.

Experimental Procedures

cDNA Constructs and Mutagenesis

Several epitope-tagged versions of the cloned murine delta opioid receptor (δOR(86)) and the human beta 2 adrenergic receptor (β_2AR (87)) were used in these studies: mutant receptors containing a FLAG epitope in the amino-terminal extracellular domain (SFδOR, SFβ_2AR, respectively) were described previously and demonstrated to be functional(88-90). Mutant delta opioid receptors containing a FLAG epitope in the amino-terminal extracellular domain and the last six carboxyl-terminal cytoplasmic residues (NH_2-GGGAAA-COOH) replaced with ten or eleven amino acids were generated (see table below). This was accomplished by insertion of a synthetic linker-adapter (Operon Technologies) encoding the ten-residue or eleven-residue sequence followed by a stop codon into an SrfI site present near the 3' end of the sequence encoding the δOR tail. Receptor cDNAs were cloned into pcDNA3 (Invitrogen) or pIRES (Clontech) and all constructs were verified by dideoxynucleotide sequencing (UCSF Genetics Core Sequencing Facility and UCSF Biomolecular Resource Center). EGFP tagged β-arrestin 2 (Bovine arrestin 3) was generously provided by Dr. Marc Caron.

Chimeric Receptor	Linker Amino Acid sequence
β_2[10]	NH_2-RNCSTNDSLL-COOH
β_2[10]-Ala	NH_2-RNCSTNDSLLA-COOH
β_2[10] DSAL	NH_2-RNCSTNDSAL-COOH
β_2[10] ASLL	NH_2-RNCSTNASLL-COOH
β_1[10]	NH_2-RPGFASESKV-COOH
β_1[10]-Ala	NH_2-RPGFASESKVA-COOH
CFTR[10]	NH_2-TEEEVQDTRL-COOH
CFTR[10]-Ala	NH_2-TEEEVQDTRLA-COOH
PDZ II[10] (GLP C[10])	NH_2-GDSSRKEYFI-COOH
GluR2[10]	NH_2-KRMKVAKNPQ-COOH

Cell Culture and Transfection

Human embryonic kidney 293 cells (ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (University of California San Francisco Cell Culture Facility). Cells grown in 6-cm dishes were transfected with ~5 μg of plasmid DNA containing the indicated receptor or arrestin construct by calcium phosphate precipitation (91,92). For studies of receptor trafficking in transiently transfected cells, cells were transfected as above, plated onto coverslips 24 hr post transfection and experiments were conducted 48 hr post transfection. Stably transfected cells expressing epitope tagged receptors were generated by transfecting 293 cells in 6-cm dishes as above. Cell clones expressing transfected receptors were selected in 500 μg/mL Geneticin (Life Technologies, Inc.) and colonies were isolated and selected to have similar levels of receptor expression.

Examination of Receptor Endocytosis by Fluorescence Microscopy

Endocytic trafficking of receptors labeled initially in the plasma membrane was visualized by fluorescence microscopy using a minor modification of a previously described method (93). Briefly, stably or transiently transfected 293 cells expressing the indicated receptor were grown on glass coverslips (Corning) and treated with M1 anti-FLAG antibody (2.5 ug/ml, Sigma) directly conjugated to Alexa 488 or 594 dye (Molecular Probes) at 37 °C for 25 min to label receptors. The cells were treated with 10 mM isoproterenol (Research Biochemicals) or 10 mM DADLE (Research Biochemicals International) for the indicated times at 37 °C. Following this incubation, cells were fixed immediately for determining internalization of FLAG-tagged receptors. The cells were fixed with 3.7% formaldehyde in PBS, pH 7.4, for 15 min and then quenched with three washes of TBS with 1 mM CaCl2. Specimens were mounted to glass slides (Vectashield) and visualized. Conventional fluorescence microscopy was performed using inverted Nikon Diaphot microscope equipped with a Nikon 60X NA1.4 objective and epifluorescence optics. Images were collected using a 12-bit cooled charge-coupled device camera (Princeton Instruments) interfaced to a Macintosh computer.

Quantitation of Receptor Endocytosis by Fluorescence Flow Cytometry

Endocytosis of epitope-tagged receptors was estimated by assaying the loss of immunoreactive receptors accessible at the cell surface to monoclonal antibody recognizing the extracellular epitope tag (FLAG). This assay is a variant of a previously described flow cytometric assay for estimating receptor internalization and recycling (90). Briefly, monolayers of cells stably expressing the indicated FLAG tagged receptor

were incubated in the presence of 10 uM of the appropriate agonist (10 μM isoproterenol or DADLE) for the indicated times min at 37 °C to drive agonist-induced internalization. At the indicated time points, cell monolayers were chilled on ice to stop membrane trafficking, and cells were lifted with PBS containing 0.04% EDTA and lacking Ca++ or Mg++ (PBS/EDTA University of California San Francisco Cell Culture Facility). Cells were washed twice in 1 ml PBS and incubated at 4 °C for 45-60 min in 0.5 ml PBS with 2.5 ug/ml M1 anti-FLAG antibody that had been conjugated with fluorescein isothiocyanate or Alexa 488 (Molecular Probes) using standard methods. Receptor immunoreactivity was quantitated by fluorescence flow cytometry (FACScan, Becton Dickinson, Palo Alto, CA). Fluorescence intensity of 10,000 cells was collected for each sample. Cellquest software (Becton Dickinson) was used to calculate the mean fluorescence intensity of single cells in each population. All experiments were conducted ≥5 times with similar results. The mean values for each experiment were averaged to obtain the overall mean fluorescence intensity and standard error of the mean reported in the figure.

β-Arresin Recruitment Assay via Fluorescence Microscopy

The ability of various chimeric receptors to recruit an EGFP tagged version of β-Arresin 2 (Bovine Arrestin 3) were assayed using fluorescence microscopy using a modification of a previously described technique (94,95). Briefly, cells expressing the indicated receptor and tranfected with EGFP-arrestin 3 were incubated with the monoclonal anti-FLAG M1 antibody (Sigma) directly conjugated to Alexa 594 (Molecular Probes) at a 1:1000 dilution for 30 min at 37 °C. Cells were treated with the appropriate agonist (10

μM isoproterenol or DADLE) and fixed as mentioned above. β-Arrestin 2-EGFP was detected by GFP fluorescence (green fluorescence). Immuno-stained coverslips were examined by epifluorescence microscopy using a Nikon 60× NA1.4 objective and Chroma filter sets optimized for these fluorophores. Images were collected using a 12-bit cooled charge-coupled device camera (Princeton Instruments) interfaced to a Macintosh computer.

Visualization of Receptor and β-arrestin clustering via Total Internal Reflection Fluorescence Microscopy (TIRF)

Cells expressing the indicated receptor were transiently transfected with EGFP-arrestin 3 and plated on 22 mm coverslips the following day. The cells were stained as above with M1 anti-FLAG antibody (Sigma) directly conjugated to Alexa 594 (Molecular Probes) for 30 min at 37 °C. Cells were then treated with the appropriate agonist (10 μM isoproterenol or DADLE) for the indicated times. The cells were immediately fixed in 3.7 % formaldehyde in PBS for 15 min. Coverslips were washed three times in TBS and kept at 4 °C in TBS (with 0.02 % NaN_3) until they were visualized. Coverslips were mounted on a peltier stage (kept at ambient temperature) with PBS in contact with the cells. Receptor and arrestin 3 were visualized using a Nikon TE 2000 microscope with 60 X Apo TIRF objective NA 1.45 illuminated with both green (HeNe, 543 nm) and red (Ar 488 nm) laser sources. Some images were collected using a video-rate SIT camera (Hamamatsu) interfaced to a Macintosh computer. Imaged were captured using an LG-3 (ScionCorp) video grabber board. Images were analyzed using ScionImage program (v. 4.02 beta for windows XP, ScionCorp). All other images were collected using a cooled

CCD camera (Princeton Instruments) interfaced to a PC compatible computer and captured and analyzed using either MetaMorph (Universal Imaging) or IPLab Spectrum (Scanalytics). Quantification of receptor clustering within individual cells was performed using still images captured at each time point following agonist treatment [0, 45 sec, 2 min, 3 min, 5 min, and 10 min]. Clustering was defined as greater than 20 receptor positive punctae per cell. At least 10 cells per time point were visualized and the results are representative of one experiment for δ/β_1AR and δ/β_1AR-Ala and two experiments for the δ OR.

Results

Previous studies (84,85) have shown that certain class I PDZ ligands can function as autonomous recycling sequences. Carboxyl terminal residues from a variety of membrane proteins including the β_1AR, β_2AR, and CFTR are capable of re-routing the δ opioid receptor from a degradative pathway into a rapidly recycling one. In the course of those studies it was noted that the absolute value of recycling efficiency obtained depended on the method used to obtain the measurement. One of the key differences in the two methods used, ratiometric recycling assay and fluorescence flow cytometry, is that the ratiometric assay specifically measures the recycling efficiency of receptors that have undergone endocytosis and discards receptors which remain on the cell surface. This suggested to us that the PDZ ligands may have an effect on the initial endocytosis of the δ OR. It has been reported previously that the PDZ ligand from the β_1AR is responsible for the poor internalization of this receptor in response to agonist treatment (81-83).

We investigated whether the PDZ ligand from the β_1AR was indeed capable of reducing the internalization rate of the δ OR when appended to the carboxyl terminus of the opioid receptor by epi-fluorescence microscopy. The wild-type δ opioid receptor is readily internalized following exposure to the peptide agonist DADLE (top panel of (A) in Figure 1). After 2 minutes of agonist exposure, receptors can be seen clustered on the plasma membrane of HEK-293 cells and a few vesicles can also be seen at this time. By 5 minutes after DADLE is added, there are multiple receptor containing vesicles within the cell and by 10-20 minutes post-treatment endocytosis of the δ OR is maximal. In

contrast, the chimeric δ/β_1AR shows no appreciable receptor clustering or endocytosis until 10 minutes post-agonist addition. After 20 minutes in the continuous presence of DADLE, there is a marked increase in the number of endocytic vesicles containing the δ/β_1AR. This effect is dependent upon an intact PDZ ligand from the β_1AR. When a single alanine residue is added to the sequence derived from the β_1AR which is predicted to disrupt PDZ domain mediated interactions, the chimeric δ OR clusters and internalizes as well as the wild-type opioid receptor.

We confirmed the internalization differences initially observed by fluorescence microscopy using fluorescence flow cytometry to track the loss of surface receptor following agonist treatment for 10 or 25 minutes. Approximately 20% of cell surface δ OR is internalized following 10 minutes of DADLE treatment (panel (B) in Figure 1). After 25 minutes of continuous agonist presence, the internalization of the δ opioid receptor is nearly maximal (approximately 80% loss of surface receptors). As expected from the visual lack of endocytosis, the chimeric δ/β_1AR shows no appreciable loss of surface receptor following 10 minutes of agonist exposure. After a longer time course of DADLE treatment (25 minutes), a majority of the δ/β_1AR has been taken up into endocytic vesicles. Again, this effect of appending the sequence derived from the β_1AR requires a functional PDZ ligand as the addition of a single alanine to the carboxyl end of the chimeric receptor allows for rapid receptor internalization similar to that evinced by the wild-type δ opioid receptor. Thus it appears that the sequence taken from the β_1AR in addition to its ability to act as a rapid recycling sequence can also act as an "endocytic brake."

In an effort to investigate the brake's mechanism, we utilized total internal reflection fluorescence (TIRF) microscopy to examine events occurring on or very near the basal surface of the HEK-293 cells. This technique takes advantage of the physical properties of light to sharply illuminate only those objects within about 100 nm of the surface of the coverslip. We observed the clustering of antibody-labeled receptors into clathrin coated pits on the basal surface and noted the effects of appending the brake sequence from the β_1AR on the clustering rate of the δ opioid receptor. Following treatment with 10 μM DADLE, the wild-type δ OR is rapidly clustered on the surface of HEK-293 cells. Even after only a brief (45 sec) exposure to agonist, the receptor is visibly clustered into cell surface structures (see top panel Figure 2A). These structures colocalize with fluorescent clathrin (data not shown), suggesting that they are clathrin coated pits and the precursors to clathrin coated vesicles. Much of the cell surface δ OR remains concentrated in clathrin coated structures during agonist treatments up to 10 minutes in duration. In contrast to the rapid clustering exhibited by the wild-type δ opioid receptor, the majority of the chimeric δ/β_1AR remain smoothly spread over the surface of the cell with few if any puncta visible until 5 minutes post-agonist. Chimeric δ/β_1AR-Ala, which has a terminal alanine appended to the sequence from the β_1AR, is clustered rapidly to clathrin coated pits following agonist treatment. The "endocytic brake" of the β_1AR appears to retard receptors from entering clathrin coated pits. Quantification of the percentage of cells with greater than 20 visible punctae (representing clustered receptors) further illustrates the differences between the wild-type δ OR and the chimeric δ/β_1AR (Figure 2B). Appending a terminal alanine residue to the β_1AR derived sequence disrupts its ability to act as an endocytic brake (Figure 2C, '45 sec').

We next asked whether PDZ ligand sequences from membrane proteins distinct from the β_1AR were also able to act as "brake" sequences and inhibit the internalization of the δ OR. We did not observe any effect of adding the last ten residues of the β_2AR (which contains the class I PDZ ligand sequence –DSLL-COOH) on the internalization rate of the δ OR by fluorescence microscopy (data not shown). The distal carboxyl terminus of the β_2AR interacts with the ATPase protein NSF (N ethyl maleimide sensitive factor) in addition to the PDZ domain containing protein hNHERF / EBP 50 (human Na^+/H^+ exchanger regulatory factor / ezrin/moesin/radixin binding phosphoprotein of 50 kDa) (ref cong). Two previously described point mutations selectively disrupt interaction with hNHERF / EBP50 or NSF (-ASLL-COOH and –DSAL-COOH respectively) (96). We found that appending the β_2AR derived point mutant sequence –DSAL-COOH which binds selectively to the PDZ domain containing protein hNHERF / EBP50 inhibited δ OR internalization when appended to the carboxyl terminus of the opioid receptor. In fact, a decreased internalization for the point mutant in the context of the β_2AR was observed in the original paper (96). The converse point mutant, -ASLL which binds selectively to NSF did not decrease the internalization of the δ OR when appended to the distal carboxyl terminus of the δ opioid receptor. We also saw a decrease in the initial rate of endocytosis of the δ OR when we appended the carboxyl terminal PDZ ligand sequence from the CFTR (cystic fibrosis transmembrane conductance regulator). By flow cytometry (see Figure 3), both the CFTR and β_2AR point mutant DSAL decrease the initial endocytosis of the δ OR following 10 minutes of agonist exposure. The β_2AR point mutant ASLL did not affect the internalization of the δ OR when assayed by the

flow cytometric assay. Thus, it appears that binding to several different class I PDZ domain containing proteins can cause a receptor to be endocytosed less efficiently.

How might the clustering of receptors be delayed? One possibility would be for the receptors, when bound to scaffolding proteins via PDZ domain mediated interactions, to be a weaker substrate for GRK (G protein-coupled receptor kinase) mediated phosphorylation. Or phosphorylated receptors might exhibit a reduced ability to bind β-arrestin and thus be less efficiently directed to clathrin coated pits and internalized. We tested various chimeric δ opioid receptors for their ability to efficiently recruit β-arrestin to the membrane. Using epi-fluorescence microscopy, no differences were observed among the various receptors for the ability to recruit β-arrestin from its cytosolic localization in the absence of agonist to the plasma membrane following agonist treatment (data not shown). In fact, the β-arrestin could even be seen to be localized into discrete punctae on the membrane even at time points where there was no similar clustering of certain receptors (e.g. δ/β_1AR or δ/CFTR). To investigate this further, we utilized total internal reflection fluorescence microscopy to visualize events occurring at the basal membrane. TIRF microscopy has the advantage of being able to illuminate objects on or very near to the plasma membrane, potentially simplifying the interpretation of clustering events. When visualized via TIRF microscopy, the δ OR rapidly clusters into punctae (see Figure 2 and also Figure 4: red staining in merged imaged) that colocalize with overexpressed EGFP-β-arrestin (Figure 4: green staining). In contrast, the chimeric δ/β_1AR does not enter clathrin coated pit structures until relatively late time points, after 10 minutes of continuous agonist exposure. When a single alanine is added to the end of the β_1AR derived sequence (δ/β_1AR-Ala in Figure 4), the chimeric receptor

now clusters rapidly into the clathrin and β-arrestin punctae on the cell surface. The PDZ ligand of the β_1AR appears to be capable of retarding the movement of activated δ OR into clathrin coated pits, but does not seem to affect the ability of the receptor to recruit β-arrestin to the plasma membrane. β-arrestin recruited to the plasma membrane by δ/β_1AR moves into clathrin coated pits rapidly. This effect is not limited to the PDZ ligand from the β_1AR. Similar results were obtained using PDZ ligands from CFTR and the DSAL point mutant of the β_2AR derived sequence. These results suggest that β-arrestin, once activated and recruited to the plasma membrane by an activated receptor, does not require direct binding to GPCRs to become associated with and can precede receptors into clathrin coated pits.

Discussion

In the present study, we explored the ability of multiple class I PDZ ligands to act as transplantable "endocytic brake" sequences. Previous results have suggested that interaction between PDZ domain containing proteins and the PDZ ligand sequence from the β_1AR can modulate the internalization of the receptor (81-83). We found that this PDZ ligand is capable of inhibiting the internalization of a heterologous GPCR, the δ opioid receptor, when appended to the distal carboxyl terminus of the receptor. Other class I PDZ ligands, from the CFTR and a point mutation to a β_2AR derived sequence (DSAL), can also function as "endocytic brake" sequences. Interaction with PDZ domain containing proteins at the cell surface may decrease the mobility of the receptors, effectively locking them into position. The protein : protein interaction may also preclude interaction between an activated receptor and GRKs resulting in a decreased receptor phosphorylation efficiency. PDZ domain mediated interactions could also interfere with the receptor's interaction with β-arrestin, decreasing the number of arrestin molecules recruited to the plasma membrane by activated phosphorylated receptors.

We tested for several of these cases and found that certain class I PDZ interactions can indeed decrease the rate at which receptors cluster into clathrin coated pits following agonist stimulation. This effect could have been due to a decreased phosphorylation of the receptor or a decrease in the receptor's ability to recruit β-arrestin to the plasma membrane. We found, however, that arrestin recruitment was normal in the mutant receptors. In fact, we saw rapid β-arrestin recruitment and clustering into clathrin coated pits following stimulation of chimeric δ opioid receptors which contain class I PDZ

ligands at their distal carboxyl terminus. The recruitment and clustering of β-arrestin appears to proceed without any visible clustering of receptors into those clathrin coated structures occupied by the arrestin molecules (see Figure 5). This result seems to violate the accepted order of events that lead to the internalization of G protein-coupled receptors.

The current model for GPCR endocytosis can be summarized as follows. A naïve receptor on the surface of the cell encounters its cognate ligand and undergoes a conformational change (97-102). This structural rearrangement in the receptor is coupled through the membrane where guanine nucleotide exchange occurs on a G protein bound to the receptor (99). The activated G protein is released to drive further downstream signaling (99). The activated GPCR is now a better substrate for GRKs and becomes phospholylated on cytoplasmic ser / thr residues which uncouple the receptor from their associated G protein (37-43). Phosphorylated GPCRs often recruit β-arrestin molecules to the plasma membrane (38,45,48-59). The receptor / arrestin complex is then thought to move laterally in the membrane to clathrin coated structures via the clathrin and adapter interaction motifs present on the β-arrestin molecule (38,45,48-59). β-arrestin is thought to act as a shuttling molecule, promoting the motion of activated G protein-coupled receptors into clathrin coated pits where the receptors can be internalized (38,45,48-59). Following internalization, receptors can be trafficked to lysosomes where they are degraded and response to further agonist exposure is muted (38,45,48-59). Other receptors recycle back to the plasma membrane in a naïve state, ready to undergo further rounds of signaling (74). A more complete understanding of the roles of the various

molecules involved in these processes could lead to better control of the processes pharmacologically.

Our surprising finding that β-arrestin molecules can cluster into clathrin coated pits independently of receptor binding calls into question the role of one of the prominent players in the internalization of GPCRs. β-arrestin may initially be recruited to the plasma membrane by activated receptors and then become clustered into clathrin coated pits regardless of whether they are bound to receptors. Instead of dragging or shuttling receptors directly to the clathrin coated pits as previously posited (38,45,48-59), the arrestin may remain in the coated pits waiting to capture passing activated GPCRs. In most cases, the receptors would not be tightly bound to structural proteins at the cell surface and would be free to move with β-arrestin and would appear to cluster in clathrin coated structures concurrently with the arrestin. In those cases where the GPCR was bound to proteins at the plasma membrane, the arrestin would appear to move independently of the receptor. Eventually, due to the transient nature of PDZ domain mediated interactions, receptors with a PDZ ligand would be released, move laterally in the plasma membrane, and finally be "captured" by β-arrestin molecules resident in clathrin coated pits.

Alternatively, β-arrestin may be recruited to the plasma membrane by activated receptors and move laterally to clathrin coated structures independent of the activating GPCR. Once in the coated pits, the arrestin molecule may then undergo some conformational change, possibly due to binding with clathrin and / or other adapter molecules, which would be required to maintain long term binding to the activated receptors. Thus activated, the β-arrestin would bind to activated GPCRs that entered the

coated pits, retain them there, and effectively concentrate the receptors in the coated pits. We can test the validity of this model by studying those receptors which rapidly enter into clathrin coated pits following agonist stimulation (e.g. the wild-type β_2AR). At very early time points, it should be possible to determine whether β-arrestin is clustered prior to visible receptor clustering in cells expressing receptors that rapidly cluster. Any delay in the receptor clustering as compared to clustering of β-arrestin for these receptors is not seen at time points as soon after agonist treatment as 45 seconds, so rapid live imaging of both receptor and arrestin would be needed to visualize the difference in clustering rates. Alternatively, it may be possible to slow down the endocytic events sufficiently, by chilling the cells or through other means, to exaggerate the differences in the clustering rates of the receptor and β-arrestin such that they could be visualized.

References

1. Beron, W., Alvarez-Dominguez, C., Mayorga, L., and Stahl, P. D. (1995) *Trends Cell Biol* **5**, 100-104
2. Conner, S. D., and Schmid, S. L. (2003) *Nature* **422**, 37-44
3. Nichols, B. J., and Lippincott-Schwartz, J. (2001) *Trends Cell Biol* **11**, 406-412
4. Lamaze, C., and Schmid, S. L. (1995) *Curr Opin Cell Biol* **7**, 573-580
5. Besterman, J. M., and Low, R. B. (1983) *Biochem J* **210**, 1-13
6. Steinman, R. M., Mellman, I. S., Muller, W. A., and Cohn, Z. A. (1983) *J Cell Biol* **96**, 1-27
7. Michl, J. (1980) *Am J Clin Nutr* **33**, 2462-2471
8. Silverstein, S. C., Steinman, R. M., and Cohn, Z. A. (1977) *Annu Rev Biochem* **46**, 669-722
9. May, R. C., and Machesky, L. M. (2001) *J Cell Sci* **114**, 1061-1077
10. Swanson, J. A., and Hoppe, A. D. (2004) *J Leukoc Biol* **76**, 1093-1103
11. Aderem, A., and Underhill, D. M. (1999) *Annu Rev Immunol* **17**, 593-623
12. Allen, L. A., and Aderem, A. (1996) *Curr Opin Immunol* **8**, 36-40
13. Gagnon, A. W., Kallal, L., and Benovic, J. L. (1998) *Journal of Biological Chemistry* **273**, 6976-6981
14. Le Roy, C., and Wrana, J. L. (2005) *Nat Rev Mol Cell Biol* **6**, 112-126
15. Man, H. Y., Lin, J. W., Ju, W. H., Ahmadian, G., Liu, L., Becker, L. E., Sheng, M., and Wang, Y. T. (2000) *Neuron* **25**, 649-662.
16. Vieira, A. V., Lamaze, C., and Schmid, S. L. (1996) *Science* **274**, 2086-2089

17. Wilde, A., Beattie, E. C., Lem, L., Riethof, D. A., Liu, S. H., Mobley, W. C., Soriano, P., and Brodsky, F. M. (1999) *Cell* **96**, 677-687

18. Anderson, R. G. (1998) *Annu Rev Biochem* **67**, 199-225

19. Steer, C. J., and Klausner, R. D. (1983) *Hepatology* **3**, 437-454

20. Brodsky, F. M. (1985) *Journal of Cell Biology* **101**, 2047-2054

21. Rothberg, K. G., Heuser, J. E., Donzell, W. C., Ying, Y. S., Glenney, J. R., and Anderson, R. G. (1992) *Cell* **68**, 673-682

22. Shaul, P. W., and Anderson, R. G. (1998) *Am J Physiol* **275**, L843-851

23. Mineo, C., Gill, G. N., and Anderson, R. G. (1999) *J Biol Chem* **274**, 30636-30643

24. Liu, P., Ying, Y., and Anderson, R. G. (1997) *Proc Natl Acad Sci U S A* **94**, 13666-13670

25. Wu, C., Butz, S., Ying, Y., and Anderson, R. G. (1997) *J Biol Chem* **272**, 3554-3559

26. Mineo, C., James, G. L., Smart, E. J., and Anderson, R. G. (1996) *J Biol Chem* **271**, 11930-11935

27. Liu, P., Ying, Y., Ko, Y. G., and Anderson, R. G. (1996) *J Biol Chem* **271**, 10299-10303

28. Collawn, J. F., Kuhn, L. A., Liu, L. F., Tainer, J. A., and Trowbridge, I. S. (1991) *Embo J* **10**, 3247-3253

29. Dautry-Varsat, A., Ciechanover, A., and Lodish, H. F. (1983) *Proceedings of the National Academy of Sciences of the United States of America* **80**, 2258-2262

30. Hopkins, C. R., Miller, K., and Beardmore, J. M. (1985) *Journal of Cell Science. Supplement* **3**, 173-186

31. Klausner, R. D., Ashwell, G., van Renswoude, J., Harford, J. B., and Bridges, K. R. (1983) *Proceedings of the National Academy of Sciences of the United States of America* **80**, 2263-2266

32. Miller, K., Shipman, M., Trowbridge, I. S., and Hopkins, C. R. (1991) *Cell* **65**, 621-632

33. Trowbridge, I. S., Newman, R. A., Domingo, D. L., and Sauvage, C. (1984) *Biochem Pharmacol* **33**, 925-932

34. Goldstein, J. L., Brown, M. S., Anderson, R. G., Russell, D. W., and Schneider, W. J. (1985) *Annu Rev Cell Biol* **1**, 1-39

35. Haglund, K., Di Fiore, P. P., and Dikic, I. (2003) *Trends Biochem Sci* **28**, 598-603

36. Haglund, K., Sigismund, S., Polo, S., Szymkiewicz, I., Di Fiore, P. P., and Dikic, I. (2003) *Nat Cell Biol* **5**, 461-466

37. Sibley, D. R., Strasser, R. H., Caron, M. G., and Lefkowitz, R. J. (1985) *J Biol Chem* **260**, 3883-3886

38. Benovic, J. L., Kuhn, H., Weyand, I., Codina, J., Caron, M. G., and Lefkowitz, R. J. (1987) *Proc Natl Acad Sci U S A* **84**, 8879-8882

39. Benovic, J. L., Strasser, R. H., Caron, M. G., and Lefkowitz, R. J. (1986) *Proc Natl Acad Sci U S A* **83**, 2797-2801

40. Gurevich, V. V., and Benovic, J. L. (1997) *Mol Pharmacol* **51**, 161-169

41. Gurevich, V. V., and Benovic, J. L. (2000) *Methods Enzymol* **315**, 422-437

42. Gurevich, V. V., Pals, R. R., Benovic, J. L., Hosey, M. M., and Onorato, J. J. (1997) *J Biol Chem* **272**, 28849-28852

43. Gurevich, V. V., and Benovic, J. L. (1993) *J Biol Chem* **268**, 11628-11638

44. von Zastrow, M., and Kobilka, B. K. (1994) *J Biol Chem* **269**, 18448-18452.

45. Goodman, O. B., Jr., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1998) *Advances in Pharmacology* **42**, 429-433

46. Ferguson, S. S., Zhang, J., Barak, L. S., and Caron, M. G. (1998) *Life Sci* **62**, 1561-1565

47. Lefkowitz, R. J., Pitcher, J., Krueger, K., and Daaka, Y. (1998) *Advances in Pharmacology* **42**, 416-420

48. DeGraff, J. L., Gagnon, A. W., Benovic, J. L., and Orsini, M. J. (1999) *Journal of Biological Chemistry* **274**, 11253-11259

49. Ferguson, S. S., Barak, L. S., Zhang, J., and Caron, M. G. (1996) *Can J Physiol Pharmacol* **74**, 1095-1110

50. Goodman, O. J., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1996) *Nature* **383**, 447-450

51. Krupnick, J. G., Goodman, O. J., Keen, J. H., and Benovic, J. L. (1997) *J Biol Chem* **272**, 15011-15016

52. Krupnick, J. G., and Benovic, J. L. (1998) *Annual Review of Pharmacology and Toxicology* **38**, 289-319

53. Laporte, S. A., Oakley, R. H., Holt, J. A., Barak, L. S., and Caron, M. G. (2000) *J Biol Chem* **275**, 23120-23126

54. Lohse, M. J., Benovic, J. L., Codina, J., Caron, M. G., and Lefkowitz, R. J. (1990) *Science* **248**, 1547-1550

55. Miller, W. E., and Lefkowitz, R. J. (2001) *Curr Opin Cell Biol* **13**, 139-145.

56. Oakley, R. H., Laporte, S. A., Holt, J. A., Barak, L. S., and Caron, M. G. (1999) *Journal of Biological Chemistry* **274**, 32248-32257

57. Pippig, S., Andexinger, S., Daniel, K., Puzicha, M., Caron, M. G., Lefkowitz, R. J., and Lohse, M. J. (1993) *J Biol Chem* **268**, 3201-3208

58. Zhang, J., Ferguson, S. S., Barak, L. S., Aber, M. J., Giros, B., Lefkowitz, R. J., and Caron, M. G. (1997) *Receptors and Channels* **5**, 193-199

59. Zhang, J., Barak, L. S., Winkler, K. E., Caron, M. G., and Ferguson, S. S. (1997) *J Biol Chem* **272**, 27005-27014

60. Altschuler, Y., Barbas, S. M., Terlecky, L. J., Tang, K., Hardy, S., Mostov, K. E., and Schmid, S. L. (1998) *Journal of Cell Biology* **143**, 1871-1881

61. Baba, T., Damke, H., Hinshaw, J. E., Ikeda, K., Schmid, S. L., and Warnock, D. E. (1995) *Cold Spring Harb Symp Quant Biol* **60**, 235-242

62. Muhlberg, A. B., Warnock, D. E., and Schmid, S. L. (1997) *Embo Journal* **16**, 6676-6683

63. Schmid, S. L., McNiven, M. A., and De Camilli, P. (1998) *Current Opinion in Cell Biology* **10**, 504-512

64. Damke, H., Baba, T., Warnock, D. E., and Schmid, S. L. (1994) *J Cell Biol* **127**, 915-934

65. Hinshaw, J. E., and Schmid, S. L. (1995) *Nature* **374**, 190-192

66. Oh, P., McIntosh, D. P., and Schnitzer, J. E. (1998) *Journal of Cell Biology* **141**, 101-114

67. Henley, J. R., Krueger, E. W., Oswald, B. J., and McNiven, M. A. (1998) *Journal of Cell Biology* **141**, 85-99

68. Sever, S., Muhlberg, A. B., and Schmid, S. L. (1999) *Nature* **398**, 470-471

69. Sweitzer, S. M., and Hinshaw, J. E. (1998) *Cell* **93**, 1021-1029

70. Takel, K., McPherson, P. S., Schmid, S. L., and De, C. P. (1995) *Nature* **374**, 186-190

71. Warnock, D. E., and Schmid, S. L. (1996) *Bioessays* **18**, 885-893

72. Warnock, D. E., Hinshaw, J. E., and Schmid, S. L. (1996) *J Biol Chem* **271**, 22310-22314

73. Zhang, J., Ferguson, S., Barak, L. S., Menard, L., and Caron, M. G. (1996) *J Biol Chem* **271**, 18302-18305

74. Hausdorff, W. P., Caron, M. G., and Lefkowitz, R. J. (1990) *FASEB J* **4**, 2881-2889

75. Innamorati, G., Sadeghi, H., and Birnbaumer, M. (1999) *Journal of Receptor and Signal Transduction Research* **19**, 315-326

76. Shenoy SK, L. R. (2003) *Biochem J.* **375(Pt 3)**, 503-515

77. Beattie, E. C., Carroll, R. C., Yu, X., Morishita, W., Yasuda, H., von Zastrow, M., and Malenka, R. C. (2000) *Nat Neurosci* **3**, 1291-1300.

78. DeFea, K. A., Zalevsky, J., Thoma, M. S., Dery, O., Mullins, R. D., and Bunnett, N. W. (2000) *Journal of Cell Biology* **148**, 1267-1281

79. Gaidarov, I., Krupnick, J. G., Falck, J. R., Benovic, J. L., and Keen, J. H. (1999) *Embo Journal* **18**, 871-881

80. Xia, Z., Gray, J. A., Compton-Toth, B. A., and Roth, B. L. (2003) *J Biol Chem* **278**, 21901-21908

81. Xu, J., Paquet, M., Lau, A. G., Wood, J. D., Ross, C. A., and Hall, R. A. (2001) *J. Biol. Chem.* **276**, 41310-41317

82. Hall, R. A. (2004) *Semin Cell Dev Biol* **15**, 281-288

83. Hu, L. A., Tang, Y., Miller, W. E., Cong, M., Lau, A. G., Lefkowitz, R. J., and Hall, R. A. (2000) *J. Biol. Chem.* **275**, 38659-38666

84. Gage, R. M., Kim, K. A., Cao, T. T., and von Zastrow, M. (2001) *J Biol Chem* **276**, 44712-44720.

85. Gage, R. M., Matveeva, E. A., Whiteheart, S. W., and von Zastrow, M. (2005) *J. Biol. Chem.* **280**, 3305-3313

86. Evans, C. J., Keith, D. J., Morrison, H., Magendzo, K., and Edwards, R. H. (1992) *Science* **258**, 1952-1955

87. Kobilka, B. K., Dixon, R. A., Frielle, T., Dohlman, H. G., Bolanowski, M. A., Sigal, I. S., Yang-Feng, T. L., Francke, U., Caron, M. G., and Lefkowitz, R. J. (1987) *Proceedings of the National Academy of Sciences of the United States of America* **84**, 46-50

88. Keith, D. E., Murray, S. R., Zaki, P. A., Chu, P. C., Lissin, D. V., Kang, L., Evans, C. J., and von Zastrow, M. (1996) *J Biol Chem* **271**, 19021-19024.

89. von Zastrow, M., and Kobilka, B. K. (1992) *J Biol Chem* **267**, 3530-3538.

90. Tsao, P. I., and von Zastrow, M. (2000) *J Biol Chem* **275**, 11130-11140.

91. Tsao, P., Cao, T., and von Zastrow, M. (2001) *Trends Pharmacol Sci* **22**, 91-96.

92. Okayama, H., and Berg, P. (1983) *Molecular and Cellular Biology* **3**, 280-289

93. Cao, T. T., Deacon, H. W., Reczek, D., Bretscher, A., and von Zastrow, M. (1999) *Nature* **401**, 286-290.

94. Klein, U., Muller, C., Chu, P., Birnbaumer, M., and von Zastrow, M. (2001) *J Biol Chem* **276**, 17442-17447.

95. Barak, L. S., Ferguson, S. S., Zhang, J., and Caron, M. G. (1997) *Journal of Biological Chemistry* **272**, 27497-27500

96. Cong, M., Perry, S. J., Hu, L. A., Hanson, P. I., Claing, A., and Lefkowitz, R. J. (2001) *J. Biol. Chem.* **276**, 45145-45152

97. Bourne, H. R. (1997) *Current Opinion in Cell Biology* **9**, 134-142

98. Collins, S., Caron, M. G., and Lefkowitz, R. J. (1992) *Trends Biochem Sci* **17**, 37-39

99. Schoneberg, T., Schultz, G., and Gudermann, T. (1999) *Mol Cell Endocrinol* **151**, 181-193

100. Hall, D. A. (2000) *Mol Pharmacol* **58**, 1412-1423

101. Wreggett, K. A., and De Lean, A. (1984) *Mol Pharmacol* **26**, 214-227

102. De Lean, A., Stadel, J. M., and Lefkowitz, R. J. (1980) *J Biol Chem* **255**, 7108-7117

Figure Legends

Figure 1

The PDZ ligand from the β_1AR inhibits the internalization of the δ opioid receptor when appended to its distal carboxyl terminus. **A** HEK-293 cells stably transfected with the indicated FLAG-tagged receptor were incubated in the presence of M1 anti-FLAG antibody directly conjugated to Alexa 594 for 30 min. Cells were next treated for the time indicated with 10 μM DADLE and then immediately fixed prior to visualization using epi-fluorescence microscopy. **B** Fluorescence flow cytometry was performed on the same stably transfected cells as in (**A**). Cells were treated with 10 μM DADLE for either 10 or 25 minutes and then chilled at 4 °C. FLAG-tagged receptors were stained using M1 anti-FLAG antibody directly conjugated to Alexa 488 for 45 min. Internalization is expressed as the % of surface brightness lost after the indicated time as compared to control, untreated specimens. Each data point was observed in triplicate with the error bars representing the standard error of the mean of ≥ 5 experiments.

Figure 2

The PDZ ligand from the β_1AR inhibits the clustering of the δ OR on the basal membrane of the cell. **A** TIRF microscopy was performed using HEK-293 cells stably transfected with the indicated receptor. Cells were pre-incubated for 30 minutes with M1 anti-FLAG antibody directly conjugated to Alexa 594 before treatment with 10 μM DADLE for the indicated times. Cells were immediately fixed before cell surface receptor visualization by TIRF microscopy. **B** Quantification of receptor clustering within individual cells was

performed at the indicated times. Clustering was defined as greater than 20 receptor positive punctae per cell. At least 10 cells per time point were visualized and the results are representative of one experiment for δ/β_1AR and two experiments for the δ OR.

C The delay in receptor clustering evident at 45 sec post agonist treatment for the δ/β_1AR does not appear when clustering of the δ/β_1AR-Ala is examined. At least 10 cells per time point were visualized and the results are representative of one experiment for δ/β_1AR and δ/β_1AR-Ala and two experiments for the δ OR.

Figure 3

Certain other class I PDZ ligands can also inhibit the internalization of the δ OR. Fluorescence flow cytometry was performed on cells stably transfected with the indicated receptor. Cells were treated with 10 μM DADLE for either 10 minutes and then chilled at 4 °C. FLAG-tagged receptors were stained using M1 anti-FLAG antibody directly conjugated to Alexa 488 for 45 min. Internalization is expressed as the % of surface brightness lost after the indicated time as compared to control, untreated specimens. Each data point was observed in triplicate with the error bars representing the standard error of the mean of ≥ 5 experiments.

Figure 4

The PDZ ligand from the β_1AR inhibits the clustering of the δ opioid receptor without altering the ability of the receptor to recruit β-arrestin or altering the rate at which β-arrestin clusters into clathrin coated pits. TIRF microscopy was performed using HEK-293 cells stably transfected with the indicated receptor and transiently transfected with

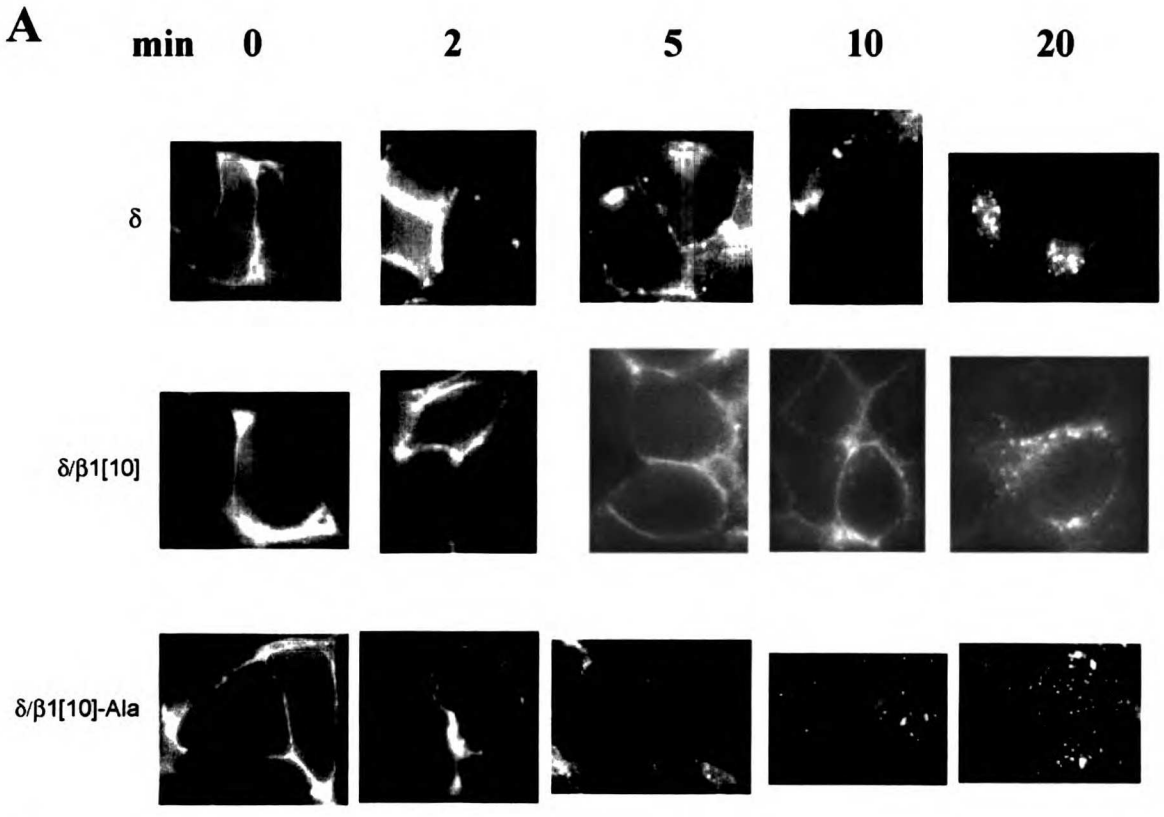
EGFP β-arrestin 2 (arrestin 3). Cells were pre-incubated for 30 minutes with M1 anti-FLAG antibody directly conjugated to Alexa 594 before treatment with 10 μM DADLE for the indicated times. Cells were immediately fixed before cell surface receptor and EGFP β-arrestin 2 visualization by TIRF microscopy.

Figure 5

Model of initial steps in G protein-coupled receptor endocytosis showing the step inhibited by certain class I PDZ ligands. The PDZ ligands from the β_1AR, CFTR, and a point mutant of the β_2AR that disrupts NSF binding are all capable of decreasing the amount of δ OR endocytosed following 10 minutes of agonist exposure. Following a longer agonist treatment of 25 minutes, the δ OR is internalized to an extent similar to that of the wild-type δ OR. Clustering of the chimeric receptors into clathrin coated pits seems to be delayed relative to clustering of the wild-type δ OR. Further, although receptor clustering seems to be affected, membrane recruitment and clustering of β-arrestin appears normal in cells transfected with these receptors. This demonstrates that β-arrestin recruitment and clustering may be a separable event from clustering of receptors into clathrin coated pits.

Figures

Figure1



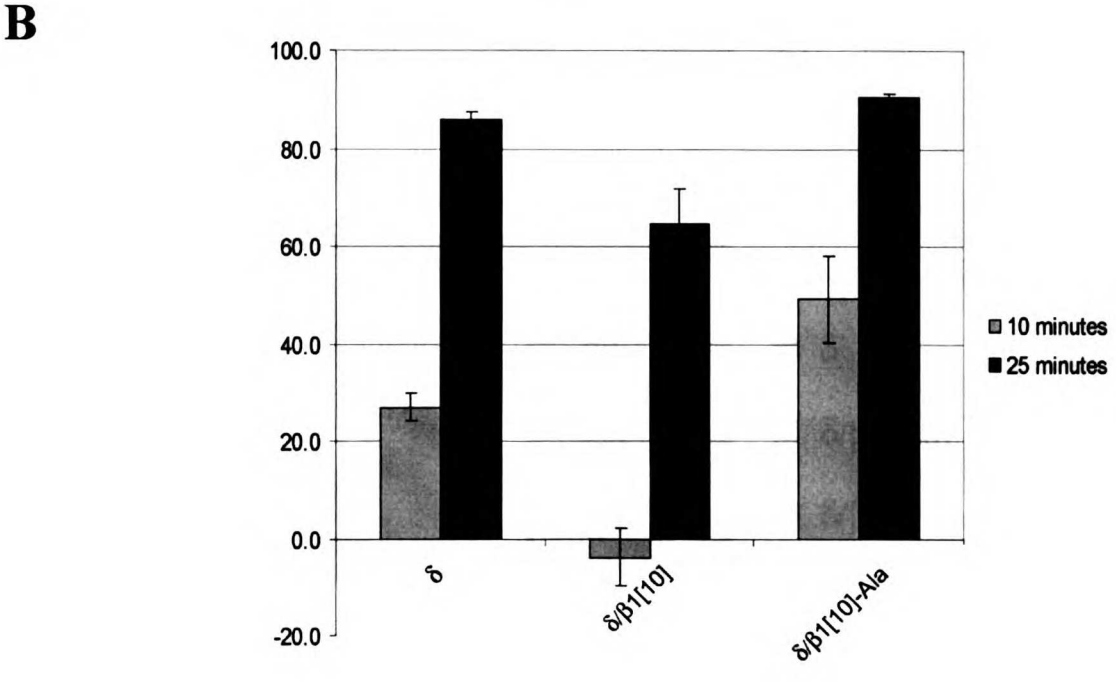
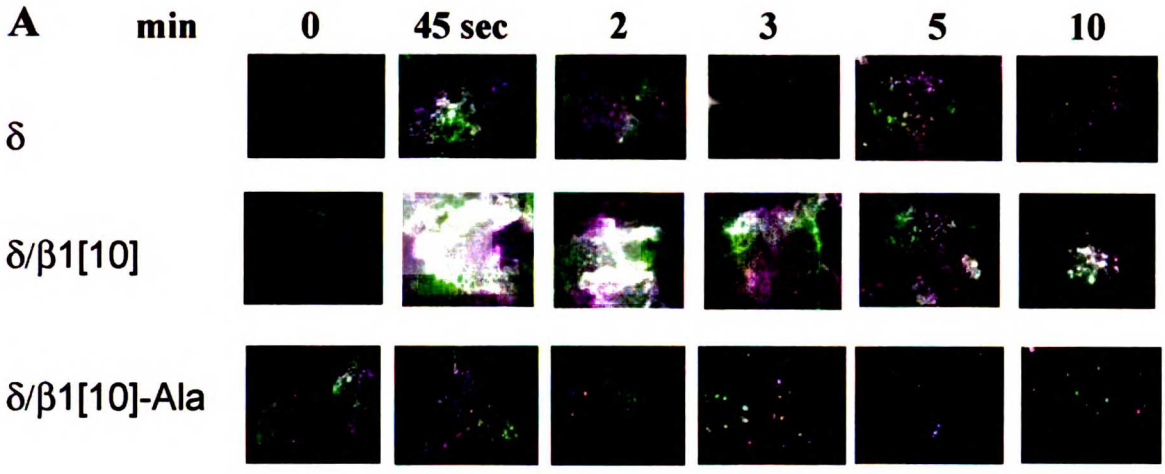
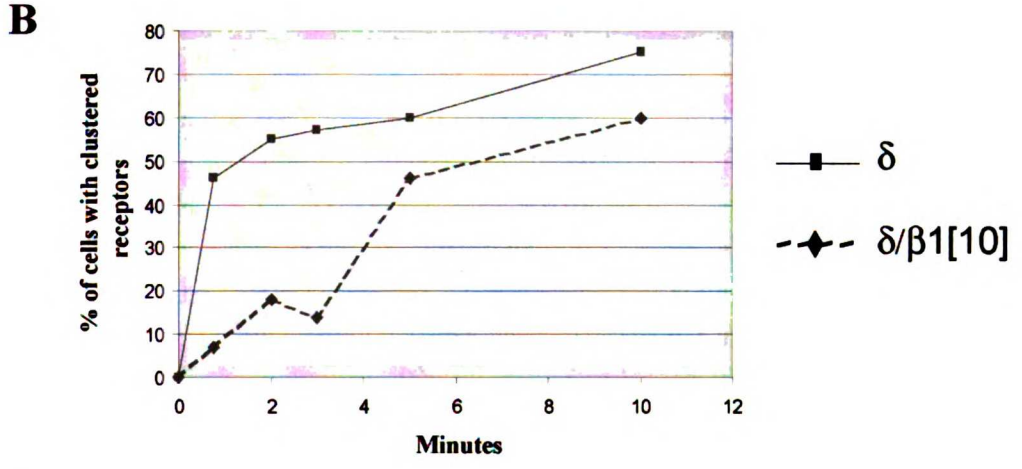


Figure 2


A
min
0
45 sec
2
3
5
10
δ
δ/β1[10]
δ/β1[10]-Ala


B
% of cells with clustered receptors
Minutes
δ
δ/β1[10]

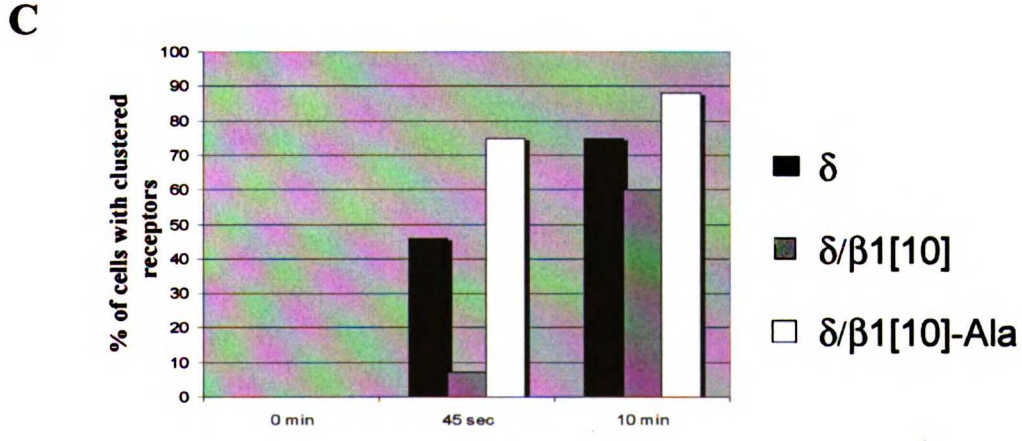

C
% of cells with clustered receptors
0 min
45 sec
10 min
δ
δ/β1[10]
δ/β1[10]-Ala

Figure 3

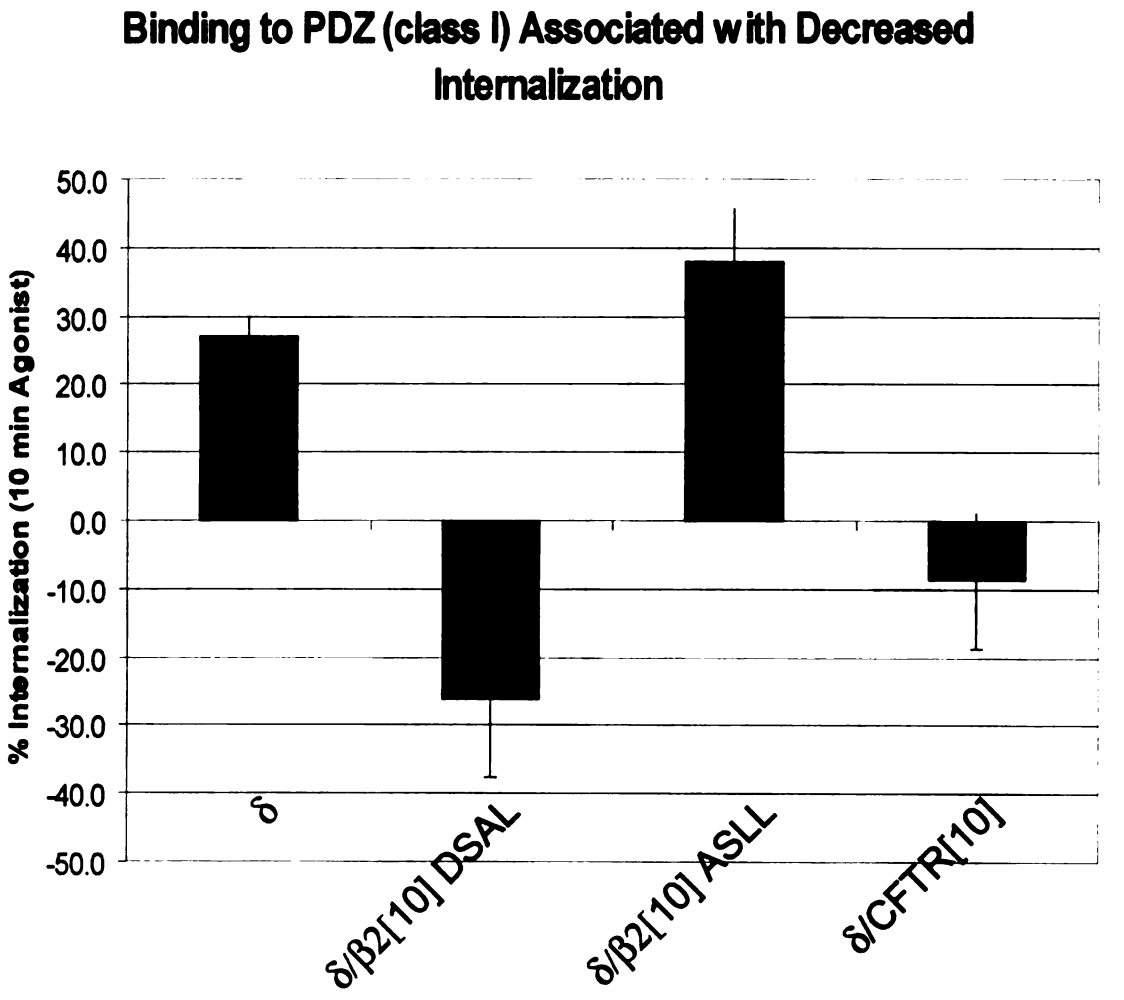
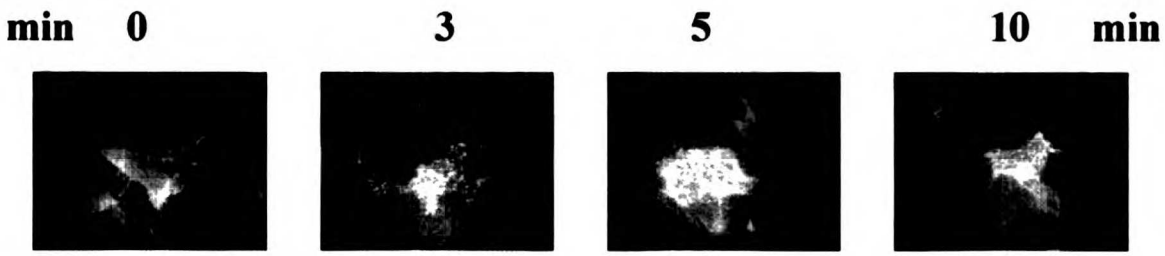
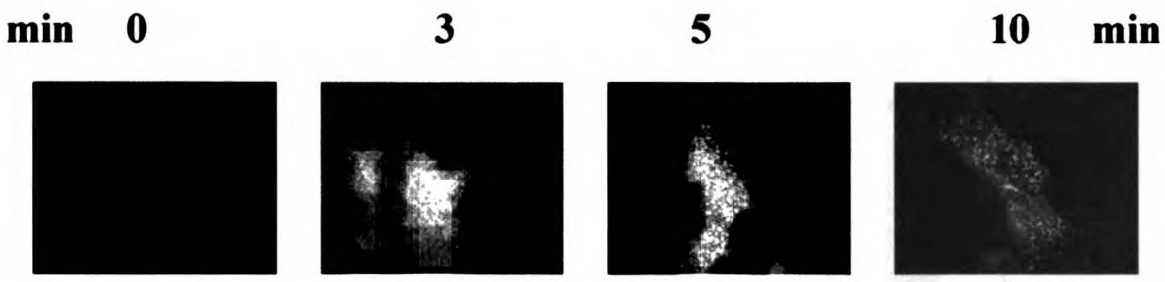

Binding to PDZ (class I) Associated with Decreased Internalization
% Internalization (10 min Agonist)
50.0
40.0
30.0
20.0
10.0
0.0
-10.0
-20.0
-30.0
-40.0
-50.0
δ
δ/β2[10] DSAL
δ/β2[10] ASLL
δ/CFTR[10]

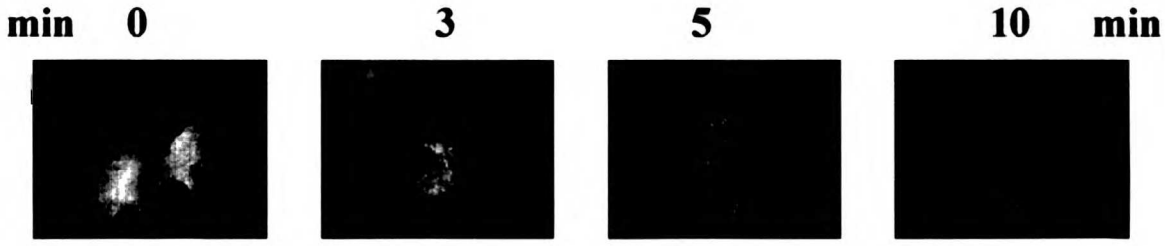
Figure 4



δ (red)
β-arrestin (green)



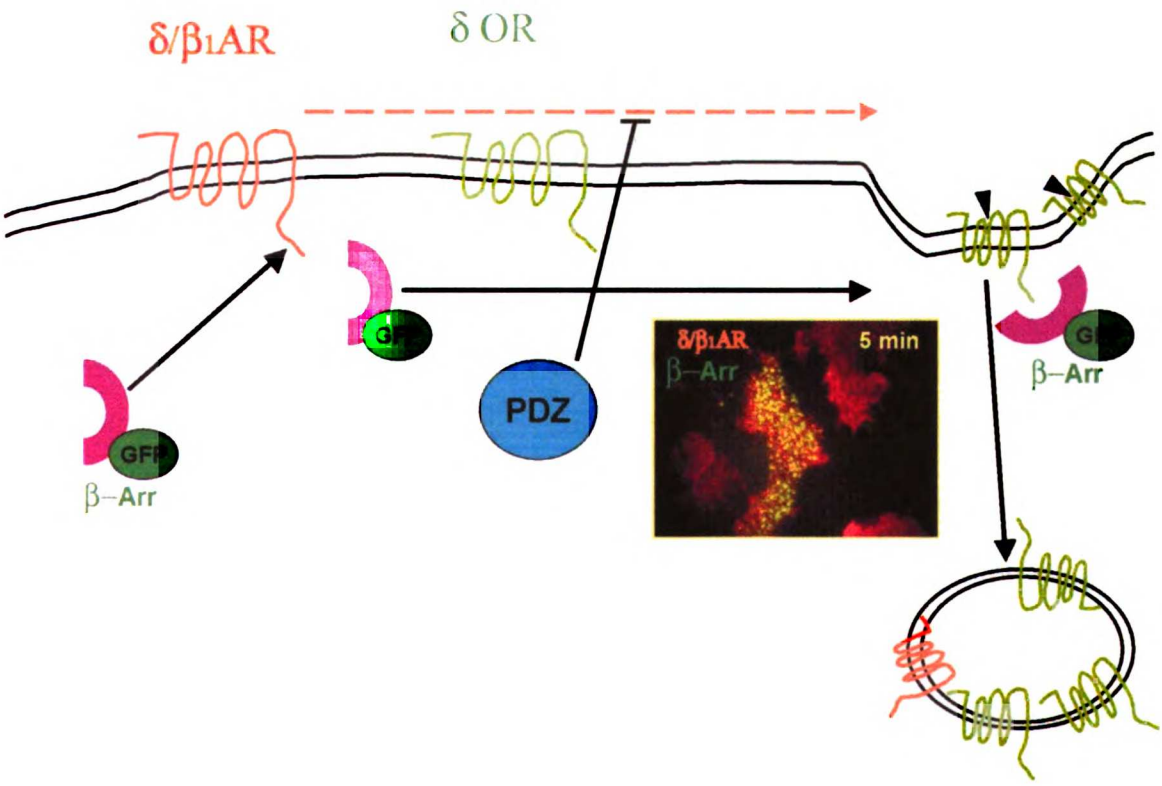
δ/β1[10] (red)
β-arrestin (green)



δ/β1[10]-Ala (red)
β-arrestin (green)

Figure 5

Certain Class I PDZ Domain Containing Proteins can Inhibit Internalization of Membrane Receptors by Limiting Receptor Clustering into Clathrin Coated Pits



Chapter 5: Overall Conclusions

Robert Michael Gage

Chapter 5: Overall Conclusions

Many membrane proteins and bulk lipid traverse the endocytic pathway and rapidly recycle back to the plasma membrane without the need for a specific cytosolic signal or protein interaction (1-3). For example, the transferrin (tfn) receptor rapidly recycles to the plasma membrane even when stripped of all cytoplasmic amino acid residues (4,5). Certain fluorescently labeled lipids also recycle rapidly to the plasma membrane with similar kinetics to those exhibited by the transferrin receptor (6). So it was an unexpected result when the lab discovered that certain G protein-coupled receptors (GPCRs) contain "recycling signals" within their carboxyl terminal tails that are required for their proper membrane trafficking (7-10). So far the β_2 adrenergic receptor (β_2AR), β_1 adrenergic receptor (β_1AR), cystic fibrosis transmembrane conductance regulator (CFTR), and μ opioid receptor (μ OR) have been found to contain such peptide recycling motifs. We determined that the peptide sequences were sufficient to function outside the context of their cognate receptor and positively affected the recycling efficiency of the δ OR (8-10). It is possible that other GPCRs also contain such recycling sequences and future experiments will elucidate which receptors require specific recycling sequences in order to recycle. This information will be critical in determining the machinery responsible for signal mediated recycling.

Three of these recycling sequences, those from the β_2AR, β_1AR, and CFTR, correspond to class I PDZ ligands. Although it has been reported that direct receptor interaction with N-ethyl maleimide sensitive factor (NSF) is responsible for the enhanced recycling of the β_2AR (11), we found that instead PDZ domain mediated interactions had

the dominant effect on receptor recycling (9). In fact for the β_1AR and CFTR, which is not a GPCR but a 12 membrane spanning Cl- channel, there are no detectable interactions with NSF but both membrane proteins have a robust interaction with PDZ domain containing proteins (9,12). PDZ domain mediated interactions have been implicated in the formation and stabilization of numerous signaling complexes at the cell surface (13-18). PDZ domain mediated interactions have also been implicated in anchoring the β_1AR to the plasma membrane (19,20). Many PDZ domain containing proteins are localized to the cell periphery directly or indirectly by interaction with cortical actin. Enhancing recycling efficiency could be due simply to retaining vesicles containing receptors which bind PDZ domain containing proteins near the periphery of the cell where they could recycle rapidly back to the plasma membrane.

Further work to study the mechanism of PDZ domain mediated recycling could lead to pharmaceutical agents to disrupt or enhance receptor recycling. Enhancing the recycling efficiency of a GPCR could reduce tolerance, the process in which increasing doses of receptor agonist is required in order to elicit the same response. In cases where a prolonged decrease in signaling (down-regulation) is desired, blocking receptor recycling with a small molecule inhibitor would be advantageous. We have begun investigations of inhibiting the recycling of the β_2AR using a small molecule PDZ inhibitor (21,22). The inhibitor decreases the recycling efficiency of the β_2AR by 20-40% in the short term, but does not seem to affect long term receptor protein levels (see Appendix 1). A drug which increases degradation of the β_2AR in addition to inhibiting recycling would be required in order for it to act as a long acting β blocker.

As mentioned above, PDZ domain mediated interactions can also modulate the internalization of GPCRs such as the β_1AR (19,20). We found that PDZ binding sequences from the membrane proteins β_1AR and CFTR are sufficient to inhibit the initial internalization of the δ OR. Point mutants of the β_2AR which fail to bind NSF yet retain binding to the PDZ domain containing protein hNHERF / EBP50 also show a decreased internalization (11). Wild type β_2AR shows significant internalization following agonist treatment. This suggested that NSF may play a role in increasing the internalization rate of those receptors which bind to PDZ domain containing proteins at the cell surface. NSF, in a manner analogous to its ATP dependent function of separating SNARE (soluble NSF attachment protein receptor) proteins, may interrupt PDZ mediated interactions at the cell surface to facilitate receptor internalization. NSF and hNHERF / EBP50 interactions are competitive (11) and NSF's ability to accelerate receptor internalization may not be energy dependent. Experiments conducted to determine if the β_2AR is a substrate for NSF have so far shown no requirement for either α-SNAP (soluble NSF attachment protein) or ATP (S.W. Whiteheart personal communication). Interfering with either the PDZ domain mediated or NSF interaction with membrane receptors may present yet another avenue for pharmacological intervention in the membrane trafficking of select GPCRs. Altering the initial internalization rate may significantly affect signaling through the receptor. Many receptors, such as the β_2AR (23), are thought to require a trip through the endocytic and recycling compartments in order to be de-phosphorylated and functionally resensitized.

We investigated the mechanism of PDZ domain mediated inhibition of internalization and were surprised to find an inversion in the previously described order

of events in GPCR internalization in clathrin coated pits. Previous work suggested that following agonist stimulation, GPCRs become phosphorylated by GRKs (GPCR kinases), recruit β-arrestin to the surface, and then receptors and arrestin become clustered in clathrin coated pits at the cell surface (24-29). β-arrestin molecules bind to activated phosphorylated G protein coupled receptors and are thought to simultaneously bind clathrin and / or AP-2 (adapter protein 2), molecules present in clathrin coated structures at the plasma membrane that serve as foci of internalization (30-36). We found that receptors which bind to certain PDZ domain containing proteins were able to recruit β-arrestin to the plasma membrane and that the arrestin would cluster into clathrin coated structures without visible clustering of receptors. This interesting finding suggests a change is needed in the model of β-arrestin mediated receptor internalization.

Future Directions

Transplantable sorting signal and hierarchy of sorting sequences

We were able to reroute the δ opioid receptor from a degradative non-recycling pathway to a recycling pathway by simply appending the last ten residues of the β_2AR, β_1AR, and the CFTR to the carboxyl terminus of the δ OR (8,9). The delta opioid receptor can also be made to recycle when the last seventeen residues of the μ OR are added to its carboxyl terminal tail (10). However, when the recycling sequence from the β_2AR is appended to carboxyl terminus of another GPCR, the V2 vasopressin receptor, the chimeric V2R does not rapidly recycle and exhibits behavior indistinguishable from wild-type V2R (Appendix 2). The V2R contains a ser / thr cluster that is associated with the receptor's ability to retain binding to β arrestin for prolonged periods after endocytosis and slowly recycling back to the plasma membrane ($t_{1/2}$ > 2 hr) (37-39). Thus there appears to be a hierarchy of signals within GPCRs with some being dominant to others.

It would be interesting to see if the recycling sequences from GPCRs other than the β_2AR are capable of inducing the V2R to rapidly recycle. The sequences from the β_2AR, β_1AR, and CFTR are all class I PDZ ligands and may affect the behavior of the V2R differently than the peptide recycling sequence from the μ OR. This would allow us to determine whether the slow recycling ser / thr cluster is dominant to all recycling sequences so far examined. Such information would be useful in developing a physical model of the mechanism(s) behind signal mediated recycling.

There are a number of other GPCRs which do not rapidly recycle or whose trafficking itinerary is unknown. It would be useful to know how broadly these recycling sequences can function when placed in the context of other GPCRs. It would also be interesting to see if they could function in the context of a different class of membrane protein, such as a single membrane spanning RTK (receptor tyrosine kinase).

Type I PDZ ligands are sufficient to promote recycling

We were able to show that several class I PDZ ligands were sufficient to promote the recycling of the δ opioid receptor when appended at the distal carboxyl terminus (8,9). These PDZ ligands interact with PDZ domain containing proteins known to be localized to the cortex of the cell (12,19,20,40,41). In contrast, the one class II PDZ ligand we tested did not confer a rapidly recycling phenotype on the δ OR. There are many PDZ domain containing proteins expressed in the human genome and not all would be expected to influence the recycling of membrane proteins. Chimeric δ ORs could be constructed which contain a more representative mixture of PDZ ligands from a variety of classes and tested for their ability to increase the recycling efficiency of the δ OR. Such an investigation work help to determine the mechanism behind the PDZ domain mediated recycling. We have suggested that locally retaining receptor containing vesicles near the cortex of the cell could act to increase their recycling efficiency. Correlating PDZ ligand : PDZ domain containing protein localization within the cell with recycling efficiency could strengthen this argument.

There is evidence that an intact actin cytoskeleton is essential for the rapid recycling of the β_2AR (7). Disruption of the actin network using latrunculin caused the recycling efficiency of the β_2AR to be significantly impaired (7). The known β_2AR binding partner which affects receptor recycling, the PDZ domain containing protein hNHERF1 / EBP 50, has an ERM (Ezrin / Radixin / Moesin) binding domain which links it to the actin cytoskeleton through an intermediary protein (42-45). It would be informative to see if binding to the actin cytoskeleton through a different mechanism would be sufficient to promote the recycling of a GPCR. Preliminary evidence from Gabriel Vargus would suggest that this is indeed the case. A chimeric δ OR which contains the actin binding domain from ezrin was constructed and found to recycle more efficiently than wild-type δ OR when expressed in HEK 293 cells.

Certain class I PDZ ligands can inhibit internalization

We have observed that certain class I PDZ ligands can inhibit the internalization of the δ OR when appended to the carboxyl terminus (Chapter 4). Not all of the PDZ ligands, however, had this effect. Notably, the ligand from the β_2AR did not inhibit the internalization of the δ OR. This sequence is sufficient to bind to both the PDZ domain containing protein hNHERF1 / EBP50 and also to NSF (N-ethyl maleimide sensitive factor) (11,12,40,41). Point mutations to the wild-type sequence from the β_2AR were found which differentially bind to hNHERF1 / EBP50 and NSF (11). We found that the sequence which selectively binds only the PDZ domain containing protein hNHERF1 / EBP 50 also inhibits the internalization of the δ OR. This suggested that direct binding to

certain class I PDZ domain containing proteins could inhibit internalization and that direct binding to NSF could release this inhibition.

Further studies are necessary to investigate NSF's role in releasing the PDZ domain mediated endocytic brake. Since both NSF and hNHERF1 / EBP 50 bind to the distal carboxyl tail of the β_2AR, NSF's ability to facilitate the internalization of the β_2AR could be due to a simple competition between the two proteins for the tail sequence. NSF is an ATPase which has been implicated in the disassembly of entangled SNAREs (soluble NSF attachment protein receptors) molecules so they can be reused (46-52). The β_2AR may be a substrate for NSF in an ATP dependent manner analogous to NSF's role in SNARE complex disassembly. NSF has been reported to bind and disassemble non-SNARE complexes such as the AMPA receptor subunit GluR2 and its PDZ domain containing partner PICK1 (53-58). Preliminary results with purified NSF, α–SNAP (soluble NSF attachment protein), and the β_2AR tail would suggest that the adrenergic receptor tail is not a substrate for NSF's ATPase. But further studies in an *in vivo* situation with full length receptor may be necessary to rule out NSF's ATPase activity in releasing the endocytic brake.

Further studies to investigate the generality of class I PDZ ligands as endocytic brakes could be useful in uncovering the molecular mechanism of PDZ domain mediated inhibition of endocytosis. The PDZ ligands investigated so far are associated with PDZ domain containing proteins which are localized near the plasma membrane and this localization may be a key factor in their ability to act as an endocytic brake. The necessity of the link to the plasma membrane could also be investigated by artificially linking a receptor to the actin cortex either directly or indirectly through ERM proteins.

Small molecule inhibition of recycling

Small molecule inhibitors of PDZ domain mediated interactions were designed, synthesized, and shown to disrupt PDZ domain : PDZ ligand interactions (21,22). We tested whether these inhibitors could disrupt the PDZ domain mediated recycling of the β_2AR and various chimeric receptors (Appendix 1). It appears that the small molecule inhibitors are capable of modestly reducing the recycling efficiency of the β_2AR after a 30 minute pre-treatment with the inhibitor. Further refinements in the chemical structure of the PDZ inhibitor could yield better biological inhibition. Also, a more complete dose response analysis of the current inhibitors would give a better estimate of the EC_{50} and efficacy for inhibiting β_2AR recycling.

Another interesting observation noted while using these inhibitors was that although acute, 30 minute pre-treatment, doses of the FJ-1 inhibitor did not significantly affect the recycling efficiency of the chimeric δ/β_2AR receptor, overnight doses of FJ-1 did. Overnight doses of FJ-1 did not affect the recycling of the wild-type β_2AR, while shorter exposures did. We tested for the simple explanation that the switch in behavior of the drug was due to metabolism of the parent FJ-1 compound. Unfortunately, this simple scenario does not seem to be the case. An HPLC (high performance liquid chromatography) and MS (mass spectrometry) analysis of cell culture media containing FJ-1 before and after 18 hour in the presence of HEK-293 cells shows no significant metabolite formation or loss of parent (~50 % loss after 24 hours). This apparent loss of parent may be explained by accumulation of inhibitor within the treated cells since we did not lyse the cells incubated overnight with FJ-1. A metabolite of FJ-1 may also be

retained within the cells and not show in the supernatant. These suppositions could be tested and verified.

The inhibitors were also tested for binding to immobilized GST conjugated β_2AR tail as well as to over-expressed hNHERF1 / EBP 50 and endogenous and recombinant NSF. The inhibitor which showed the greatest inhibition of β_2AR recycling, FJ-1, did not bind strongly to the PDZ domain containing protein hNHERF1 / EBP 50. It did however bind strongly to NSF, suggesting at least a partial role for direct receptor binding to NSF in the recycling of the β_2AR. Two other related inhibitors, FJ-3 and FJ-7, did bind to hNHERF1 / EBP50 although they did not inhibit the recycling of the β_2AR. It is possible that another yet to be identified PDZ domain containing protein with overlapping ligand specificity with hNHERF1 / EBP50 could be responsible for the rapid recycling phenotype of the β_2AR. Studies have begun looking at the PDZ domain containing protein mrt1a as a possible candidate for investigation.

Motility differences between β_2AR and δ OR containing vesicles

We investigated the possibility of differences in the motility of receptor containing endosomes dependent on their cargo. We observed that in general, in HEK 293 cells expressing the δ OR, there was a population of rapidly moving vesicles not observed in cells expressing the β_2AR (Appendix 3). These observations, while not seen in every experiment, were relatively consistent across several experiments. In the future, the motility of vesicles containing various receptors could be quantified and tested for statistically significant differences. Specifically, the velocities and processivity of

receptor containing vesicles could be determined and compared. To aid in the detection and tracking or receptor containing vesicles, a more sophisticated camera could be employed to capture vesicular motion. One of the limits of using the SIT camera is increased noise levels as compared to CCD cameras. The SIT camera has an advantage in speed, video-rate detection (30 fps) and read-out. However, when integration of the raw video image is taken into account (typically ten video frames averaged per final image) newer CCD cameras can easily match this speed and with improved signal to noise ratios and much better spatial resolution.

References

1. Trowbridge, I. S., Collawn, J. F., and Hopkins, C. R. (1993) *Annual Review of Cell Biology* **9**, 129-161
2. Gruenberg, J. (2001) *Nat Rev Mol Cell Biol.* **2**, 721-730
3. Gruenberg, J., and Maxfield, F. R. (1995) *Current Opinion in Cell Biology* **7**, 552-563
4. Jing, S. Q., Spencer, T., Miller, K., Hopkins, C., and Trowbridge, I. S. (1990) *J Cell Biol* **110**, 283-294
5. McGraw, T. E., and Maxfield, F. R. (1990) *Cell Regul* **1**, 369-377
6. Mayor, S., Presley, J. F., and Maxfield, F. R. (1993) *Journal of Cell Biology* **121**, 1257-1269
7. Cao, T. T., Deacon, H. W., Reczek, D., Bretscher, A., and von Zastrow, M. (1999) *Nature* **401**, 286-290.
8. Gage, R. M., Kim, K. A., Cao, T. T., and von Zastrow, M. (2001) *J Biol Chem* **276**, 44712-44720.
9. Gage, R. M., Matveeva, E. A., Whiteheart, S. W., and von Zastrow, M. (2005) *J. Biol. Chem.* **280**, 3305-3313
10. Tanowitz, M., and von Zastrow, M. (2003) *J. Biol. Chem.* **278**, 45978-45986
11. Cong, M., Perry, S. J., Hu, L. A., Hanson, P. I., Claing, A., and Lefkowitz, R. J. (2001) *J. Biol. Chem.* **276**, 45145-45152

12. Hall, R. A., Ostedgaard, L. S., Premont, R. T., Blitzer, J. T., Rahman, N., Welsh, M. J., and Lefkowitz, R. J. (1998) *Proceedings of the National Academy of Sciences of the United States of America* **95**, 8496-8501

13. Harris, B. Z., and Lim, W. A. (2001) *J Cell Sci* **114**, 3219-3231

14. Tiffany, A. M., Manganas, L. N., Kim, E., Hsueh, Y. P., Sheng, M., and Trimmer, J. S. (2000) *J Cell Biol* **148**, 147-158

15. Sheng, M., Sala, C. (2001) *Annu Rev Neurosci* **24**, 1-29

16. Sheng, M., and Kim, E. (2000) *J Cell Sci* **113 (Pt 11)**, 1851-1856

17. Naisbitt, S., Valtschanoff, J., Allison, D. W., Sala, C., Kim, E., Craig, A. M., Weinberg, R. J., and Sheng, M. (2000) *J Neurosci* **20**, 4524-4534

18. Wang, S., Yue, H., Derin, R. B., Guggino, W. B., and Li, M. (2000) *Cell* **103**, 169-179.

19. Hu, L. A., Tang, Y., Miller, W. E., Cong, M., Lau, A. G., Lefkowitz, R. J., and Hall, R. A. (2000) *J. Biol. Chem.* **275**, 38659-38666

20. Xu, J., Paquet, M., Lau, A. G., Wood, J. D., Ross, C. A., and Hall, R. A. (2001) *J. Biol. Chem.* **276**, 41310-41317

21. Fujii, N., Haresco, J. J., Novak, K. A., Stokoe, D., Kuntz, I. D., and Guy, R. K. (2003) *J Am Chem Soc* **125**, 12074-12075

22. Novak, K. A., Fujii, N., and Guy, R. K. (2002) *Bioorg Med Chem Lett* **12**, 2471-2474

23. Hausdorff, W. P., Caron, M. G., and Lefkowitz, R. J. (1990) *FASEB J* **4**, 2881-2889

24. Bennett, N., and Sitaramayya, A. (1988) *Biochemistry* **27**, 1710-1715

25. Benovic, J. L., Kuhn, H., Weyand, I., Codina, J., Caron, M. G., and Lefkowitz, R. J. (1987) *Proc Natl Acad Sci U S A* **84**, 8879-8882

26. Ferguson, S. S., Barak, L. S., Zhang, J., and Caron, M. G. (1996) *Can J Physiol Pharmacol* **74**, 1095-1110

27. Krupnick, J. G., and Benovic, J. L. (1998) *Annual Review of Pharmacology and Toxicology* **38**, 289-319

28. Lefkowitz, R. J. (1998) *Journal of Biological Chemistry* **273**, 18677-18680

29. Zhang, J., Ferguson, S. S., Barak, L. S., Aber, M. J., Giros, B., Lefkowitz, R. J., and Caron, M. G. (1997) *Receptors and Channels* **5**, 193-199

30. Goodman, O. J., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1996) *Nature* **383**, 447-450

31. Krupnick, J. G., Santini, F., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1997) *J Biol Chem* **272**, 32507-32512

32. Krupnick, J. G., Goodman, O. J., Keen, J. H., and Benovic, J. L. (1997) *J Biol Chem* **272**, 15011-15016

33. Laporte, S. A., Oakley, R. H., Holt, J. A., Barak, L. S., and Caron, M. G. (2000) *J Biol Chem* **275**, 23120-23126

34. Lin, F. T., Krueger, K. M., Kendall, H. E., Daaka, Y., Fredericks, Z. L., Pitcher, J. A., and Lefkowitz, R. J. (1997) *J Biol Chem* **272**, 31051-31057

35. Oakley, R. H., Laporte, S. A., Holt, J. A., Barak, L. S., and Caron, M. G. (1999) *Journal of Biological Chemistry* **274**, 32248-32257

36. Zhang, J., Barak, L. S., Winkler, K. E., Caron, M. G., and Ferguson, S. S. (1997) *J Biol Chem* **272**, 27005-27014

37. Innamorati, G., Sadeghi, H., and Birnbaumer, M. (1999) *Journal of Receptor and Signal Transduction Research* **19**, 315-326

38. Innamorati, G., Le Gouill, C., Balamotis, M., and Birnbaumer, M. (2001) *Journal of Biological Chemistry* **276**, 13096-13103

39. Innamorati, G., Sadeghi, H. M., Tran, N. T., and Birnbaumer, M. (1998) *Proceedings of the National Academy of Sciences of the United States of America* **95**, 2222-2226

40. Hall, R. A., Premont, R. T., Chow, C. W., Blitzer, J. T., Pitcher, J. A., Claing, A., Stoffel, R. H., Barak, L. S., Shenolikar, S., Weinman, E. J., Grinstein, S., and Lefkowitz, R. J. (1998) *Nature* **392**, 626-630

41. Hall, R. A. (2004) *Semin Cell Dev Biol* **15**, 281-288

42. Reczek, D., Berryman, M., and Bretscher, A. (1997) *J Cell Biol* **139**, 169-179

43. Weinman, E. J., and Shenolikar, S. (1997) *Exp Nephrol* **5**, 449-452

44. Weinman, E. J., Steplock, D., Wang, Y., and Shenolikar, S. (1995) *J Clin Invest* **95**, 2143-2149

45. Voltz, J. W., Weinman, E. J., and Shenolikar, S. (2001) *Oncogene* **20**, 6309-6314

46. Whiteheart, S. W., Schraw, T., and Matveeva, E. A. (2001) *Int Rev Cytol* **207**, 71-112

47. May, A. P., Whiteheart, S. W., and Weis, W. I. (2001) *J Biol Chem* **276**, 21991-21994

48. Whiteheart, S. W., and Kubalek, E. W. (1995) *Trends Cell Biol* **5**, 64-68

49. Matveeva, E., and Whiteheart, S. W. (1998) *FEBS Lett* **435**, 211-214

50. Sollner, T., Bennett, M. K., Whiteheart, S. W., Scheller, R. H., and Rothman, J. E. (1993) *Cell* **75**, 409-418

51. Weber, T., Parlati, F., McNew, J. A., Johnston, R. J., Westermann, B., Sollner, T. H., and Rothman, J. E. (2000) *J Cell Biol* **149**, 1063-1072

52. Nichols, B. J., and Pelham, H. R. (1998) *Biochim Biophys Acta* **1404**, 9-31

53. Whiteheart, S. W., and Matveeva, E. A. (2004) *J Struct Biol* **146**, 32-43

54. Braithwaite, S. P., Xia, H., and Malenka, R. C. (2002) *Proc Natl Acad Sci U S A* **99**, 7096-7101

55. Hanley, J. G., Khatri, L., Hanson, P. I., and Ziff, E. B. (2002) *Neuron* **34**, 53-67

56. Osten, P., and Ziff, E. B. (1999) *Ann N Y Acad Sci* **868**, 558-560

57. Osten, P., Srivastava, S., Inman, G. J., Vilim, F. S., Khatri, L., Lee, L. M., States, B. A., Einheber, S., Milner, T. A., Hanson, P. I., and Ziff, E. B. (1998) *Neuron* **21**, 99-110

58. Nishimune, A., Isaac, J. T., Molnar, E., Noel, J., Nash, S. R., Tagaya, M., Collingridge, G. L., Nakanishi, S., and Henley, J. M. (1998) *Neuron* **21**, 87-97

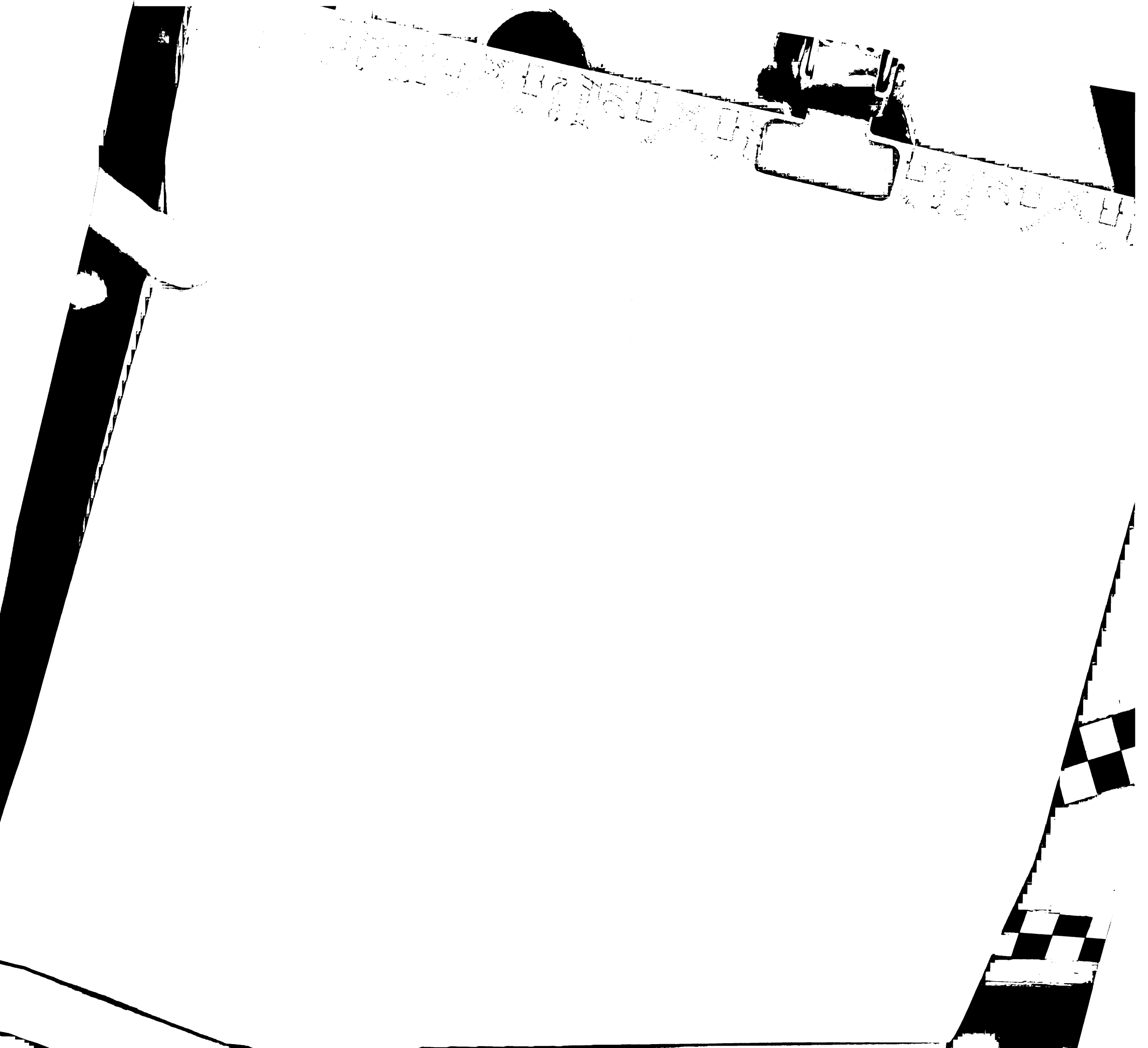
Appendix 1: Small molecule inhibition of recycling (FJ-1, FJ-3, FJ-7, and FJ-9)

All work presented in this appendix was completed by Robert Michael Gage with the exceptions noted:

(1) All synthesis of inhibitor molecules was performed by Naoaki Fuji in the R. Kip Guy lab, UCSF.

(2) All inhibitor binding and competition experiments (figures 5, 6, and 7) were performed by Naoaki Fuji in the R. Kip Guy lab, UCSF.

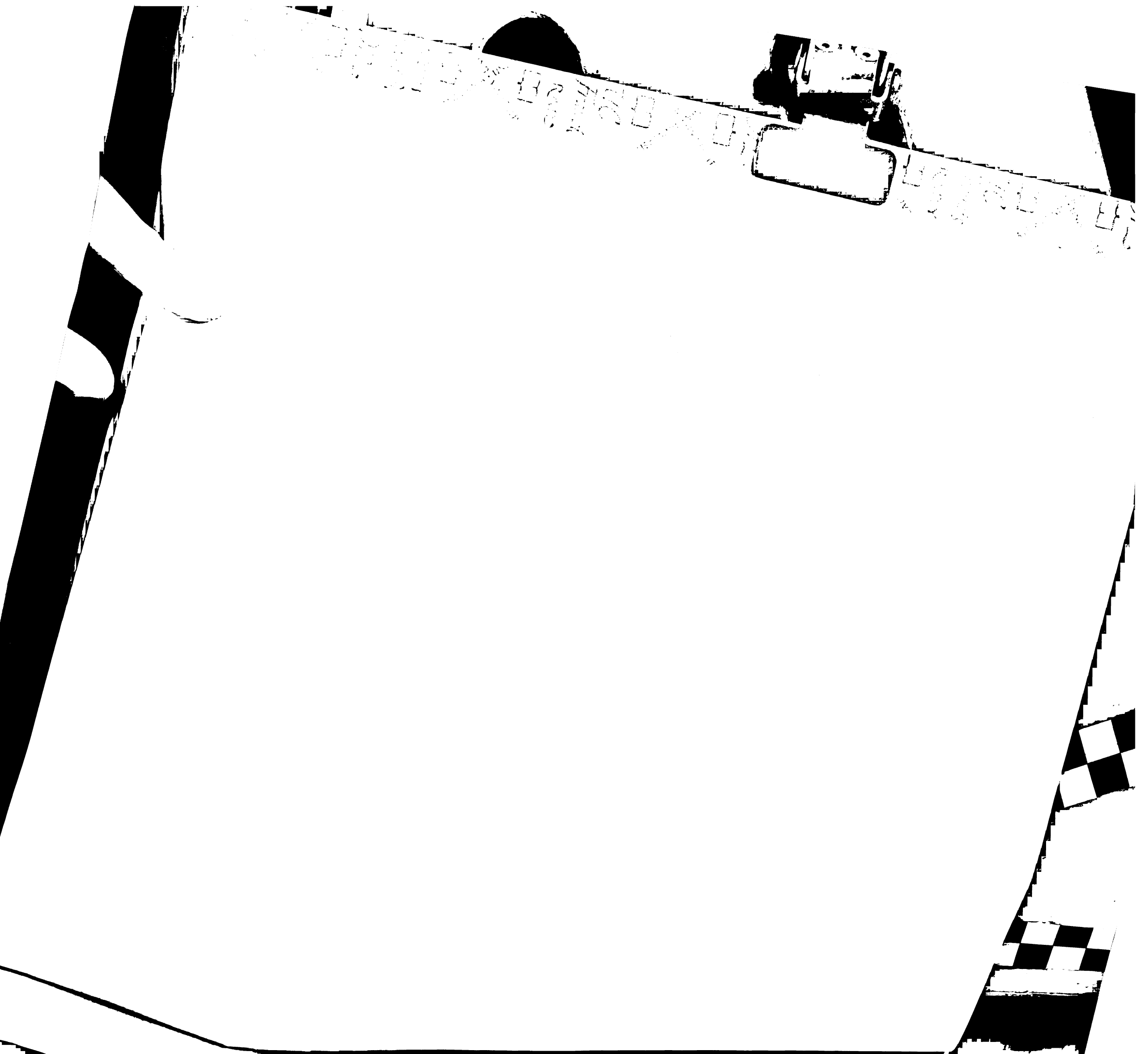
(3) LC / MS analysis of conditioned media (figure 3A) was performed by Naoaki Fuji in the R. Kip Guy lab, UCSF.



Introduction

The β_2 adrenergic receptor (β_2AR) contains a sequence within its distal carboxyl terminus that is required for the rapid return of the receptor following agonist induced internalization (1). Alterations of the carboxyl terminal sequence greatly impair the ability of the β_2AR to recycle and increase the degradation rate of the receptor (1,2). This sequence is sufficient to promote the recycling of a heterologous G protein-coupled receptor (GPCR), the δ opioid receptor (δ OR), when appended to the carboxyl terminus (3). The terminal four residues of the β_2AR constitute a canonical ligand for class I PDZ (PSD-95, Discs large, Zona Occludens homology) domain containing proteins.

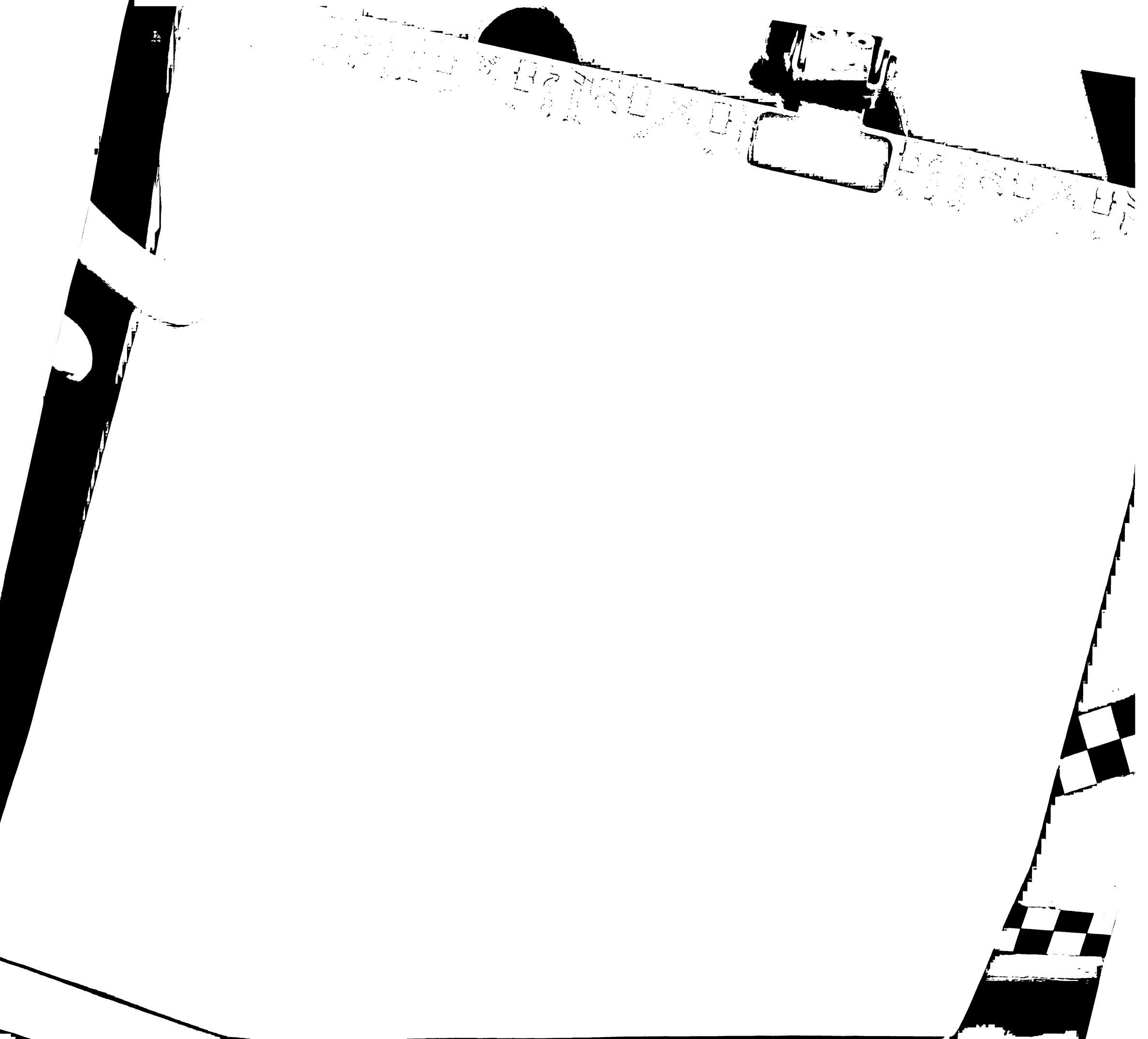
In addition to a PDZ domain mediated interaction with hNHERF1 / EBP50 (Na^+ / H^+ exchanger regulatory factor / ERM binding phosphoprotein of 50 kDa), the β_2AR also interacts with the N-ethyl maleimide sensitive factor (NSF) with the same carboxyl terminal region (2). Two point mutations within the carboxyl terminal four amino acids were found which discriminate between binding to hNHERF1 / EBP50 and NSF (2). Although it was initially reported that selective binding to NSF conferred a rapid recycling phenotype (2), further study showed that the converse was true (4). The recycling sequence from the β_2AR was appended in-frame to the carboxyl terminus of the δ OR where it was sufficient to re-route the opioid receptor from a degradative pathway to a rapidly recycling pathway (3). Placing the putative recycling sequences in the context of the δ OR, a non-recycling degrading receptor, shows the sufficiency of the sequence to confer a rapidly recycling phenotype. The results of this study (4) showed



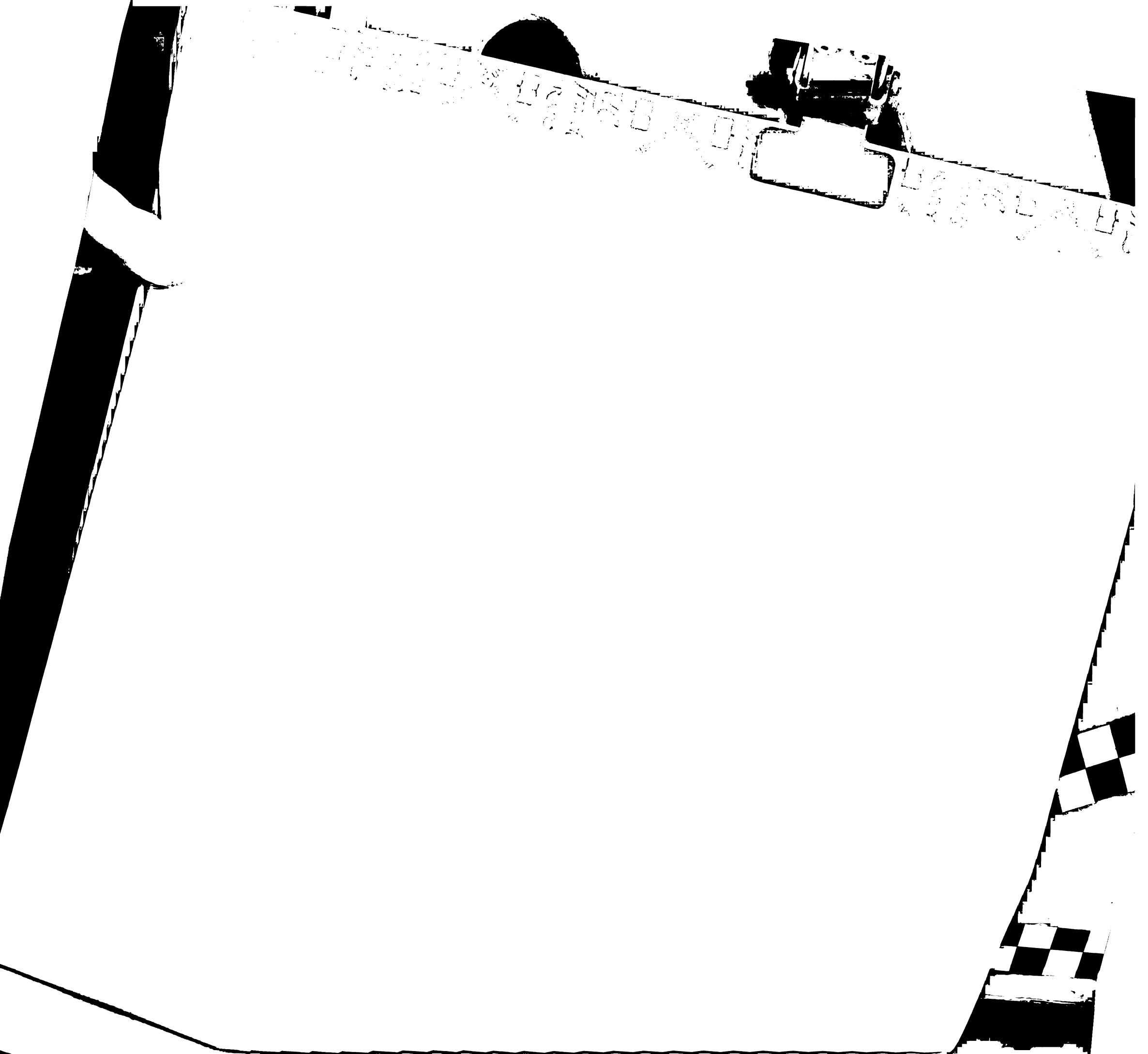
that in addition to sequences derived from the β_2AR, other class I PDZ ligands can also function as autonomous "recycling signals."

PDZ domain mediated protein interactions are believed to play a role in the formation and localization of numerous signaling complexes (5-9). They also have been reported to affect the intracellular trafficking of several membrane proteins (1,10,11). PDZ domains are a structurally similar group of protein folds, usually consisting of a relatively small ($\geq$ 90 amino acids) domain which have their carboxyl and amino termini close to one another in their folded structure (5,12-18). Thus they are highly modular and can integrate into a protein with minimal disruption to the original structure (5). They are often present in multiple copies within a single protein and each PDZ domain may have different ligand specificity (5). The structure of a PDZ domain consists of six β-strands (βA-βF) and two α-helices (αA and αB). Peptide ligands, usually the carboxyl terminal four or five residues of the PDZ binding partner, bind in a groove formed between one helix and one strand of the beta sheet in a process termed β-strand addition (5,19).

The affinity of PDZ domain containing proteins for their ligands is usually in the low micromolar (1-10 μM) range (5,20,21). This relatively weak affinity, by protein interaction standards, allows for dynamic and transient interactions among PDZ domain containing proteins and their ligands. The specificity of a PDZ domain and its cognate ligand is principally determined by the last four to five carboxyl amino acids of the ligand (5,14,22,23). This specificity and small binding surface has made it possible to rationally design small molecule inhibitors of PDZ domain mediated interactions (24). The synthesized inhibitors (see figure 1) were capable of inhibiting PDZ domain interaction in an *in vitro* fluorescent polarization assay (24,25). We sought in the present study to



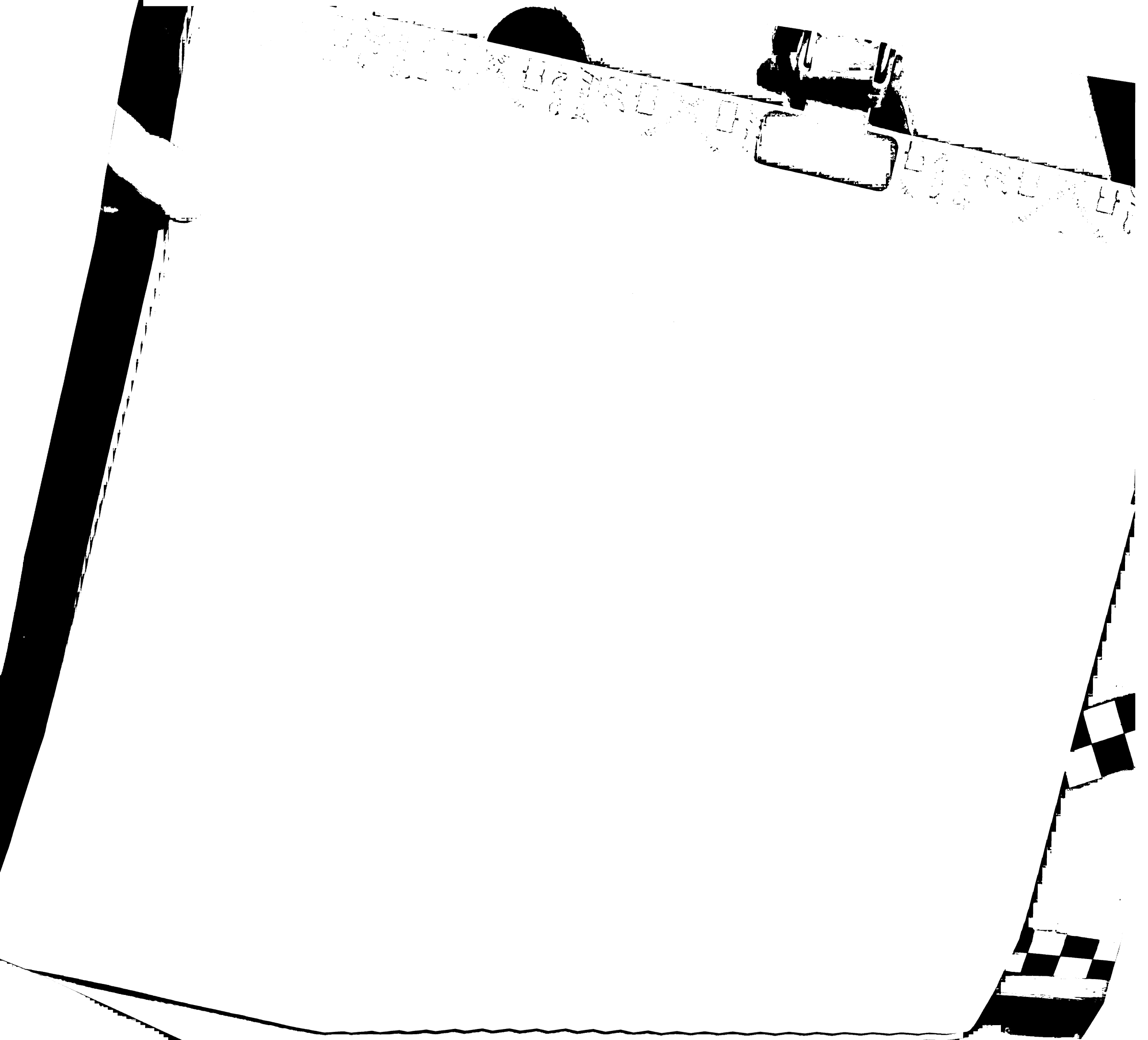
determine whether a small molecule inhibitor of the interaction between the β_2AR and its PDZ domain containing protein interaction partner could alter the trafficking of the receptor.



Experimental Procedures

cDNA Constructs and Mutagenesis

Several epitope-tagged versions of the cloned murine delta opioid receptor (δ OR (26)) and the human beta 2 adrenergic receptor (β_2AR (27)) were used in these studies: mutant receptors containing a FLAG epitope in the amino-terminal extracellular domain (SFδ OR and SFβ_2AR, respectively) were described previously and demonstrated to be functional (28-30). Mutant delta opioid receptors containing a FLAG epitope in the amino-terminal extracellular domain and the last six carboxyl-terminal cytoplasmic residues (NH_2-GGGAAA-COOH) deleted, replaced with the ten carboxyl-terminal residues from the β_1AR (NH_2-RPGFASESKV-COOH), ten carboxyl-terminal residues from the β_2AR (NH_2-RNCSTNDSLL-COOH) or the ten β_2AR residues plus an alanine (NH_2-RNCSTNDSLLA-COOH). This was accomplished by insertion of a synthetic linker-adapter (Operon Technologies) encoding the ten-residue or eleven-residue sequence followed by a stop codon into an Srf I site present near the 3' end of the sequence encoding the δ OR tail. Receptor cDNAs were cloned into pcDNA3 (Invitrogen) or pIRES (Clonetech) and all constructs were verified by dideoxynucleotide sequencing (UCSF Genetics Core Sequencing Facility). The HA tagged version of the hNHERF1 / EBP50 receptor was cloned into pcDNA3 and verified by dideoxynucleotide sequencing (UCSF Genetics Core Sequencing Facility).



Cell Culture and Transfection

Human embryonic kidney 293 cells (ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (University of California San Francisco Cell Culture Facility). Cells grown in 6-cm dishes were transfected with ~5 ug of plasmid DNA containing the indicated receptor by calcium phosphate precipitation (31,32). For studies of receptor trafficking in transiently transfected cells, cells were transfected as above, plated onto coverslips 24 h post transfection and experiments were conducted 48 h post transfection. Stably transfected cells expressing epitope tagged receptors were generated by transfecting 293 cells in 6-cm dishes as above. Cell clones expressing transfected receptors were selected in 500 ug/ml Geneticin (Life Technologies, Inc.) and colonies were isolated and selected to have similar levels of receptor expression, as estimated by radioligand binding assay conducted as described previously (31). Receptor levels in stably transfected cell lines ranged from 0.7 to 4.2 pmol/mg of total protein.

Examination of Receptor Endocytosis and Recycling by Fluorescence Microscopy

Endocytic trafficking of receptors labeled initially in the plasma membrane was visualized by fluorescence microscopy using a minor modification of a previously described method (1). Briefly, stably or transiently transfected 293 cells expressing the indicated receptor were grown on glass coverslips (Corning) treated with M1 anti-FLAG antibody (2.5 ug/ml, Sigma) directly conjugated to Alexa-488 or Alexa-594 (Molecular Probes) at 37 °C for 25 min to label receptors. The cells were treated, at 37 °C for 25 min, in the presence of 10 mM isoproterenol (Research Biochemicals) or 10 mM DADLE

([D-Ala^2, D-Leu^5 enkephalin], Research Biochemicals International). Following this incubation, cells were either fixed immediately, for determining internalization of FLAG-tagged receptors, or were subsequently washed twice in DMEM supplemented with 10% fetal bovine serum. After washing in DMEM, the cells were further incubated in DMEM (an additional 45 min. at 37°C) to allow receptor recycling to occur before fixation. The cells were fixed with 3.7% formaldehyde in PBS, pH 7.4, for 10 min and then quenched with three washes of TBS with 1 mM CaCl2. Conventional fluorescence microscopy was performed using inverted Nikon Diaphot microscope equipped with a Nikon 60X NA1.4 objective and epifluorescence optics. Images were collected using a 12-bit cooled charge-coupled device camera (Princeton Instruments) interfaced to a Macintosh computer.

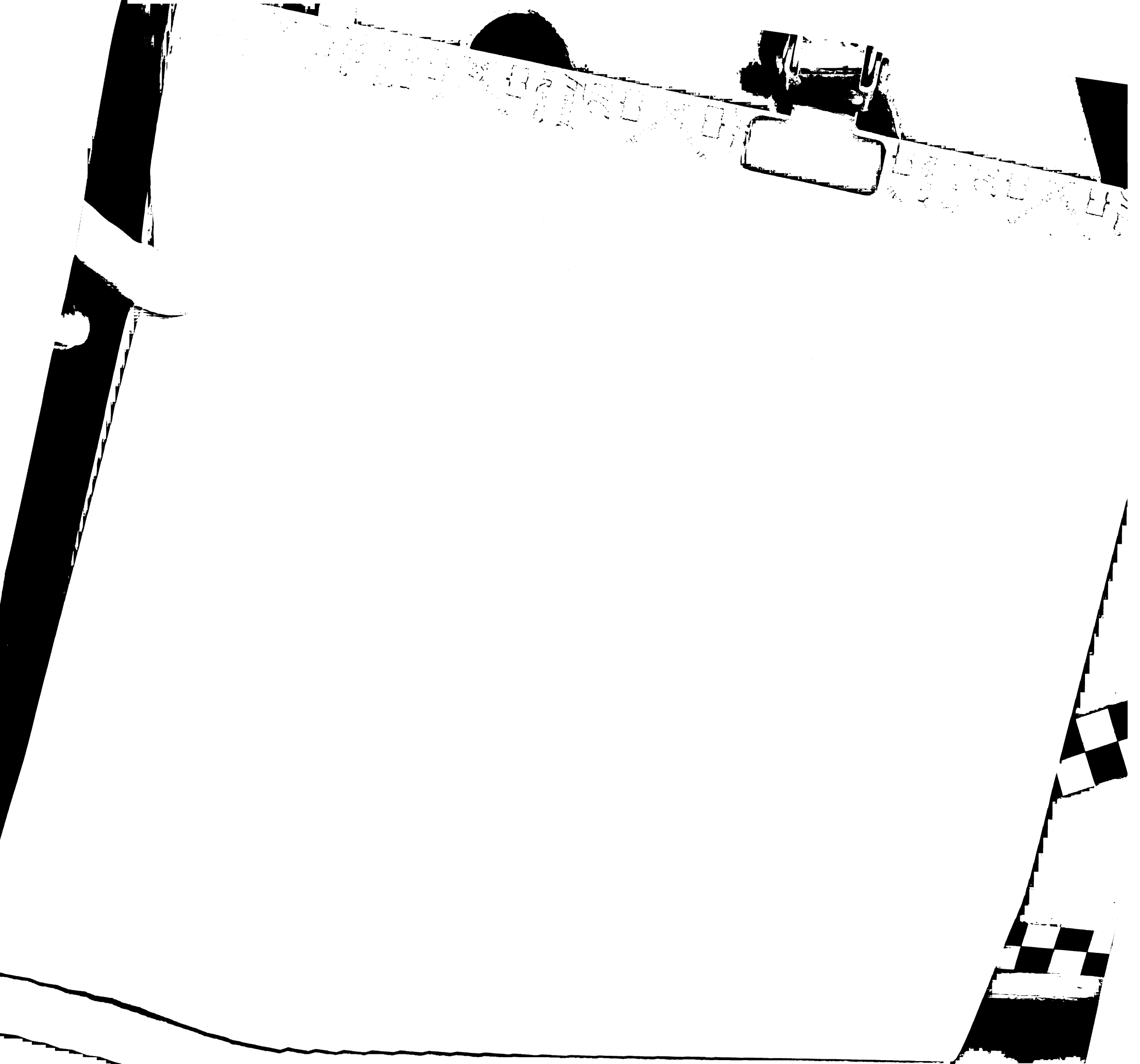
In vitro inhibitor binding assays

ELIZA-based detection of protein binding (Figure 5). 96 well dishes were coated with streptavidin (125 pmol per well). Next, biotinylated versions of the inhibitors FJ-1, FJ-3, and FJ-7 (1 nmol per well) were incubated in the wells for 1 hr at rt in PBST. After washing in PBST, lysate from HEK-293 cells overexpressing an HA-tagged version of the hNHERF1 / EBP50 protein and representing 10 μg of total protein was incubated in the wells for 3 hr at rt. Alternately, 100 nM of purified recombinant NSF protein (1.2 pmol) was incubated in the wells for 18 hr at rt. Following several washes in PBST, bound proteins were probed with either 2E5 mouse monoclonal anti-NSF antibody or HA.11 mouse anti-HA antibody (2E5 antibody kindly provided by S. W. Whiteheart and HA.11 purchased from Babco). Bound primary antibody was detected by goat anti-

mouse antibody directly conjugated to horseradish peroxidase (HRP, Jackson Immunoresearch) and quantified by enzyme-linked immunosorbant assay (ELISA) using chemi-luminescent detection.

Immunoprecipitation of inhibitor bound hNHERF1 / EBP50 (Figure 6A & 6B). Lysate representing 50 μg of total protein was incubated with the indicated biotinylated inhibitor at 50 μM concentration for 17 hr at 4 °C. Proteins bound to the biotinylated inhibitors were immunoprecipitated using monomeric avidin or streptavidin coated agarose beads for 3 hr at 4 °C. Immuno-precipitated protein corresponding to 4 μg of total protein was loaded per lane on SDS-PAGE gel and separated before transfer to nitrocellulose membrane. Blots were blocked in TBSTM (TBS, 0.1 % v/v tween-20, 3% w/v dry milk). Blots were incubated with HA.11 anti-HA mouse antibody to detect HA-hNHERF1 / EBP50 followed by goat anti-mouse antibody conjugated to HRP or streptavidin-HRP to detect the probe molecule (biotinylated inhibitor FJ-3 or FJ-7). After washing in TBST, proteins bands were detected using Super Signal (Pierce). Blots were directly imaged via chemiluminescence detection on a FluorChem 8000 instrument (AlphaInnotech Corp.) using a 16-bit cooled charge-coupled device camera and analyzed using FluorChem 2.0 software (AlphaInnotech Corp.).

Co-immunoprecipitation of HA-hNHERF1 / EBP50 and FLAG-β_2AR (Figure 7). Lysate representing 50 μg of total protein was incubated with the indicated biotinylated inhibitor at 50 μM concentration for 17 hr at 4 °C. Proteins bound to the biotinylated inhibitors were immunoprecipitated using monomeric avidin coated agarose beads for 3 hr at 4 °C.



4 μg of immuno-precipitated protein was loaded per lane on SDS-PAGE gel and separated before transfer to nitrocellulose membrane. Blots were blocked in TBSTM (TBS, 0.1 % v/v tween-20, 3% w/v dry milk). Blots were incubated with HA.11 anti-HA mouse antibody to detect HA-hNHERF1 / EBP50 or M1 anti-FLAG antibody to detect FLAG-β2AR followed by goat anti-mouse antibody conjugated to HRP. After washing in TBST, proteins bands were detected using Super Signal (Pierce). Blots were directly imaged via chemiluminescence detection on a FluorChem 8000 instrument (AlphaInnotech Corp.) using a 16-bit cooled charge-coupled device camera and analyzed using FluorChem 2.0 software (AlphaInnotech Corp.).

ELIZA based inhibitor competition assay (figure 6C). GST-β2AR beads were immobilized in 96 well dishes. 100 nM of purified recombinant NSF protein (1.2 pmol) was incubated in the wells for 18 hr at rt. Several washes in PBSTwere performed and bound NSF was competed against the inhibitors FJ-1, FJ-3, FJ-7, and FJ-9 over a range of concentrations [0, 30, 300, or 1000 μM]. Alternatively a peptide corresponding to the last ten amino acids of the β2AR was used to compete bound NSF [0, 30, 100, 300 μM]. Bound proteins were probed with 2E5 mouse monoclonal anti-NSF antibody (2E5 antibody kindly provided by S. W. Whiteheart). Bound primary antibody was detected by goat anti-mouse antibody directly conjugated to horseradish peroxidase (HRP, Jackson Immunoresearch) and quantified by enzyme-linked immunosorbant assay (ELISA) using chemi-luminescent detection.

HPLC / MS determination of loss of parent drug from conditioned media

HEK-293 cells overexpressing the β_2AR were plated on 10 cm dishes and treated overnight with 100 μM inhibitor compound (FJ-1) in DMEM with 10% FBS. 10 ml of media from control cells as well as from those treated with inhibitor was filter-sterilized (0.22 μm syringe filter, Nalgene). 100 μM inhibitor was added to the untreated media and both samples were run on HPLC. Traces were examined for loss of parent compound (peak at around 6.9 min MS 366 Da) and appearance of new peaks representing metabolites (expected MS 382 Da for hydroxylated metabolites).

Quantitation of Receptor Recycling by Fluorescence Flow Cytometry

Recycling of epitope-tagged receptors back to the plasma membrane was estimated by assaying the recovery of immunoreactive receptors accessible at the cell surface to monoclonal antibody recognizing the extracellular epitope tag (FLAG). This assay is a variant of a previously described flow cytometric assay for estimating receptor internalization and recycling (28). Briefly, monolayers of cells stably expressing the indicated FLAG tagged receptor were incubated in the presence of 10 uM of the appropriate agonist (isoproterenol or DADLE) for 25 min at 37 °C to drive agonist-induced internalization, then rinsed twice with DMEM, and subsequently incubated at 37°C in the presence of the appropriate antagonist (10 uM alprenolol or naloxone (Research Biochemicals) to block additional endocytosis of receptors. At the indicated time points, cell monolayers were chilled on ice to stop membrane trafficking, and cells were lifted with PBS containing 0.04% EDTA and lacking Ca++ or Mg++ (PBS/EDTA University of California San Francisco Cell Culture Facility). Cells were washed twice in

1 ml PBS and incubated at 4 °C for 45-60 min in 0.5 ml PBS with 2.5 ug/ml M1 anti-FLAG antibody that had been conjugated with Alexa-488 (Molecular Probes) using standard methods. Receptor immunoreactivity was quantitated by fluorescence flow cytometry (FACScaliber, Becton Dickinson, Palo Alto, CA). An alternate procedure was also performed with similar results. In this variation, cells were pretreated with M1 anti-FLAG antibody for 30 min prior to agonist and antagonist treatment as above. After placing the cells on ice, the cells were treated with Alexa-488 conjugated rabbit anti-mouse antibody (Molecular Probes) (2.5 ug/ml in PBS) for 45-60 min at 4 °C. Fluorescence intensity of 10,000 cells was collected for each sample. Triplicates of each time point were taken. Cellquest software (Becton Dickinson) was used to calculate the mean fluorescence intensity of single cells in each population. All experiments were conducted ≥2 times with similar results. The mean values for each experiment were averaged to obtain the overall mean fluorescence intensity and standard error of the mean reported in the figure.

Biochemical analysis of receptor degradation

Western blotting to detect proteolysis of total cellular receptors- To determine the effect of agonist treatment on steady state levels of total receptor protein, immunoblotting was performed as described previously (28). Briefly, cells stably transfected with the indicated FLAG tagged receptors were grown in 6 well dishes and treated for 0, 1, or 4 hours with the appropriate agonist (10 uM isoproterenol or DADLE) at 37 °C. Dishes of cells containing stably transfected cells were washed with 2.5 ml PBS and the cells were dissociated and harvested in 1.5 ml PBS/EDTA for 30 min at 4 °C. After pelleting the

cells by centrifugation (1000 rpm for 5 min on benchtop microcentrifuge), the cells were lysed by placing them in 0.5 ml of hypotonic lysis solution (25 mM Tris-HCl or 25 uM Hepes buffer, pH 7.4, 1 ug/ml leupeptin, 1 ug/ml, pepstatin, and 2 ug/ml aprotinin) while vortexing for 2 min. The crude membrane fraction was separated from the cytoplasmic fraction by centrifugation at 14000 rpm for 15 min on a microcentrifuge. The supernatant was discarded and the pellet resuspended in 0.25 ml of resuspension buffer (25 mM Tris-HCl or 50 mM Hepes buffer, pH 7.4, 1 ug/ml leupeptin, 1 ug/ml, pepstatin, 2 ug/ml aprotinin, and 0.25% v/v Triton-X100). The non-soluble fraction was removed by centrifugation as above at 14000 rpm and the supernatant was decanted and analyzed for protein content by the Bradford method (33) using bovine serum albumin as standard. Lysate from the samples corresponding to ~25 ug of total protein were loaded and separated by SDS-PAGE under denaturing conditions. Resolved proteins were transferred to nitrocellulose membranes (Micron Separations, Inc.) and placed in TBSTM (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.1% v/v Tween-20, and 5% dry nonfat milk) for 60 min. Detection of receptors containing FLAG epitope was carried out by incubation of the blots with M1 anti-FLAG antibody (15 ug/ml in TBSTM) for 60 min, washing in TBST (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, and 0.1% v/v Tween-20), and incubation for 60 min in TBSTM containing 400 ng/ml goat anti-mouse conjugated to horseradish peroxidase (Jackson ImmunoResearch). After washing in TBST, proteins bands were detected using Super Signal (Pierce). Band intensities were quantitated by densitometry of films exposed in the linear range, imaged using a charge-coupled device camera, and analyzed using National Institutes of Health Image software or FluorChem 2.0 (AlphaInnotech Corp).

Alternatively, some blots were directly imaged via chemiluminescence detection on a FluorChem 8000 instrument (AlphaInnotech Corp.) using a 16-bit cooled charge-coupled device camera and analyzed using FluorChem 2.0 software (AlphaInnotech Corp.).

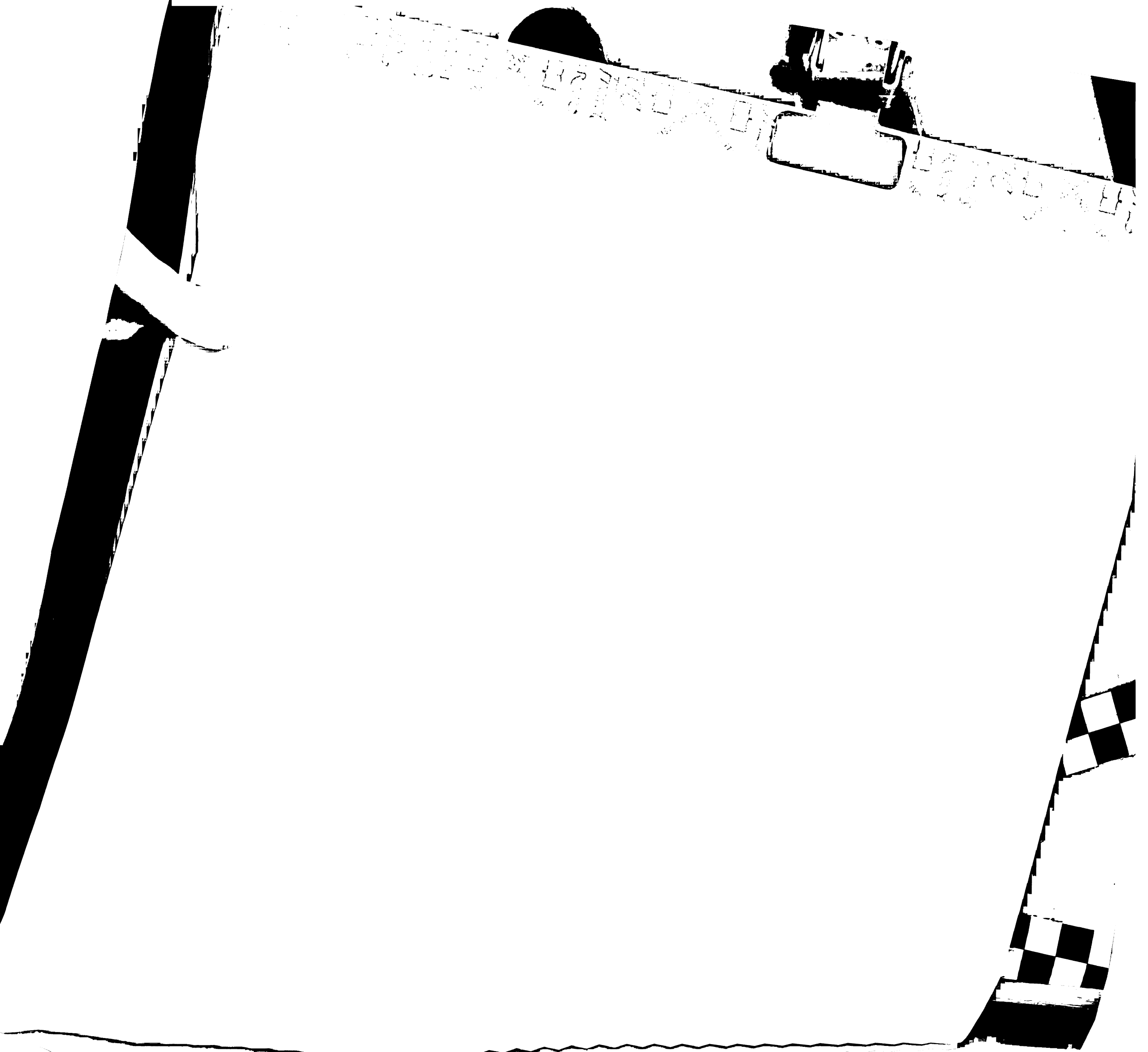
Results

Inhibitors of PDZ domain mediated protein interaction were designed and synthesized previously (24). These inhibitors, based on an indole scaffold, were designed to project functional groups in an analogous way to those presented by carboxyl terminal PDZ ligands (see figure 1A). A series of inhibitors were generated which differed in such characteristics as reversibility of inhibition and susceptibility to oxidative metabolism. The inhibitor FJ-1 was found to be a specific reversible inhibitor of PDZ domain mediated interaction (25), while the FJ-3 compound acted similarly but was irreversibly bound to the PDZ domain (25) (see Figure 1B & C). FJ-9 was designed to be less susceptible than FJ-1 to oxidative attack at an electron rich position β to the indole nitrogen (see Figure 1D). The inhibitor compounds were tested for activity in the inhibition of β_2AR and δ/β_1AR recycling both receptors that require a carboxyl terminal PDZ ligand sequence for their proper membrane trafficking after agonist-induced endocytosis.

The FJ-1 inhibitor was added acutely (30 min prior to treating the cells with agonist to drive internalization) to HEK-293 cells overexpressing either β_2AR or δ/β_1AR and the recycling efficiencies for these cells were obtained. The recycling efficiency of the β_2AR was visibly decreased by pre-treating the cells with 100 mM FJ-1 when analyzed by fluorescence microscopy as compared to untreated cells (data not shown). To determine this effect more quantitatively, we performed a flow cytometric assay. As seen in figure 2A, the inhibitor significantly decreased the recycling efficiency of the wild-type β_2AR but did not significantly alter the recycling of the chimeric δ/β_1AR receptor. Thus the

inhibitor seems to show some selectivity for the PDZ domain containing protein which binds to the ligand present in the β_2AR (-DSLL-COOH). The δ/β_1AR carboxyl terminal tail sequence, -ESKV-COOH, although still a class I PDZ ligand does not bind to the same PDZ domain containing proteins reported to bind to the β_2AR. The inhibitors are stable for prolonged periods at 37 °C in aqueous solution and we believed a longer pre-incubation may have increased the effect of the inhibitor. We were surprised to see that in contrast to experiments in which FJ-1 is applied acutely, when the inhibitor is incubated with receptor expressing cells overnight (18 hr) the recycling of the δ/β_1AR is inhibited while the β_2AR recycles normally. This raised the possibility that metabolism of FJ-1 was responsible for the switched behavior.

We next tested for the presence of an active metabolite of FJ-1 that could influence the recycling efficiency of the chimeric δ/β_1AR receptor but that was unable to influence the recycling of the β_2AR. Media conditioned by β_2AR expressing cells after an overnight treatment of 100 μM FJ-1 was filtered and analyzed by LC / MS (liquid chromatography / mass spectrometry). As shown in figure 3A this was compared to conditioned media from HEK-293 cells overexpressing the β_2AR, but not exposed to FJ-1. After filtration, FJ-1 inhibitor was added to this sample at the same concentration (100 μM) as applied to the cells in the previous analysis. Surprisingly, no new peaks in the chromatogram were seen. After integration of the FJ-1 peak (appears at approximately 6.9 min on each trace) it appears that 50 % of the FJ-1 was lost. This does not account for losses arising from FJ-1 that was concentrated inside the HEK-293 cells which were not lysed prior to removing the media or from inhibitor that was lost during filtration. Also of note, the conditioned media contained no trace of FJ-1 (mass 366 M+H) derived compounds at a



mass of 382 Da, where likely hydroxylated derivatives would be found, when analyzed by mass spectrometry. This surprising result was further corroborated by recycling assays performed using pre-conditioned media.

As demonstrated in figure 3B, media taken from cells treated overnight with 100 μM FJ-1 has no perceptible effect on the recycling of the β_2AR and only a marginal effect on the chimeric δ/β_1AR receptor. This does not rule out the possibility of a metabolite which is concentrated within the treated cells and not present in the liquid media, but further studies would be needed to determine whether this is the case. Thus there appears to be no demonstrable metabolism of the FJ-1 compound which could easily explain the phenotypic switch in recycling inhibition exhibited based on pre-incubation time.

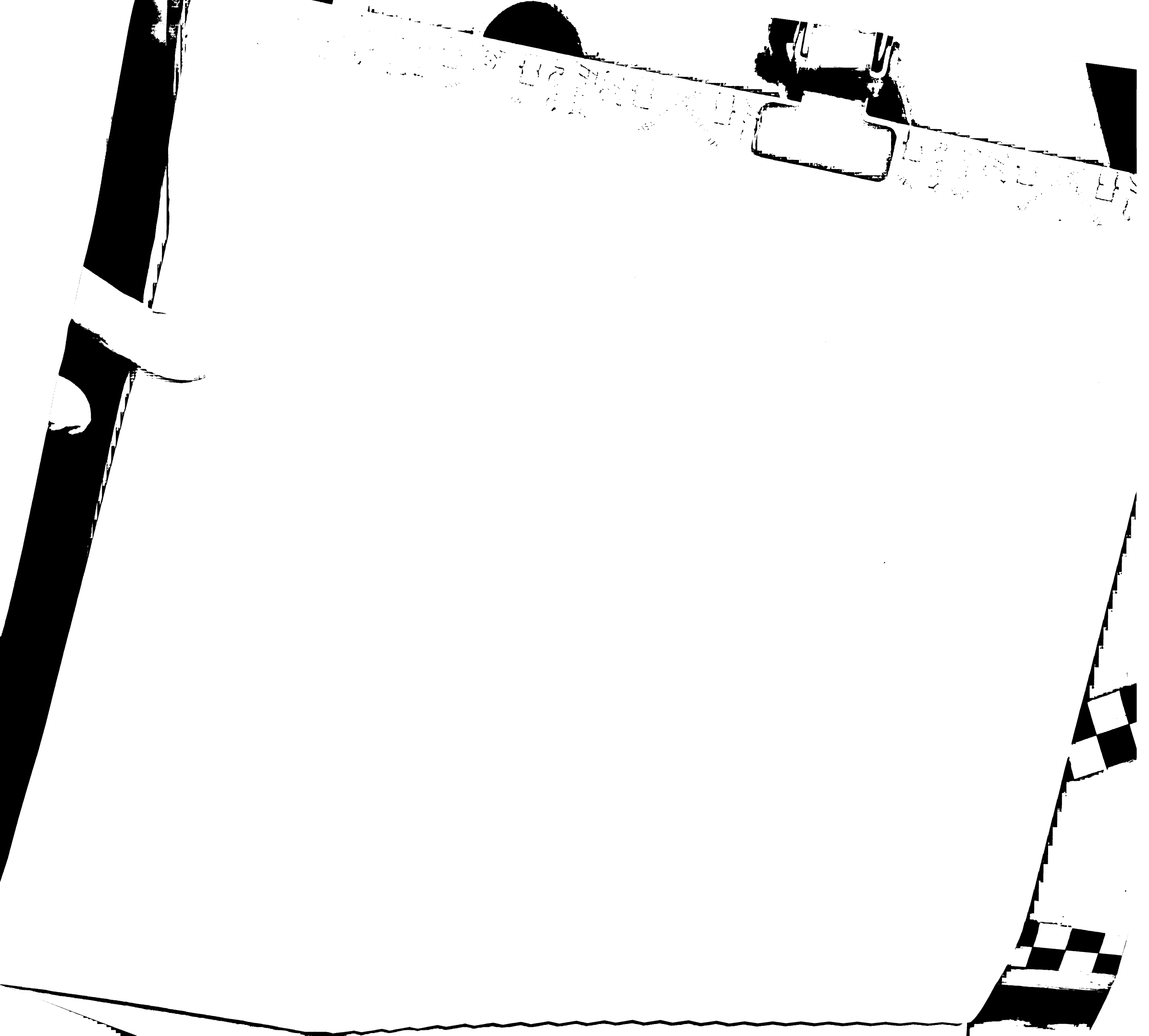
We tested a set of structurally similar inhibitors, FJ-3, FJ-9, and FJ-7, for the ability to inhibit the recycling of the β_2AR and the chimeric δ/β_1AR receptor. As illustrated in figure 4A & B, the inhibitors FJ-3 and FJ-9 do not seem to greatly inhibit the recycling of either receptor tested in a dose-dependent way. FJ-3 may inhibit the recycling of δ/β_1AR (figure 4B), but the data show that either it is not dose dependent or the EC50 must be significantly below 5 μM. Also, the data for FJ-3 are representative of only a single experiment and thus no definite conclusions can be drawn either way. In data not shown, the inhibitor FJ-7 did not inhibit the recycling efficiency of either tested receptor. Thus small changes in chemical structure can have large effects on the efficacy and / or potency of the inhibitor.

The carboxyl terminal tail of the β_2AR can bind to a variety of proteins including hNHERF1 / EBP50 and NSF (1,2,34). A likely mechanism of β_2AR recycling inhibition by FJ-1 is direct binding to the protein partner responsible for mediating the signal

dependent recycling of the β_2 adrenergic receptor. We investigated whether the inhibitors were capable of directly binding to hNHERF1 / EBP50 or NSF using an *in vitro* assay. Biotinylated derivatives of each of the inhibitory compounds were synthesized and bound to the wells of a 96 well dish coated with streptavidin. Either purified NSF or lysate from cells expressing an HA tagged variant of hNHERF1 / EBP50 was incubated with the coated wells to determine binding to immobilized compound. Biotin-FJ-1 bound well to NSF, but did not detectibly bind hNHERF / EBP50 over background (see figure 5). FJ-3 and FJ-7 seemed to bind to both proteins with FJ-3 showing a greater affinity for NSF while FJ-7 bound more tightly to hNHERF1 / EBP50. These results seem to show that FJ-1, the compound most active in the recycling inhibition assays, binds to and presumably inhibits NSF. This suggests that direct interaction between β_2AR and NSF could play a role in the proper membrane trafficking of endocytosed β_2 adrenoreceptor in agreement with previously reported results (2).

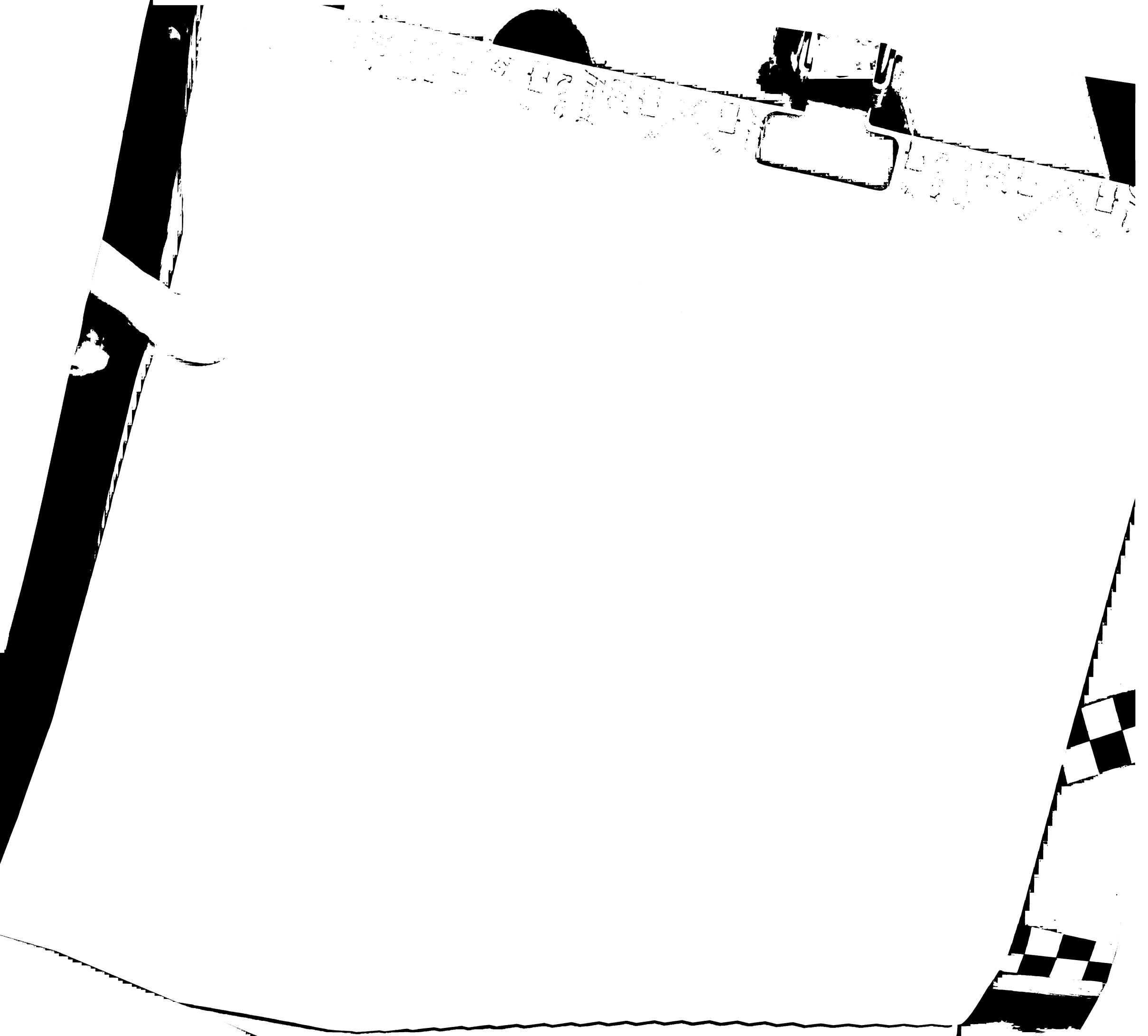
Further protein binding experiments were carried out with the FJ-3 and FJ-7 inhibitors. FJ-3 and FJ-7 are both capable of strongly pulling down the HA-hNHERF1 / EBP50 protein (see figure 6A & B) by immunoprecipitation from crude lysate derived from cells overexpressing HA-hNHERF1 / EBP50. ELIZA based competition of free inhibitor to NSF bound to immobilized GST-β2AR showed that both FJ-1 and FJ-7 are capable of competing NSF away from the β2AR tail (see figure 6C). Two similar molecules, FJ-3 and FJ-9, were unable to compete NSF away from the β2AR tail even at concentrations as high as 1000 μM.

We next wanted to test for an *in vivo* interaction between the inhibitors and NSF. We further tested whether the inhibitors FJ-3 and FJ-7 were capable of co-

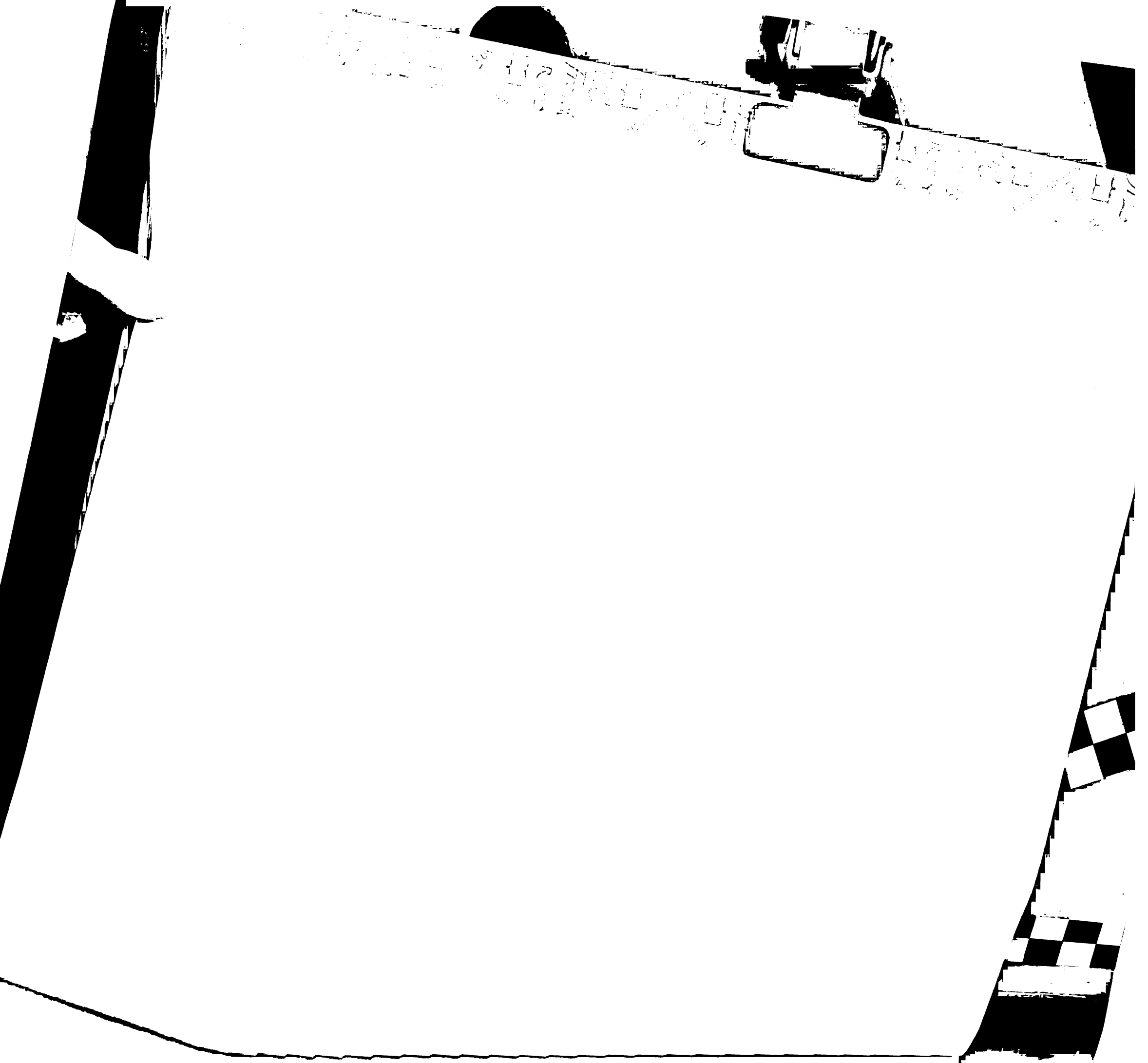


immunoprecipitating the β_2AR through their interaction with hNHERF1 / EBP50. Lysate from cells expressing both FLAG tagged β_2AR and HA-hNHERF1 / EBP50 was incubated with biotinylated versions of FJ-3 and FJ-7. After immunoprecipitation with monomeric avidin, it appears that although both inhibitors could pull down HA-hNHERF1 / EBP50, only FJ-7 was able to pull down both of the proteins (see Figure 7). This result is consistent with FJ-3 binding to the 1st PDZ domain of HA-hNHERF1 / EBP50 and FJ-7 binding to the 2nd PDZ domain. Since the β_2AR binds to the 1st PDZ domain, FJ-3 binding would effectively block concurrent receptor binding. FJ-7, by binding to the 2nd PDZ domain would not block the β_2AR from binding to HA-hNHERF1 / EBP50. Further testing with isolated PDZ domains from hNHERF / EBP50 could elucidate whether this is true.

Inhibition of the rapid recycling of the β_2AR has been shown to affect the long-term fate of the receptor (3,35). Deletion mutants of the β_2AR which lack the cytoplasmic recycling signal are targeted to lysosomes following agonist induced endocytosis and are subsequently degraded leading to a pronounced down-regulation of the receptor (3,35). We tested whether the FJ-1 inhibitor, which produced a significant decrease in the recycling of the β_2AR, was capable of increasing the rate of degradation of the wild-type β_2AR as well as a chimeric δ/β_2AR receptor. We compared the total receptor level as measured by western blot of HEK-293 cells expressing the indicated receptor at zero and four hours post agonist treatment. After four hours, mutant β_2AR that lack the carboxyl terminal recycling sequence show a marked decrease in receptor level, approximately 25 % of control levels as compared to 93 % of the receptors remaining for wild-type β_2AR (3,35). Although the FJ-1 inhibitor was capable of reducing the recycling efficiency of



the β_2AR, it was unable to increase the degradation rate of the receptor (see figure 8). And in fact the inhibitor appears to decrease the degradation rate of the β_2AR and even the δ opioid receptor which does not contain a known PDZ ligand sequence. It does so in a dose-dependent manner, such that the more FJ-1 inhibitor that is pre-incubated with the cells, the greater the protective effect on degradation rate.



Discussion

The β_2 adrenergic receptor contains a recycling signal present in its distal carboxyl cytoplasmic tail that is both necessary for its own membrane trafficking (1) and sufficient to reroute a heterologous GPCR from a degradative to a rapidly recycling membrane trafficking pathway (3). This sequence is contained within the last four amino acids of the carboxyl terminus which constitute a class I PDZ ligand. Recently, small molecule inhibitors of PDZ domain function were developed (24) that were able to inhibit PDZ domain mediated binding and also to disrupt the function of the PDZ domain containing proteins (24,25). We sought to determine if inhibition of PDZ domain mediated binding of the hNHERF1 / EBP50 protein would affect β_2AR recycling and degradation rate. A range of inhibitors were tested for both the ability to bind to hNHERF1 / EBP50 and for biological activity in altering the membrane trafficking of the β_2AR. One of the inhibitors, FJ-1, was capable of inhibiting the recycling of the β_2AR after an acute 30 minute pre-treatment of the drug but failed to inhibit receptor recycling after an overnight pre-treatment. The same molecule when applied overnight was capable of inhibiting the recycling of a chimeric δ/β_1AR receptor. The sequence taken from the carboxyl terminal tail of the β_1AR contains a class I PDZ ligand distinct from that present in the β_2AR and binds to a different subset of PDZ domain containing proteins. This curious observation shows that the inhibitors can be specific in their inhibition and that their effects are sensitive to pre-treatment conditions and times.

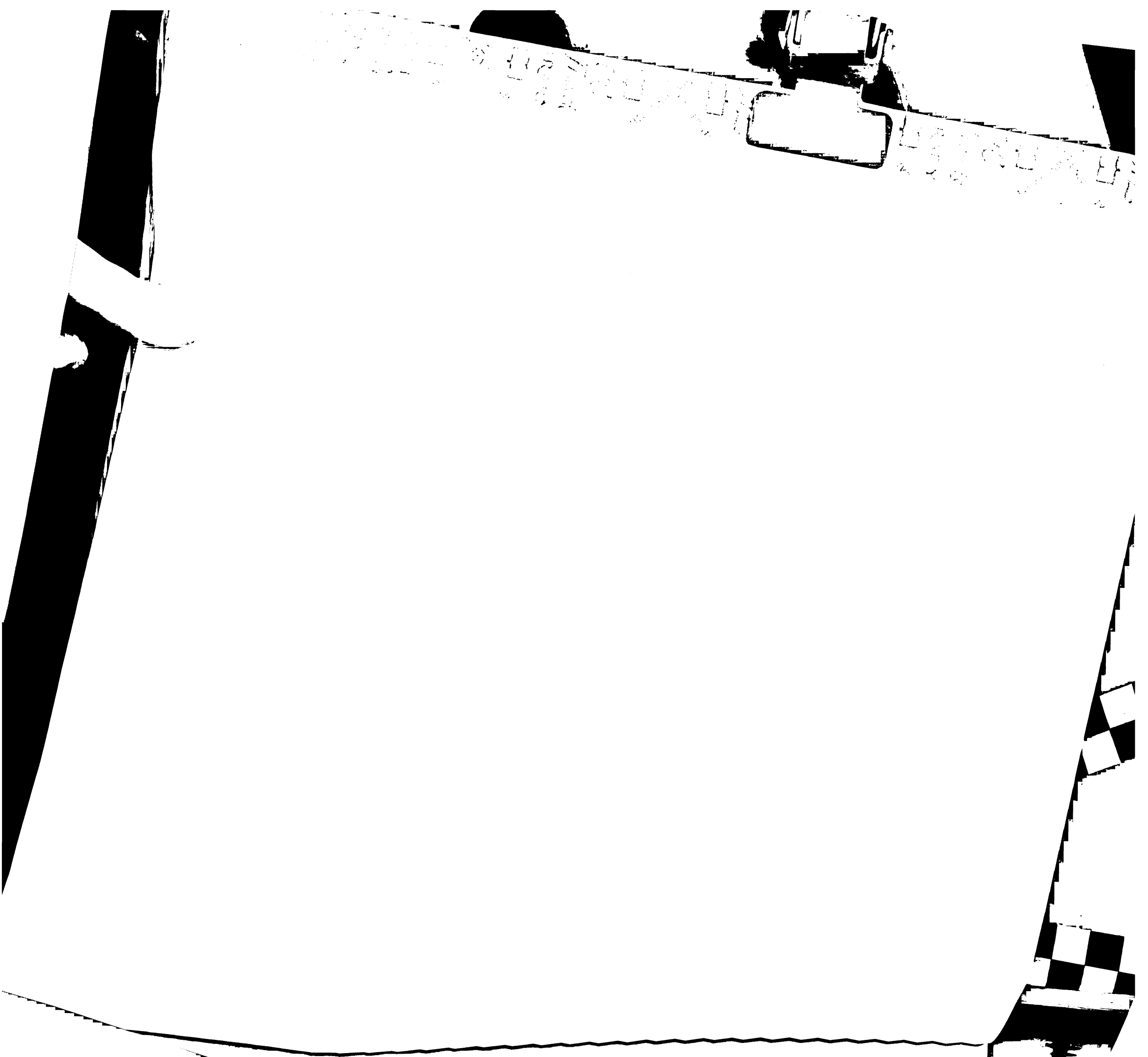
The change in efficacy after different exposure times could be explained by metabolism of the parent FJ-1 compound, either after entering the HEK-293 cells or in

the media itself. However, when we tested for the presence of a metabolite of FJ-1 in media from cells conditioned overnight with FJ-1, we did not find a significant loss of parent or the appearance of new compound peaks in either the HPLC trace or the mass spectrometry results. Concurrent with this study, conditioned media was tested for biological activity towards inhibition of β_2AR recycling. The conditioned media did not decrease receptor recycling as would have been expected if the media had contained an active metabolite of FJ-1.

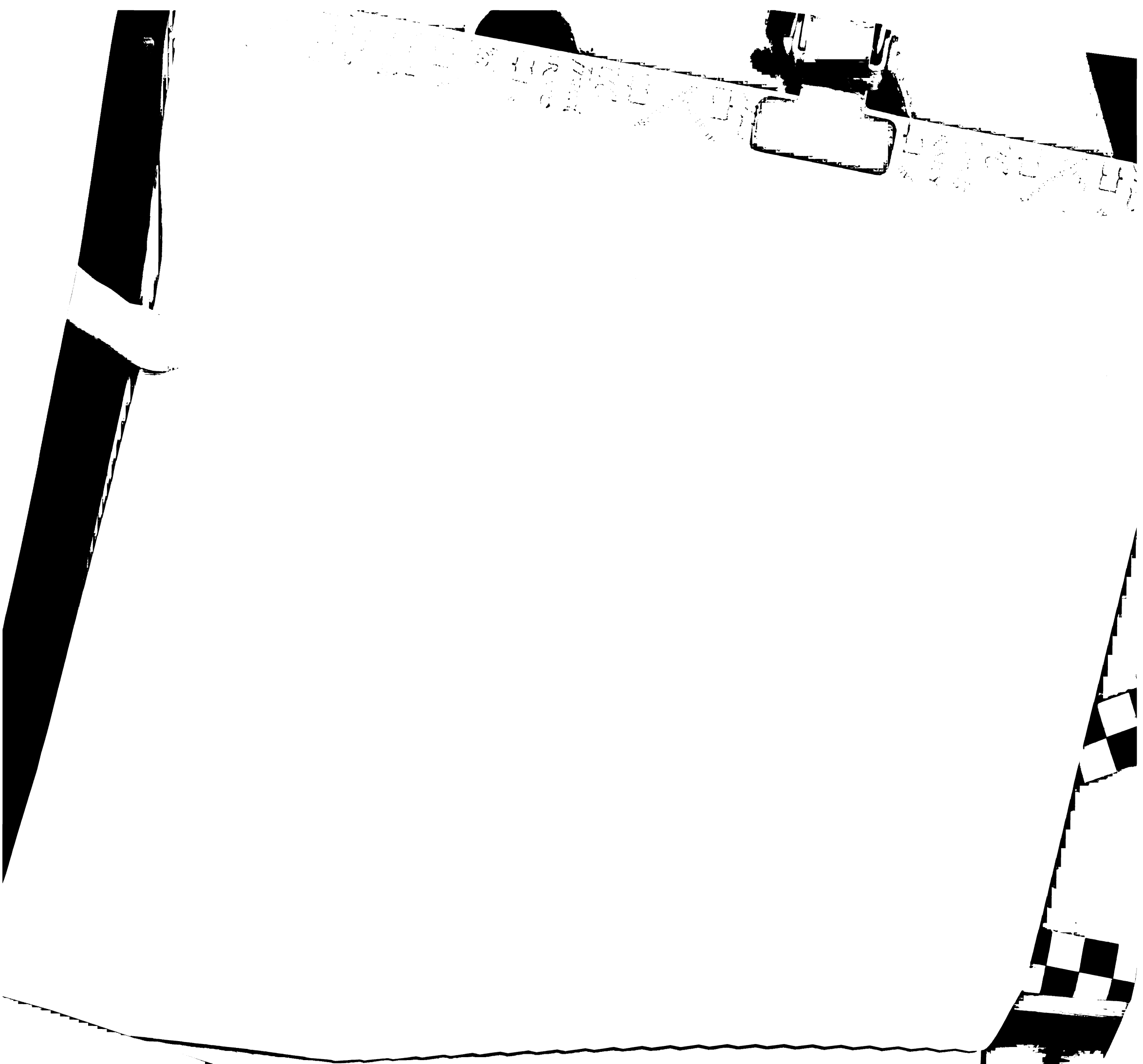
Previous studies have shown an inverse correlation between a receptor's ability to recycle back to the plasma membrane following agonist exposure and the rate of degradation following prolonged agonist exposure (1,3,4). It was expected that since FJ-1 was capable of inhibiting the recycling efficiency of the β_2AR, it would also increase the degradation rate of the receptor. Surprisingly, we observed no effect on the degradation rate of the β_2AR, with possibly an increase in receptor number after 4 hours of continuous agonist exposure at high concentrations of FJ-1. In fact, the degradation rate of the degrading receptor δ OR was actually slowed at high concentrations of the inhibitor despite the receptor lacking an obvious PDZ ligand sequence. Thus it appears that although the drug can divert a portion of the β_2AR from a rapid recycling pathway, it is not capable of altering the portion of receptors which enter lysosomes and are subsequently degraded. It is possible that a majority of receptor will recycle back to the plasma membrane, albeit more slowly. This could be tested by performing a similar set of recycling assays as those completed herein, but allowing the receptors a longer time period in which to recycle. It is also possible that a portion of internalized β_2AR in the presence of the FJ-1 inhibitor and agonist internalize and remain within the cell for

prolonged periods. The receptor may enter a subcellular compartment distinct from early endosomes or lysosomes from which they could be protected from degradation and yet not return rapidly to the plasma membrane.

It is likely that the recycling sequence from the β_2AR functions via protein-protein interaction(s). Two candidate proteins which are known to bind the very carboxyl terminus of the β_2 adrenergic receptor and have been reported to influence its recycling are hNHERF1 / EBP50 and NSF (1,2,4,34). From the results of the binding studies we conducted, it appears that the compound which was biologically active in the recycling assay, FJ-1, does not bind detectably to hNHERF1 / EBP50 but binds well to NSF. This is curious since NSF does not contain a known PDZ domain which would be capable of binding to both the carboxyl terminal tail of the β_2AR and FJ-1 inhibitor. NSF may play a role in the recycling of the β_2AR, but this does not rule out the possibility of an as yet unidentified PDZ domain containing protein being responsible for enhancing the recycling efficiency of the β_2AR. PDZ domain interactions have been shown to be important for the rapid recycling of the β_2AR and the β_1AR whose last four carboxyl terminal residues correspond to class I PDZ ligands. However, PDZ domain mediated recycling may be a more general feature for GPCRs than can be initially surmised based upon sequence analysis of their distal carboxyl terminal sequences. Interactions based on other interaction motifs, such as SH2 and SH3, could potentially bridge the receptor to a PDZ domain containing protein. In fact, many PDZ domain containing proteins also contain other such interaction motifs. For example, PSD-95 contains multiple PDZ domains, an SH3 domain, and a guanylyl kinase domain (5). A recent report (11) found evidence for an internal peptide motif present in the carboxyl tail of the ET_A (endothelin

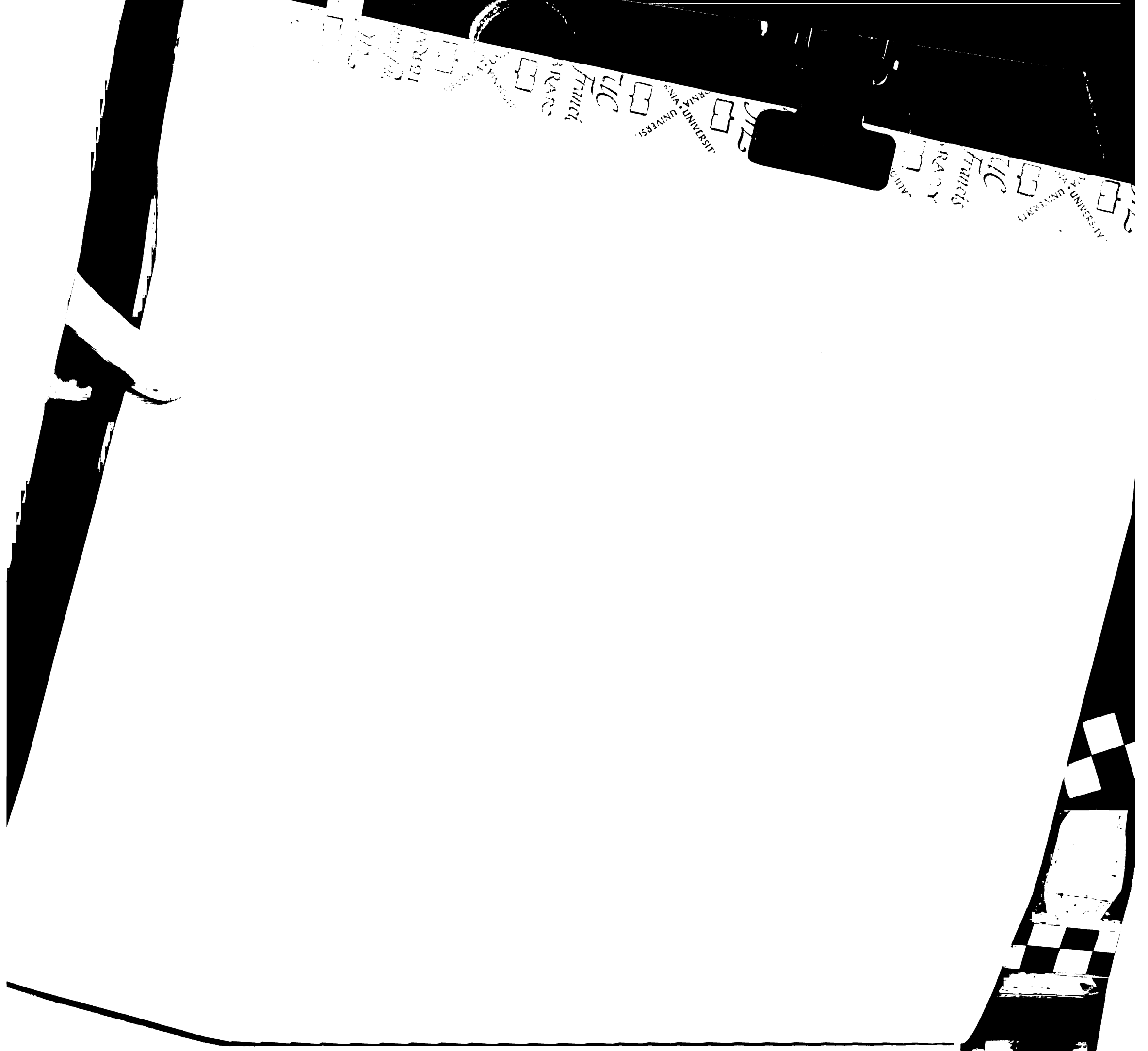


A) receptor that was essential for the proper membrane recycling of this receptor. They further suggest that this motif, similar to the internal β hairpin peptide of nNOS (neuronal nitric oxide synthase) which binds to PDZ #2 of PSD-95 (15), may reside in a number of GPCRs which lack classical distal carboxyl terminal PDZ ligands (11). Thus PDZ domain mediated protein interactions may play a role in the endocytic trafficking of a large number of GPCRs.



References

1. Cao, T. T., Deacon, H. W., Reczek, D., Bretscher, A., and von Zastrow, M. (1999) *Nature* **401**, 286-290.
2. Cong, M., Perry, S. J., Hu, L. A., Hanson, P. I., Claing, A., and Lefkowitz, R. J. (2001) *J. Biol. Chem.* **276**, 45145-45152
3. Gage, R. M., Kim, K. A., Cao, T. T., and von Zastrow, M. (2001) *J Biol Chem* **276**, 44712-44720.
4. Gage, R. M., Matveeva, E. A., Whiteheart, S. W., and von Zastrow, M. (2005) *J. Biol. Chem.* **280**, 3305-3313
5. Harris, B. Z., and Lim, W. A. (2001) *J Cell Sci* **114**, 3219-3231
6. Tiffany, A. M., Manganas, L. N., Kim, E., Hsueh, Y. P., Sheng, M., and Trimmer, J. S. (2000) *J Cell Biol* **148**, 147-158
7. Sheng, M., and Kim, E. (2000) *J Cell Sci* **113 (Pt 11)**, 1851-1856
8. Naisbitt, S., Valtschanoff, J., Allison, D. W., Sala, C., Kim, E., Craig, A. M., Weinberg, R. J., and Sheng, M. (2000) *J Neurosci* **20**, 4524-4534
9. Wang, S., Yue, H., Derin, R. B., Guggino, W. B., and Li, M. (2000) *Cell* **103**, 169-179
10. Xu, J., Paquet, M., Lau, A. G., Wood, J. D., Ross, C. A., and Hall, R. A. (2001) *J. Biol. Chem.* **276**, 41310-41317
11. Paasche, J. D., Attramadal, T., Kristiansen, K., Oksvold, M. P., Johansen, H. K., Huitfeldt, H. S., Dahl, S. G., and Attramadal, H. (2005) *Mol Pharmacol*
12. Berman, H. M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T. N., Weissig, H., Shindyalov, I. N., and Bourne, P. E. (2000) *Nucleic Acids Res* **28**, 235-242



13. Daniels, D. L., Cohen, A. R., Anderson, J. M., and Brunger, A. T. (1998) *Nat Struct Biol* **5**, 317-325

14. Doyle, D. A., Lee, A., Lewis, J., Kim, E., Sheng, M., and MacKinnon, R. (1996) *Cell* **85**, 1067-1076

15. Hillier, B. J., Christopherson, K. S., Prehoda, K. E., Bredt, D. S., and Lim, W. A. (1999) *Science* **284**, 812-815

16. Karthikeyan, S., Leung, T., Birrane, G., Webster, G., and Ladias, J. A. (2001) *J Mol Biol* **308**, 963-973

17. Webster, G., Leung, T., Karthikeyan, S., Birrane, G., and Ladias, J. A. (2001) *Acta Crystallogr D Biol Crystallogr* **57**, 714-716

18. Karthikeyan, S., Leung, T., and Ladias, J. A. (2001) *J Biol Chem* **276**, 19683-19686

19. Harrison, S. C. (1996) *Cell* **86**, 341-343

20. Harris, B. Z., Hillier, B. J., and Lim, W. A. (2001) *Biochemistry* **40**, 5921-5930

21. Niethammer, M., Valtschanoff, J. G., Kapoor, T. M., Allison, D. W., Weinberg, T. M., Craig, A. M., and Sheng, M. (1998) *Neuron* **20**, 693-707

22. Karthikeyan S, L. T., Birrane G, Webster G, Ladias JA. (2001) *J Mol Biol.* **308**, 963-973

23. Karthikeyan, S., Leung, T., and Ladias, J. A. A. (2001) *J Biol Chem* **276**, 19683-19686

24. Fujii, N., Haresco, J. J., Novak, K. A., Stokoe, D., Kuntz, I. D., and Guy, R. K. (2003) *J Am Chem Soc* **125**, 12074-12075

25. Novak, K. A., Fujii, N., and Guy, R. K. (2002) *Bioorg Med Chem Lett* **12**, 2471-2474

26. Evans, C. J., Keith, D. J., Morrison, H., Magendzo, K., and Edwards, R. H. (1992) *Science* **258**, 1952-1955

27. Kobilka, B. K., Dixon, R. A., Frielle, T., Dohlman, H. G., Bolanowski, M. A., Sigal, I. S., Yang-Feng, T. L., Francke, U., Caron, M. G., and Lefkowitz, R. J. (1987) *Proceedings of the National Academy of Sciences of the United States of America* **84**, 46-50

28. Tsao, P. I., and von Zastrow, M. (2000) *J Biol Chem* **275**, 11130-11140.

29. von Zastrow, M., and Kobilka, B. K. (1992) *J Biol Chem* **267**, 3530-3538.

30. Keith, D. E., Murray, S. R., Zaki, P. A., Chu, P. C., Lissin, D. V., Kang, L., Evans, C. J., and von Zastrow, M. (1996) *J Biol Chem* **271**, 19021-19024.

31. Tsao, P., Cao, T., and von Zastrow, M. (2001) *Trends Pharmacol Sci* **22**, 91-96.

32. Okayama, H., and Berg, P. (1983) *Molecular and Cellular Biology* **3**, 280-289

33. Bradford, M. M. (1976) *Analytical Biochemistry* **72**, 248-254

34. Reczek, D., Berryman, M., and Bretscher, A. (1997) *Journal of Cell Biology* **139**, 169-179

35. Carman, C. V., Lisanti, M. P., and Benovic, J. L. (1999) *Journal of Biological Chemistry* **274**, 8858-8864

Figure Legends

Figure 1: Chemical structure of inhibitors of PDZ domain mediated interaction. **(A)** DSWL PDZ domain protein interaction inhibitor. The inhibitor is based on an indole scaffold and highlighted in the boxes are the chemical moieties that represent the indicated amino acid residue of the DSWL PDZ ligand sequence. **(B)** FJ-1 inhibitor, a reversible inhibitor of PDZ domain mediated interaction. **(C)** FJ-3 inhibitor, an irreversible inhibitor of PDZ domain mediated interaction. **(D)** The position β to the indole nitrogen of FJ-1 is susceptible to oxidative attack, so a derivative was designed that should be more stable, FJ-9.

Figure 2: Acute doses of FJ-1 inhibit β_2AR recycling while longer pretreatment inhibits the recycling of the chimeric δ/β_1AR receptor. **(A)** HEK-293 cells expressing the indicated receptor were treated with 10 μM isoproterenol or DADLE for 25min to drive internalization. Cells were washed in DMEM lacking agonist and receptors allowed to recycle for 45 min. Flow cytometry revealed that FJ-1 was capable of decreasing the recycling efficiency of the β_2AR but not chimeric δ/β_1AR receptor after pre-treatment of the cells for 30 min with 100 μM inhibitor. **(B)** After an overnight treatment with 100 μM FJ-1, HEK-293 cells expressing δ/β_1AR showed a decrease in recycling efficiency, while cells expressing β_2AR did not. Each graph represents ≥ 5 independent experiments with all time points taken in triplicate. Error bars show the standard error of the mean.

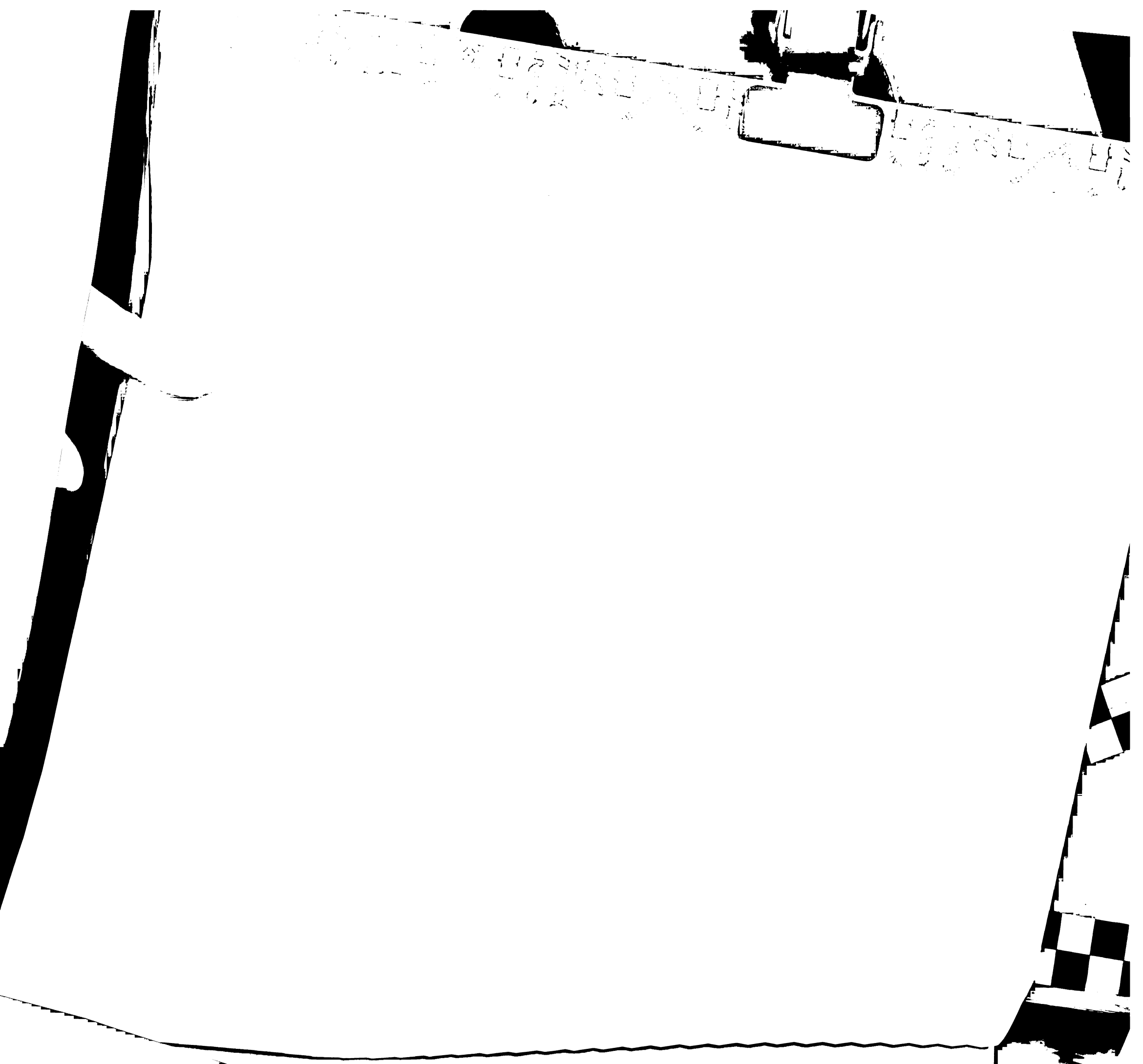
Figure 3: No active metabolite of FJ-1 could be found. **(A)** Media overlying HEK-293 cells overexpressing the β_2AR and treated overnight (18 hr) with 100 μM FJ-1 compound (top trace) and media from control cells spiked with 100 μM FJ-1 (bottom trace) were compared by HPLC and mass spectrometry. Traces from the HPLC are shown with the peak around 6.9 min corresponding to FJ-1 bracketed. No new peaks were observed following 18 hr in the presence of HEK-293 cells. Integration of the peaks showed a loss of approximately 50 % of the FJ-1 compound in this time. **(B)** Flow cytometry of HEK-293 cells overexpressing the indicated receptor was performed as in figure 2. This showed that an acute treatment of cells with conditioned media taken from β_2AR expressing cells exposed to FJ-1 overnight (18 hr) was insufficient to inhibit the recycling of the β_2AR or δ/β_1AR. This represents 2 independent experiments with all time points taken in triplicate. Error bars show the standard deviation of the mean.

Figure 4: Structurally similar molecules to FJ-1 do not inhibit recycling of either δ/β_1AR or β_2AR. **(A)** HEK-293 cells overexpressing the indicated receptor were assessed for recycling efficiency as in figure 2. FJ-9 does not significantly decrease receptor recycling as measured by flow cytometry. This represents 3 independent experiments with all time points taken in triplicate. Error bars show the standard error of the mean. **(B)** FJ-3 also does not seem to inhibit the recycling of the β_2AR. Inhibition of the recycling of the δ/β_1AR does not appear to be dose dependent or the EC50 is less than 5 μM. As this represents only a single experiment, no conclusions can be drawn.

Figure 5: Binding of HA-hNHERF1 / EBP50 and NSF to immobilized inhibitors FJ-1, FJ-3, and FJ-7. Biotinylated versions of the inhibitors FJ-1, -3, & -7 were bound to streptavidin coated 96 well dishes. Lysate from HEK-293 cells overexpressing HA-hNHERF / EBP50 or purified NSF was allowed to interact with the immobilized inhibitors. After washing, bound protein was detected by ELIZA. NSF bound well to FJ-1 and less well to FJ-3 and FJ-7. hNHERF1 / EBP50 did not bind detectably to FJ-1, bound to FJ-3, and bound well to FJ-7.

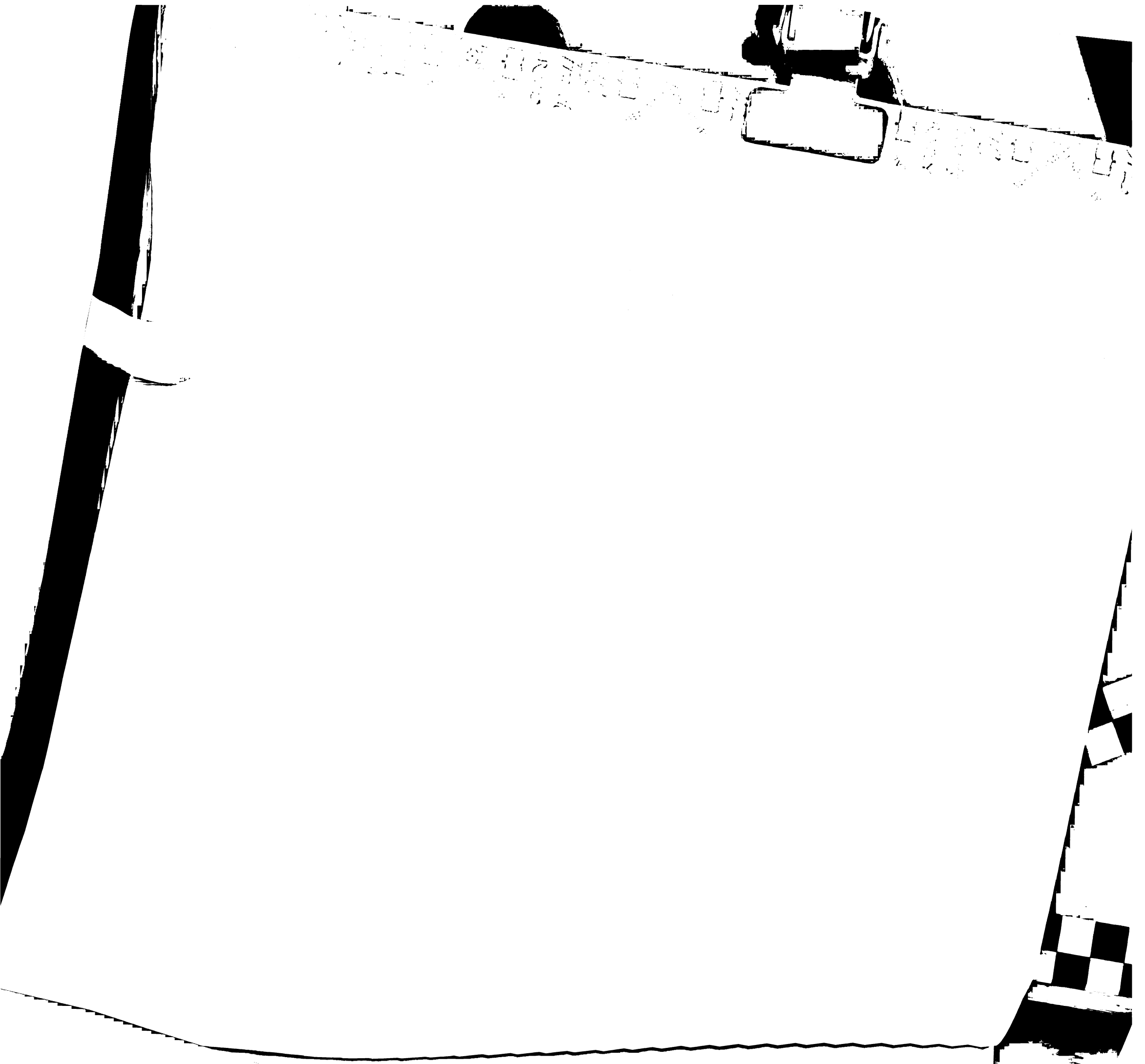
Figure 6: Immunoprecipitation of HA-hNHERF1 / EBP50 by immobilized inhibitor FJ-3 and FJ-7. **(A)** HEK-293 cells overexpressing HA-hNHERF1 / EBP50 were lysed and protein was immunoprecipitated using biotinylated FJ-3 or FJ-7 followed by streptavidin. After separation on SDS-PAGE gel and transfer to nitrocellulose, blots were probed for either streptavidin (probe for inhibitor) or HA-hNHERF1 / EBP50. Both FJ-3 and FJ-7 appear to bind well to HA-hNHERF1 / EBP50. **(B)** Immunoprecipitation was performed as above with the substitution of monomeric avidin for streptavidin. Again, both FJ-3 and FJ-7 appear to bind well to HA-hNHERF1 / EBP50. **(C)** Competition assay using FJ-1, FJ-3, FJ-7, and FJ-9 small molecule inhibitors and beta 2 adrenergic receptor peptide to compete NSF from immobilized β_2AR tail. FJ-1 and FJ-7 are capable of competing away NSF from bound β_2AR, while structurally similar molecules, FJ-3 and FJ-9, are not.

Figure 7: Co-immunoprecipitation of HA-hNHERF1 / EBP50 and FLAG-β_2AR. Lysate from cells expressing both HA-hNHERF1 / EBP50 and FLAG-β_2AR was incubated with



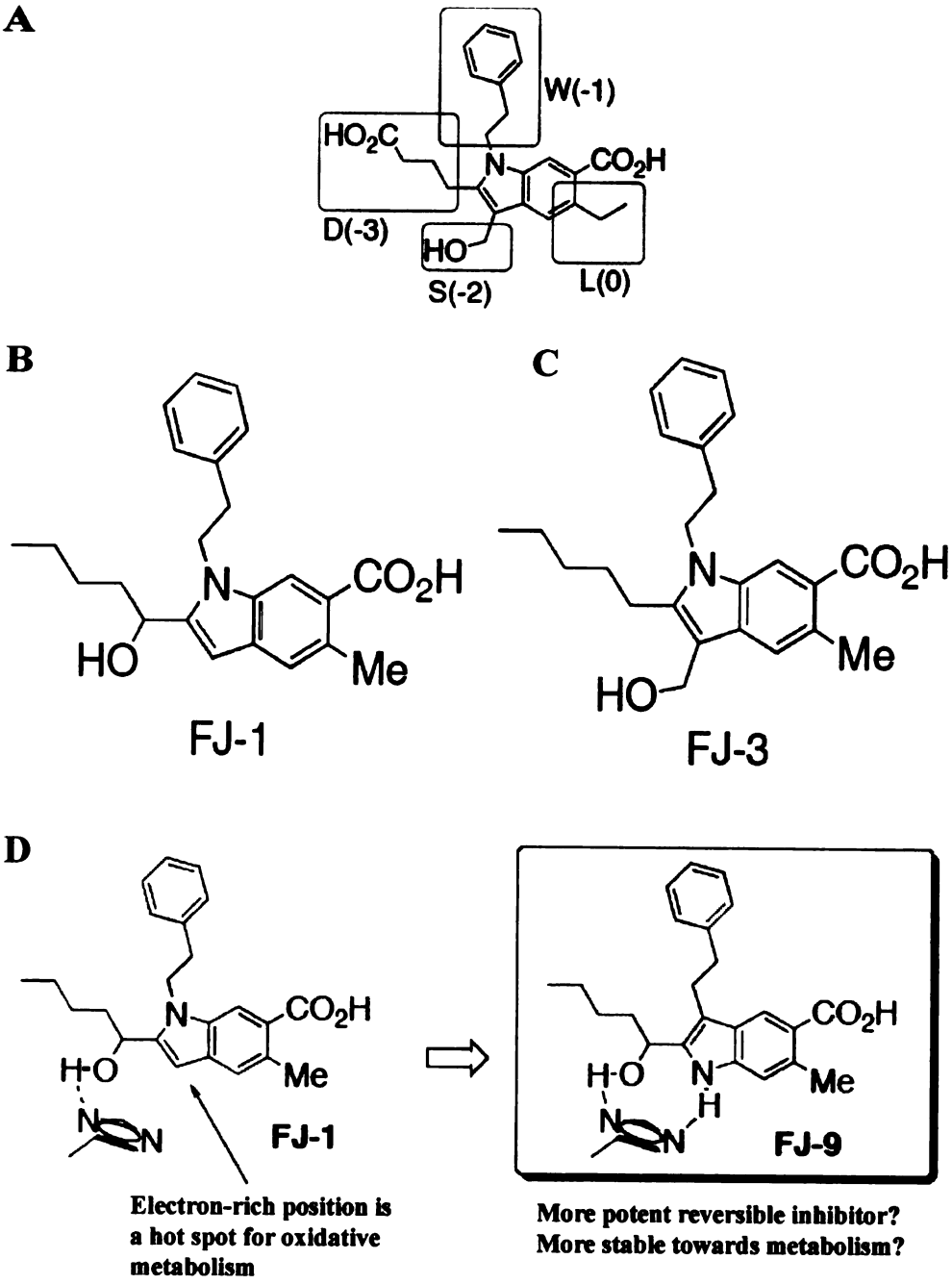
biotinylated FJ-3 or FJ-7 followed by immunoprecipitation as in figure 6B. Detection of immunoprecipitated HA-hNHERF1 / EBP50 and co-immunoprecipitated FLAG-β_2AR revealed that although both FJ-3 and FJ-7 strongly bind to HA-hNHERF1 / EBP50, only FJ-7 was capable of also pulling down FLAG-β_2AR. This suggests that the inhibitors may bind to disparate PDZ domains on HA-hNHERF1 / EBP50.

Figure 8: Receptor degradation. HEK-293 cells overexpressing the indicated receptor were pre-treated with the indicated concentration of FJ-1 PDZ inhibitor. The appropriate agonist (isoproterenol, iso or DADLE) was added for zero or four hours. FJ-1 did not increase the degradation rate of β_2AR. At the highest concentration used, 300 μM, FJ-1 decreased the degradation rate of the delta opioid receptor and the chimeric δ/β_2AR receptor.



Figures

Figure 1:



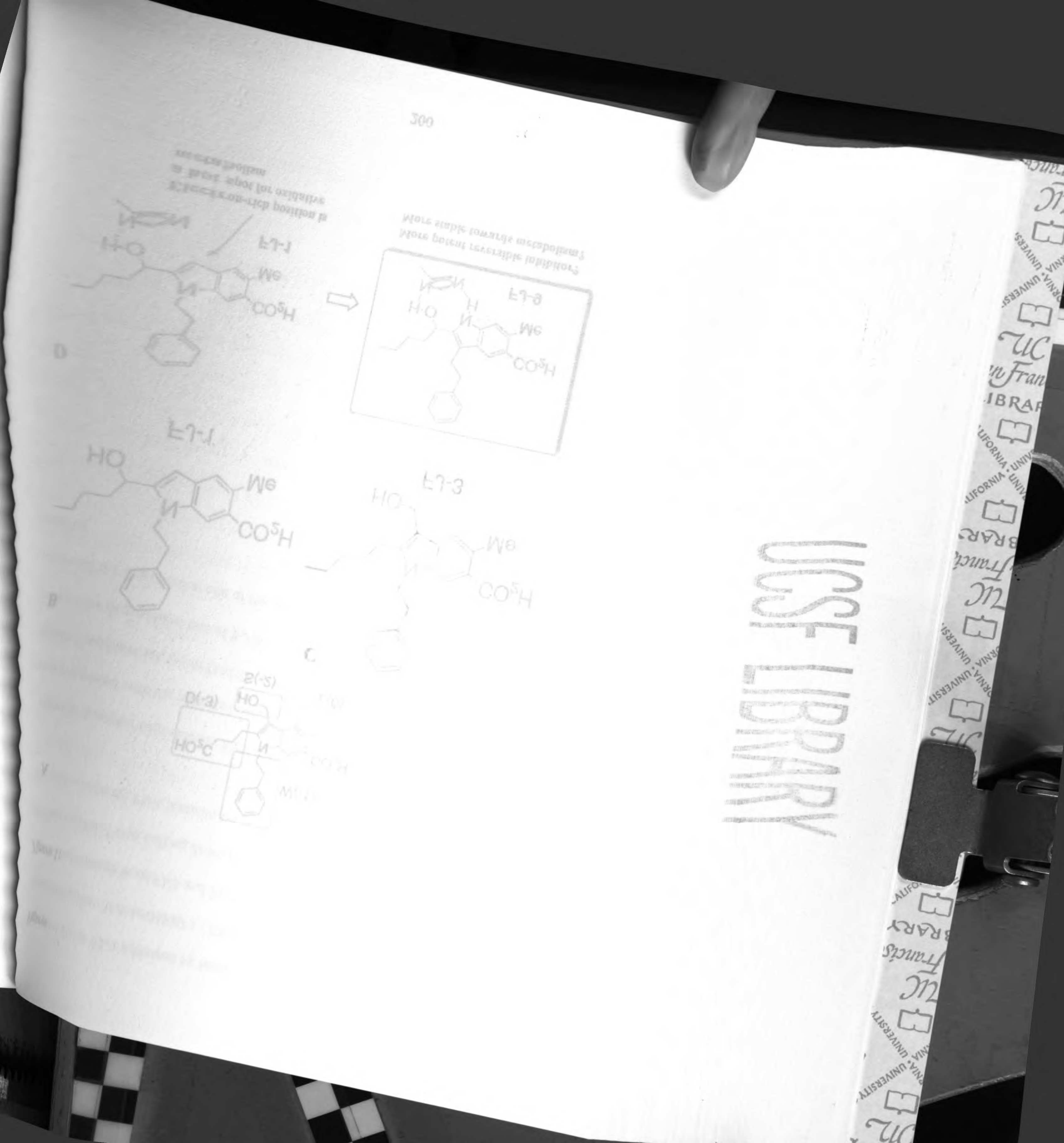
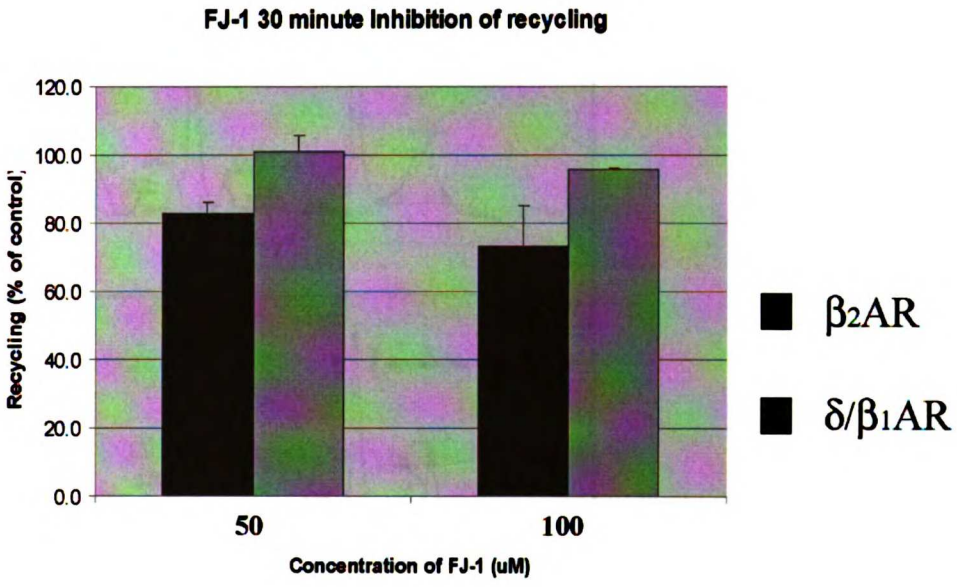
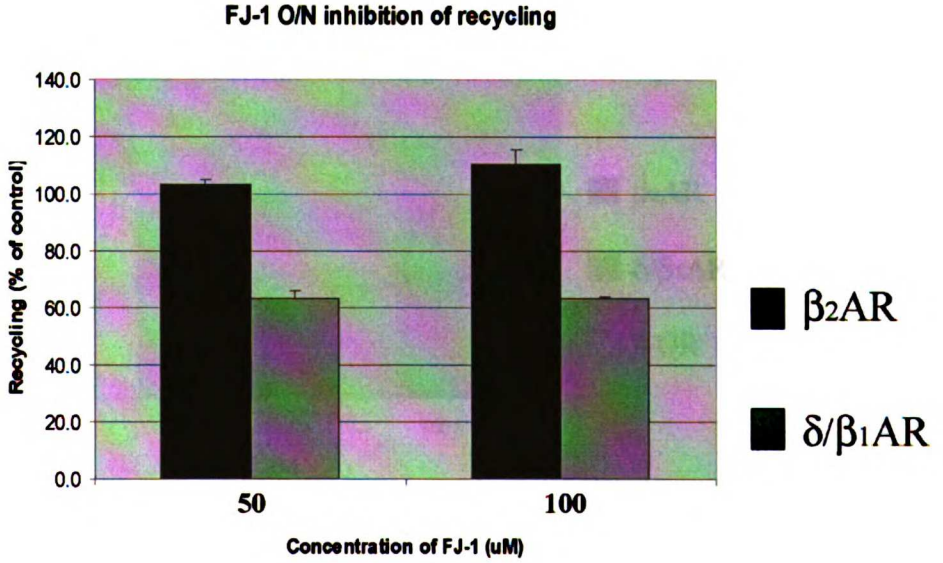


Figure 2:

A


FJ-1 30 minute inhibition of recycling
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Recycling (% of control)
50
100
Concentration of FJ-1 (uM)
β2AR
δ/β1AR

B


FJ-1 O/N inhibition of recycling
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Recycling (% of control)
50
100
Concentration of FJ-1 (uM)
β2AR
δ/β1AR

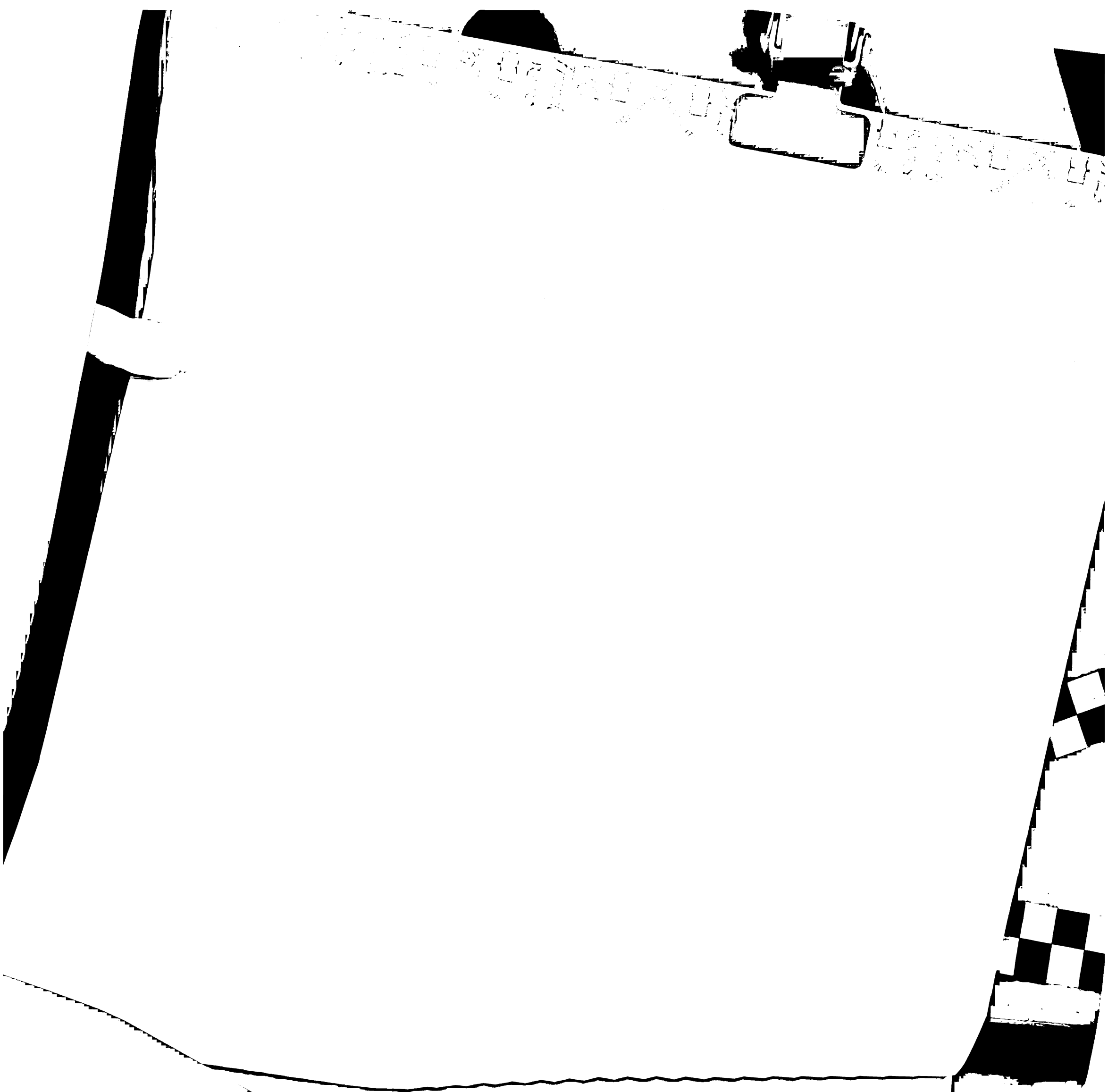
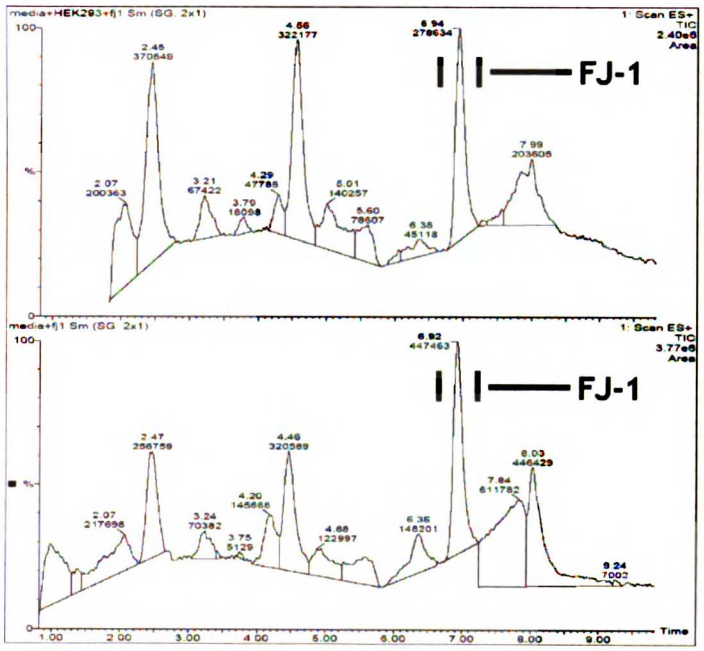


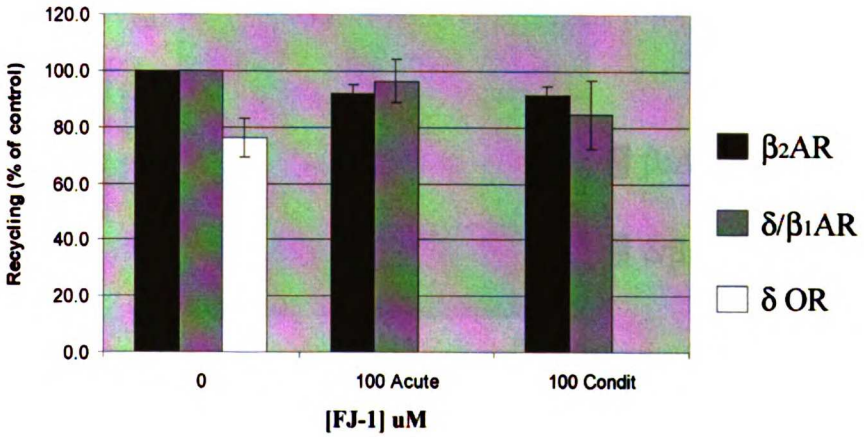
Figure 3:

A



B

Media conditioned with 100 uM FJ-1 does not significantly decrease recycling



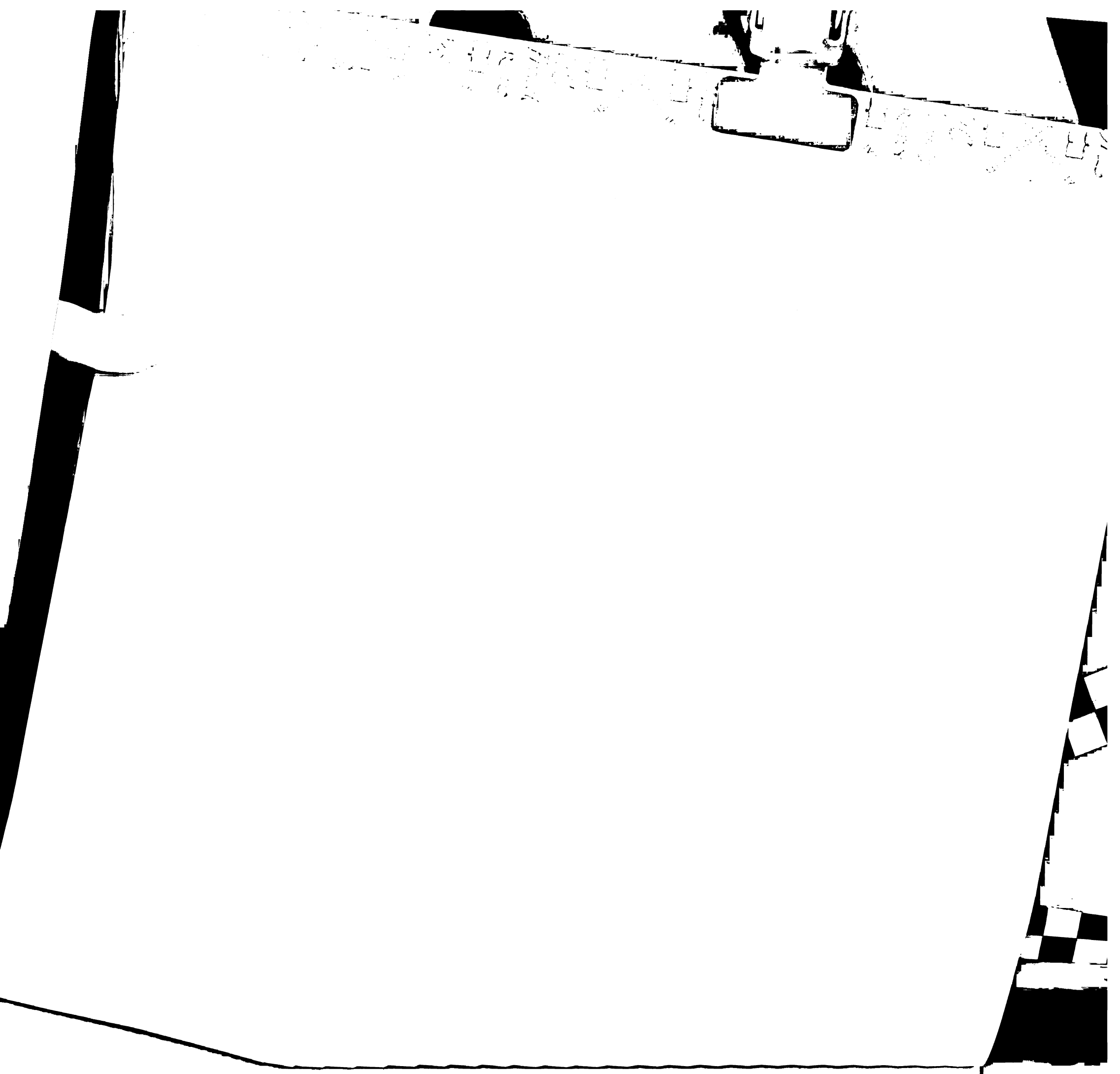
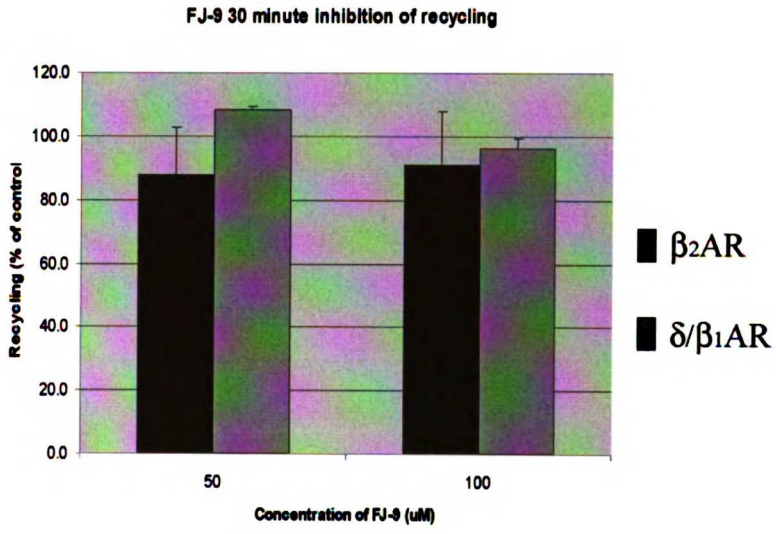
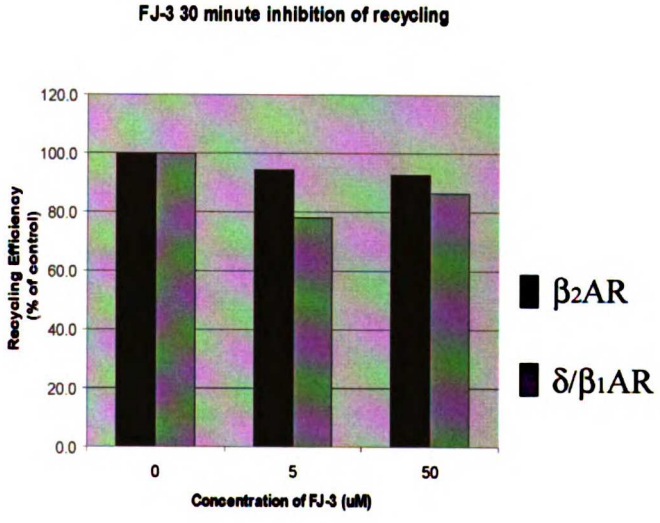


Figure 4:

A


FJ-9 30 minute inhibition of recycling
Recycling (% of control)
120.0
100.0
80.0
60.0
40.0
20.0
0.0
50
100
Concentration of FJ-9 (uM)
β2AR
δ/β1AR

B


FJ-3 30 minute inhibition of recycling
Recycling Efficiency (% of control)
120.0
100.0
80.0
60.0
40.0
20.0
0.0
0
5
50
Concentration of FJ-3 (uM)
β2AR
δ/β1AR

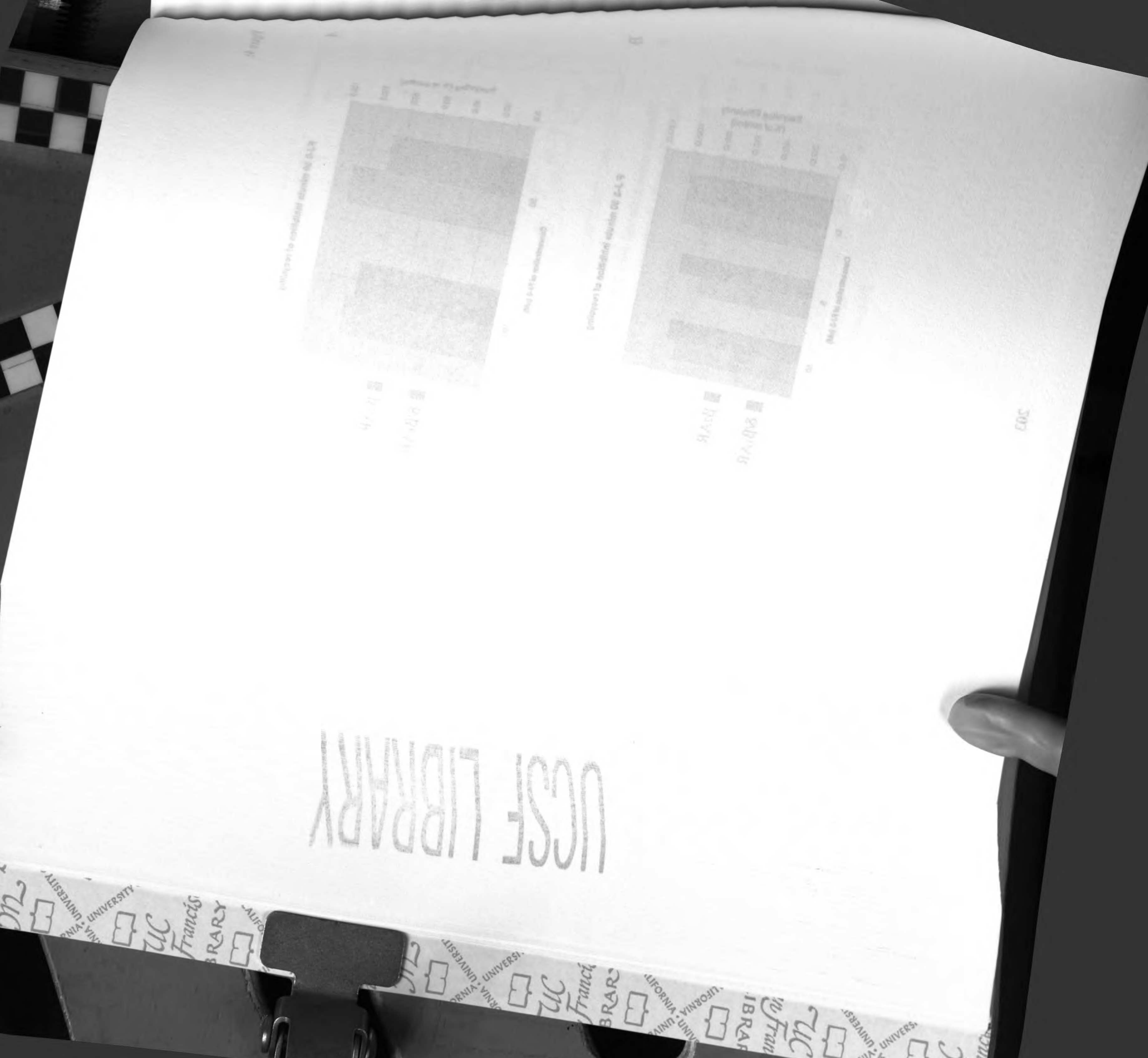
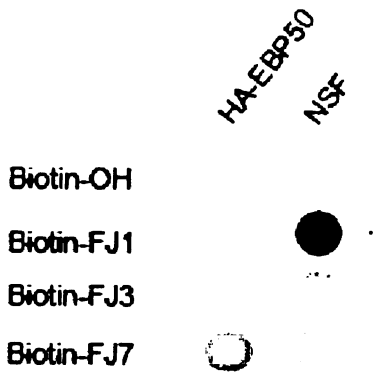


Figure 5:



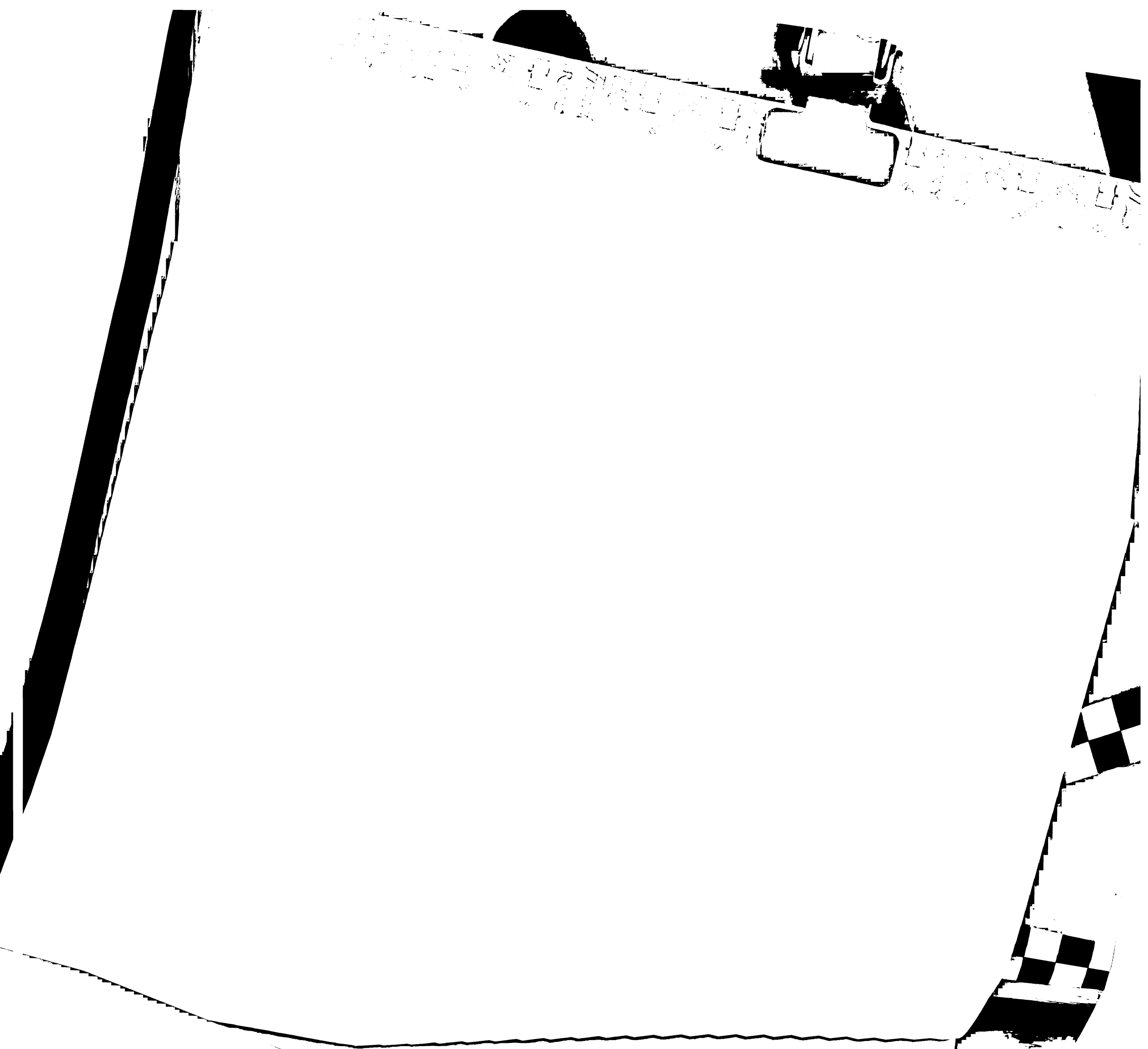
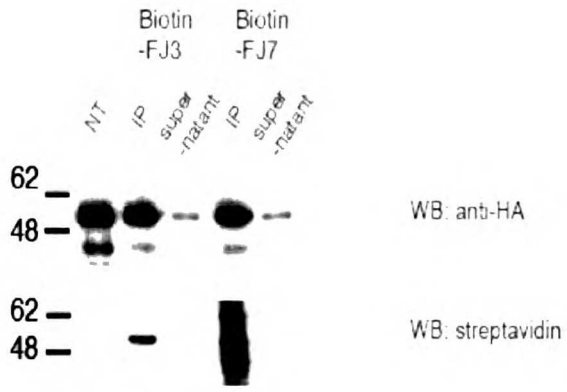
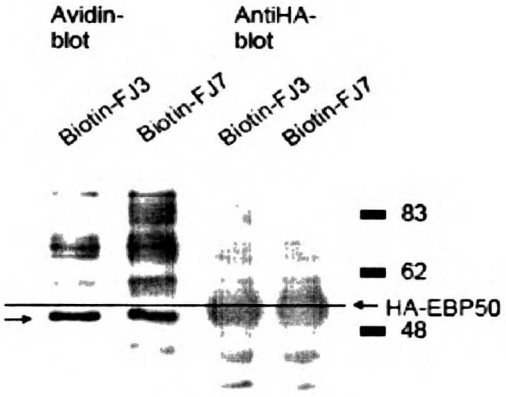


Figure 6:

A


Biotin -FJ3
Biotin -FJ7
NT
IP
super -natant
IP
super -natant
62
48
62
48
WB: anti-HA
WB: streptavidin

B


Avidin-blot
AntiHA-blot
Biotin-FJ3
Biotin-FJ7
Biotin-FJ3
Biotin-FJ7
83
62
HA-EBP50
48

C

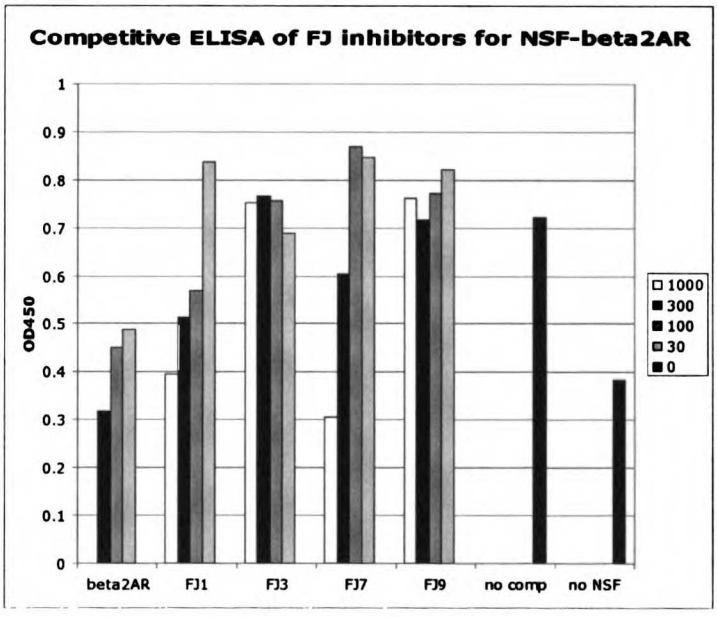
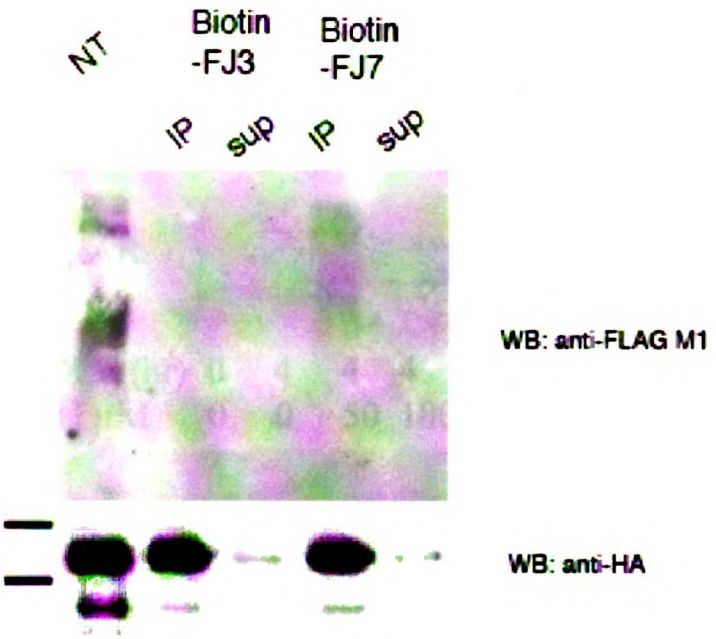

Competitive ELISA of FJ inhibitors for NSF-beta2AR
OD450
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
beta2AR
FJ1
FJ3
FJ7
FJ9
no comp
no NSF
1000
300
100
30
0

Figure 7:


NT
Biotin
-FJ3
Biotin
-FJ7
IP
sup
IP
sup
WB: anti-FLAG M1
WB: anti-HA

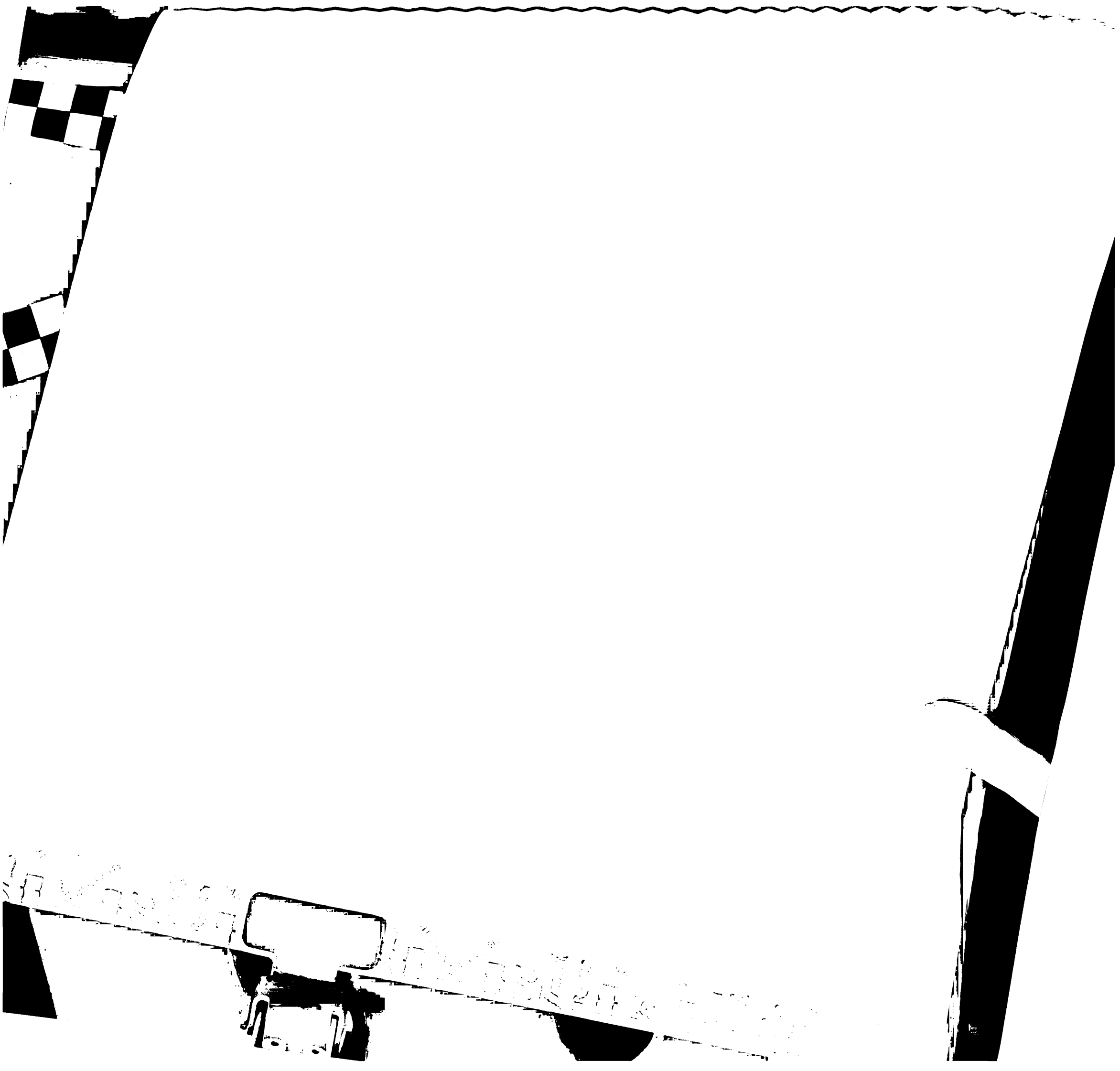
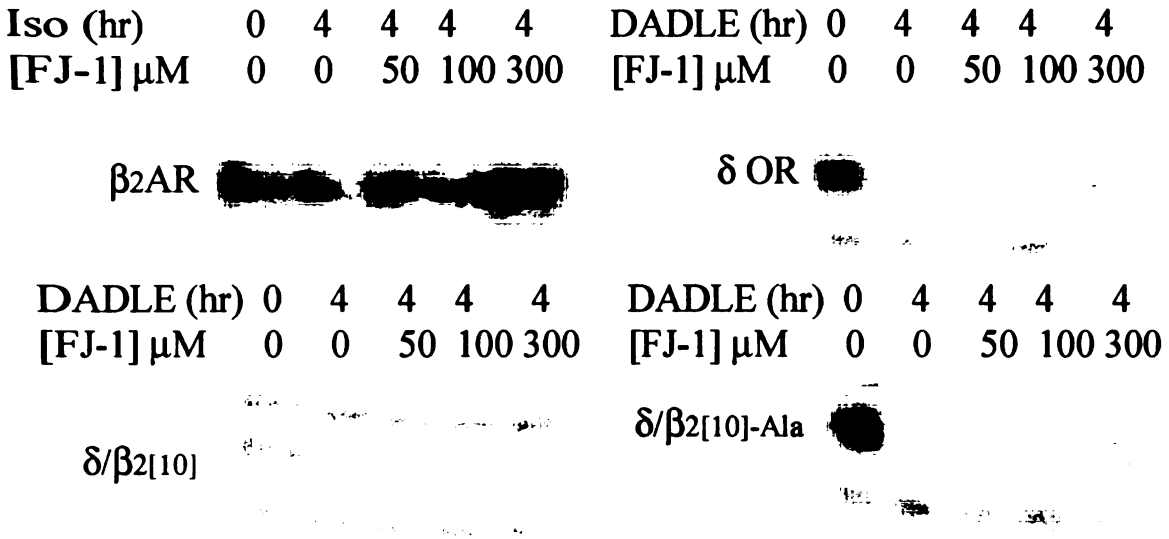
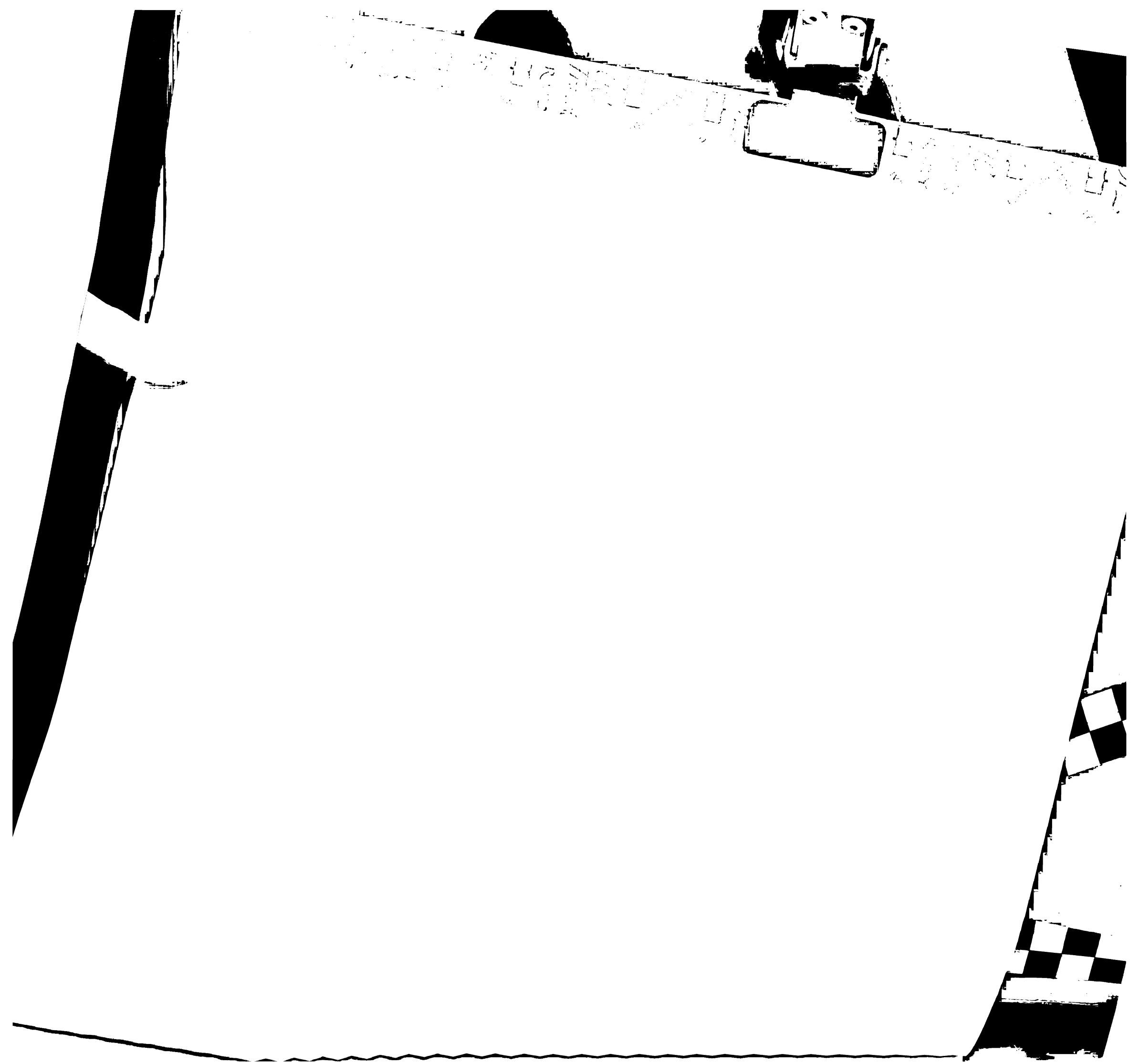


Figure 8:


Iso (hr) 0 4 4 4 4
[FJ-1] μM 0 0 50 100 300
β2AR
DADLE (hr) 0 4 4 4 4
[FJ-1] μM 0 0 50 100 300
δ OR
DADLE (hr) 0 4 4 4 4
[FJ-1] μM 0 0 50 100 300
δ/β2[10]
DADLE (hr) 0 4 4 4 4
[FJ-1] μM 0 0 50 100 300
δ/β2[10]-Ala

Appendix 2: Vassopressin 2 Receptor (V_2R) [Dominance of Slow Recycling over Rapid Signal Mediated Recycling]

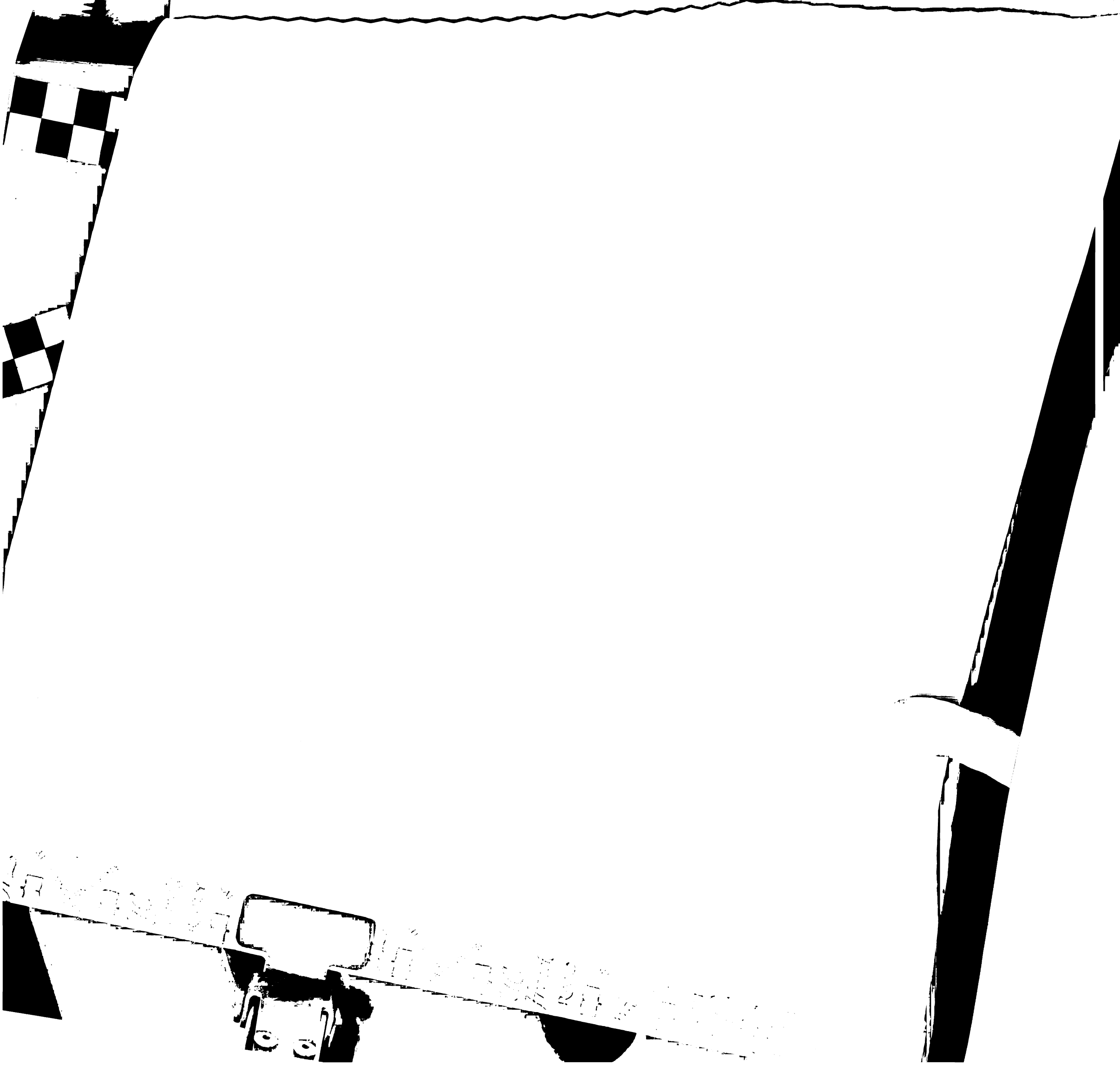
All work presented in this appendix was performed by Robert Michael Gage.



Introduction

Following stimulation by their cognate agonist, many G protein-coupled receptors (GPCRs) undergo endocytosis via a β-Arrestin and dynamin dependent mechanism (1-4). After internalization into clathrin coated vesicles, receptors can rapidly ($t_{½}$ <15 min) return to the plasma membrane and become functionally resensitized (3,4). Alternately, many receptors are targeted to lysosomes where they are degraded and this can lead to the functionally opposite process of down-regulation (5-7). Yet a third possibility exhibited by so called class I B receptors, including the vassopressin 2 receptor (V2R), is for the receptor containing vesicles to retain β-Arrestin which normally dissociates rapidly from endocytosed vesicles and then slowly ($t_{½}$ >2 hr) recycle back to the plasma membrane after passing through a peri-nuclear compartment (8). Often, there are specific cytoplasmic determinants present in a receptor which govern its endocytic trafficking route.

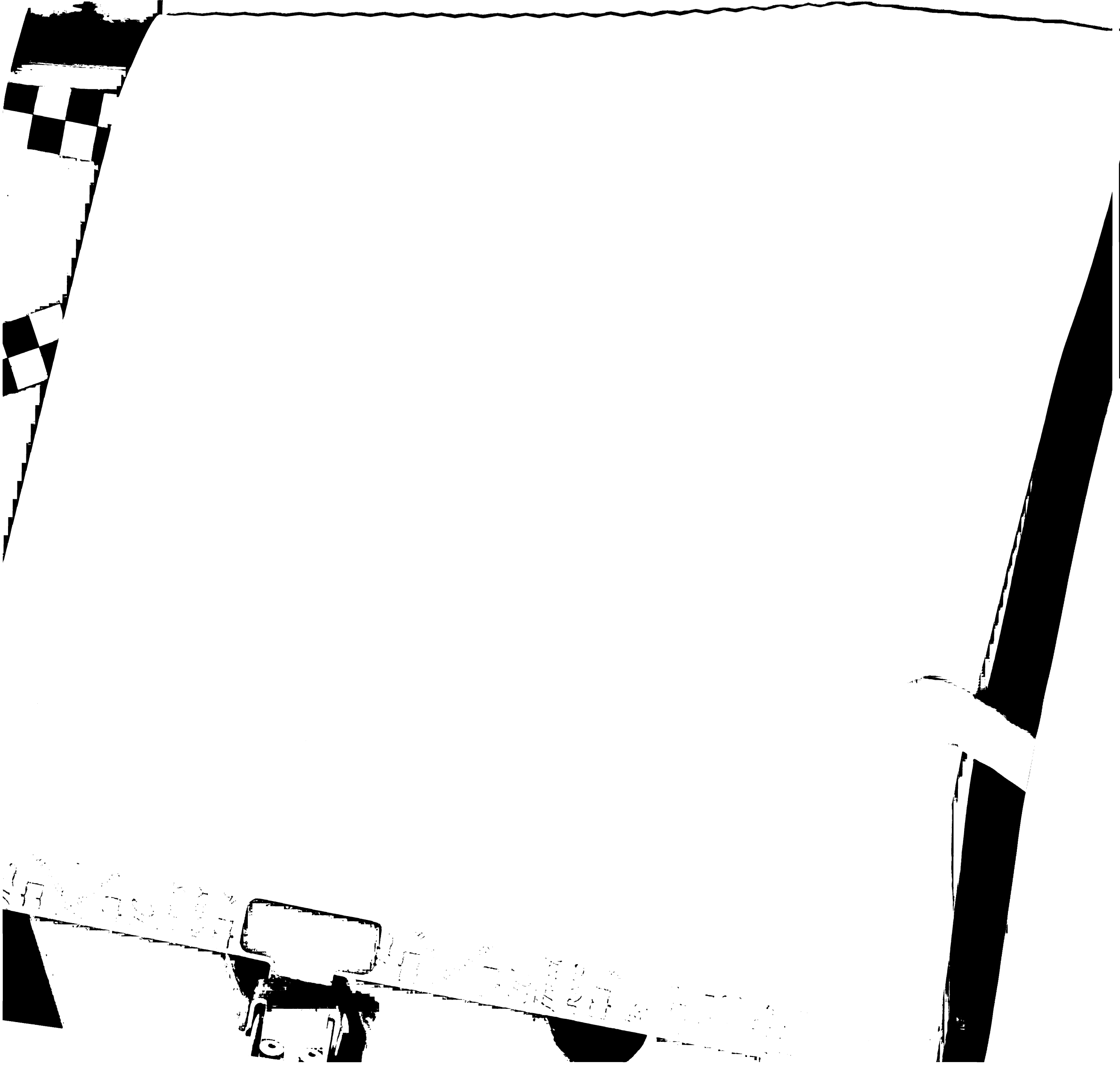
For example, the β_2 adrenergic (β_2AR) and the μ opioid (μ OR) receptors have carboxyl-terminal tail sequences which are essential for routing them into a rapidly recycling pathway (9-12). Although the sequences which govern recycling of these two receptors differ in sequence, location, and binding partners, they may share a common mechanism for enhancing recycling efficiency. In contrast, the V2R recycles more slowly as it transits the endocytic pathway through a peri-nuclear recycling compartment (13). A cluster of ser/thr residues in the V2R were shown to be crucial in maintaining β-Arrestin binding to vasopressin receptor containing vesicles and routing the receptors on a slow recycling pathway (13-15). Receptors lacking this cluster rapidly recycle to the plasma membrane following agonist induced endocytosis (13). The carboxyl terminal



tail of the vasopressin receptor was sufficient to confer a class I B phenotype on the β_2 adrenoreceptor during a tail swapping experiment (15). As mentioned previously, the fate of agonist stimulated receptors is important for their pharmacological responses and thus the study of the factors influencing a receptor's fate may be useful in the search for better, longer acting, and more selective drugs.

Rapidly recycling receptors are thought to be returned to the plasma membrane in a state capable of further signaling (3,4) while the receptors which are alternatively targeted to lysosomes usually exhibit a decrease in their ability to signal (5-7). It has been proposed that the extended route of class I B receptors and in particular the retention of the scaffolding molecule β-Arrestin on receptor containing vesicles could lead to prolonged signaling from these internal vesicles (16). β-Arrestin is known to bind to signaling molecules including MAPKs (mitogen-activated protein kinases) such as JNK3 (c-Jun N-terminal kinase 3), ERK1/2 (extracellular-signal-regulated kinase 1/2) and p38 MAPKs (16).

The present study seeks to determine whether the rapid recycling sequence from the β_2AR can override the class I B, β-Arrestin interaction stabilizing, motif from the V2R and lead to a rapidly recycling version of the vasopressin receptor. This rapid recycling sequence was previously shown to be sufficient to re-route a heterologous GPCR, the δ opioid receptor, from a degradative pathway (9). These data show that a chimeric V2R receptor with the last ten carboxyl residues from the β_2AR traffics in a manner indistinguishable from wild-type V2R. Thus it appears that the slow recycling signal of the vasopressin receptor is dominant over the rapid recycling signal of the β_2AR.



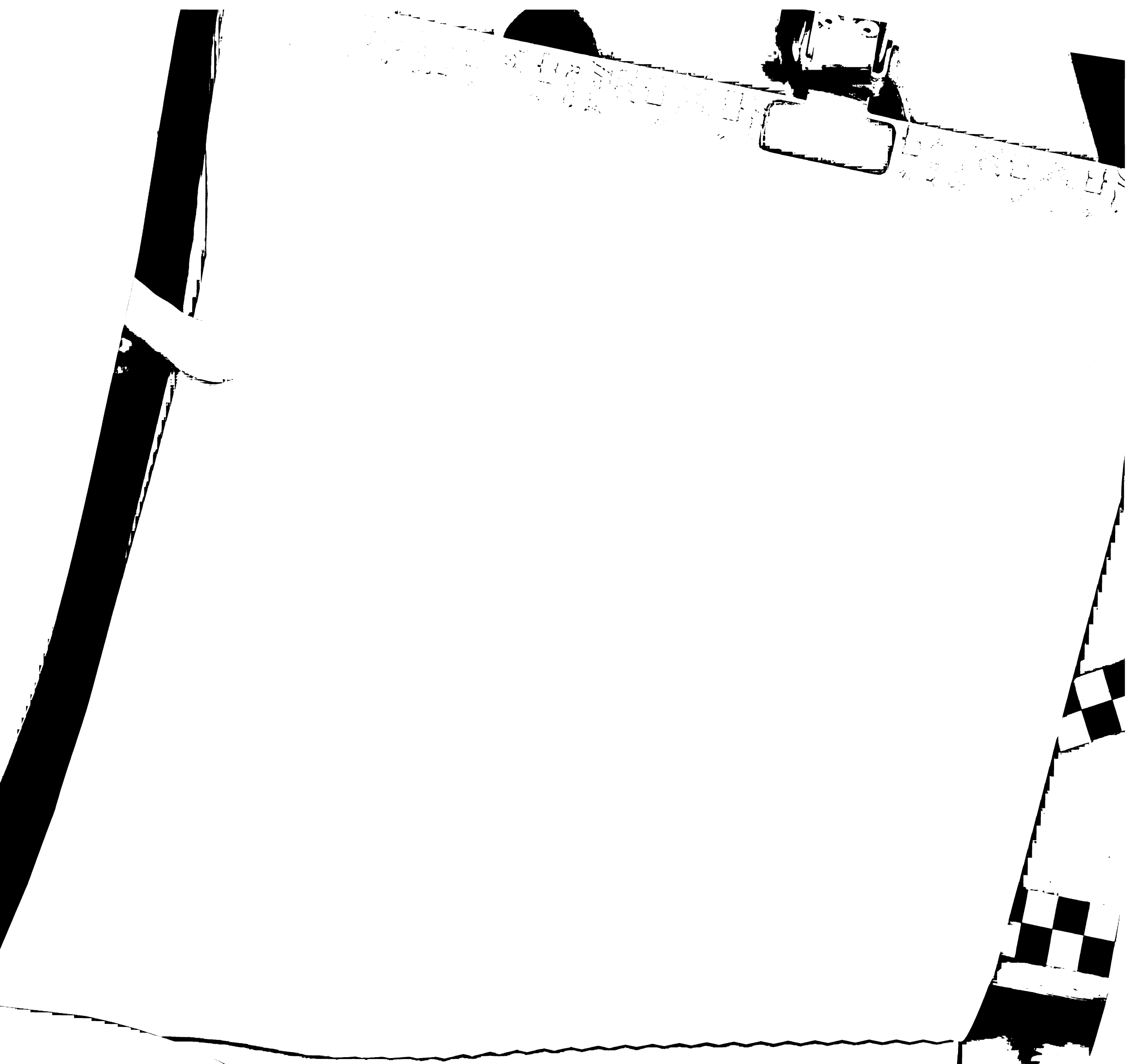
Experimental Procedures

cDNA Constructs and Mutagenesis

Several epitope-tagged versions of the cloned human vasopressin 2 receptor (V2R (17)) were used in these studies: mutant receptors containing a FLAG epitope in the amino-terminal extracellular domain (SF-V2R) were described previously and demonstrated to be functional (17). A mutant receptor containing a FLAG epitope in the amino-terminal extracellular domain and a point mutation (S370G) that added a BamH I site to the carboxyl terminus was generated by oligonucleotide (Operon) directed Quickchange (Stratagene) mutagenesis. Mutant vasopressin 2 receptors containing a FLAG epitope in the amino-terminal extracellular domain, the S370G point mutation and the addition of either the ten carboxyl-terminal residues from the β_2AR (NH_2-RNCSTNDSLL-COOH) or the ten residues plus an alanine (NH_2-RNCSTNDSLLA-COOH). This was accomplished by insertion of a synthetic linker-adapter (Operon Technologies) encoding the ten-residue or eleven-residue sequence followed by a stop codon into the BamH I site engineered into the 3' end of the sequence encoding the V2R tail.

Cell Culture and Transfection

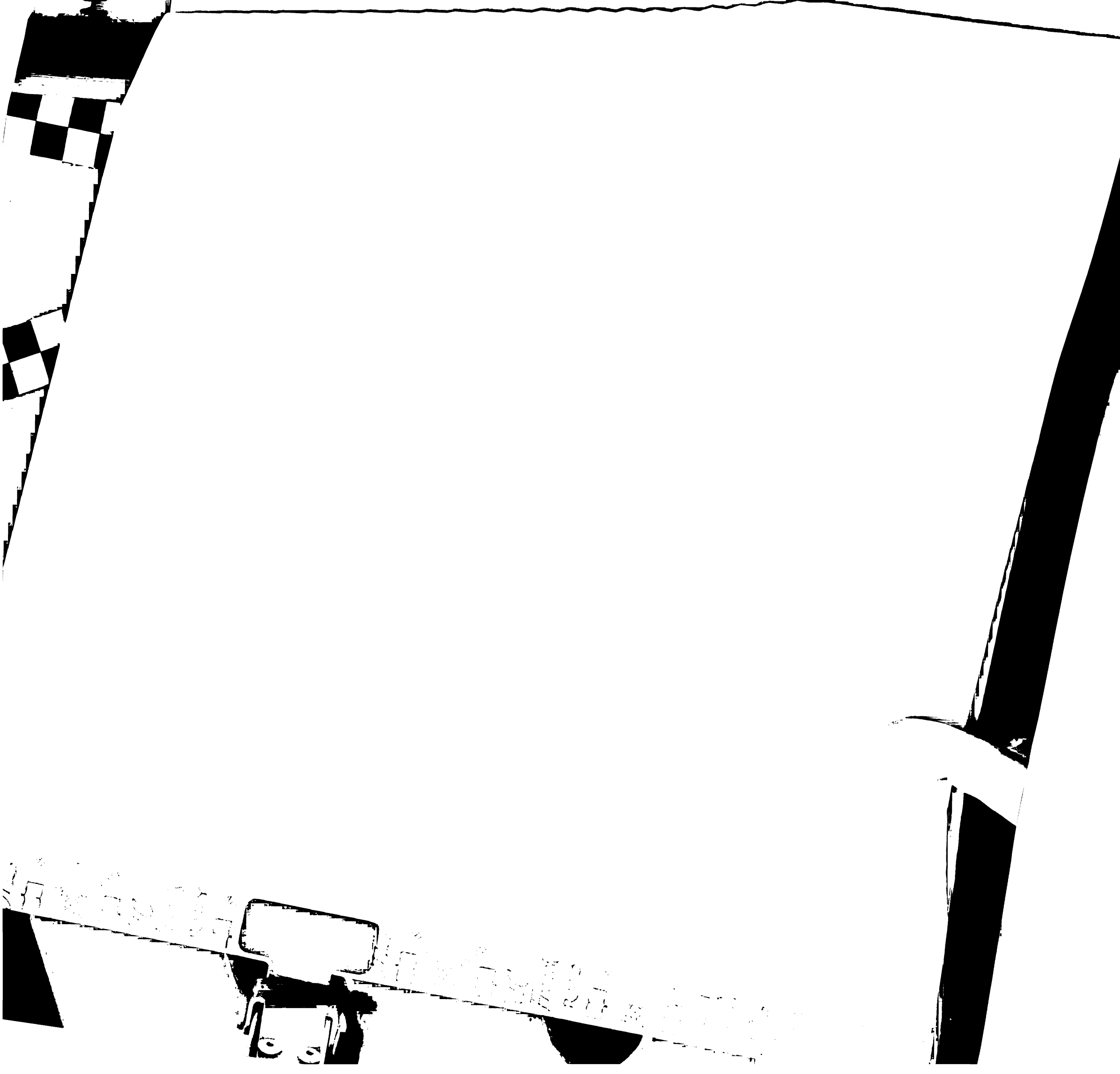
Human embryonic kidney 293 cells (ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (University of California San Francisco Cell Culture Facility). Cells grown in 6-cm dishes were transfected with ~5 ug of plasmid DNA containing the indicated receptor by calcium phosphate precipitation (7,18). For studies of receptor trafficking in transiently transfected cells, cells were transfected as above, plated onto coverslips 24 h post transfection and



experiments were conducted 48 h post transfection. Stably transfected cells expressing epitope tagged receptors were generated by transfecting 293 cells in 6-cm dishes as above. Cell clones expressing transfected receptors were selected in 500 ug/ml Geneticin (Life Technologies, Inc.) and colonies were isolated.

Examination of Receptor Endocytosis and Recycling by Fluorescence Microscopy

Endocytic trafficking of receptors was visualized by fluorescence microscopy using a minor modification of a previously described method (11). Briefly, stably or transiently transfected 293 cells expressing the indicated receptor were grown on glass coverslips (Corning). The cells were treated at the same time (37 °C for 25 min) in the presence of 10 mM [Arg^8] Vasopressin (AVP, Bachem or Sigma). Following this incubation, cells were either fixed immediately, for determining internalization of FLAG-tagged receptors, or were subsequently washed twice in DMEM supplemented with 10% fetal bovine serum. After washing in DMEM, the cells were further incubated in DMEM (for an additional 60 or 120 min. at 37°C) to allow receptor recycling to occur before the cells were fixed. The cells were fixed with 3.7% formaldehyde in PBS, pH 7.4, for 10 min and then quenched with three washes of TBS with 1 mM CaCl2. Specimens were permeabilized with 0.1% Triton X-100 (Sigma) in Blotto (3% dry milk in TBS with 1 mM $CaCl_2$) and incubated with M1 anti-FLAG antibody (2.5 ug/ml, Sigma) at for 30 min to label receptors and washed 3X in TBS. M1 labeled receptors were secondarily labeled with fluorescein isothiocyanate-conjugated donkey anti-mouse secondary antibody (1:500 dilution; Jackson ImmunoResearch) for 30 min to detect FLAG-tagged receptors. Conventional fluorescence microscopy was performed using inverted Nikon Diaphot

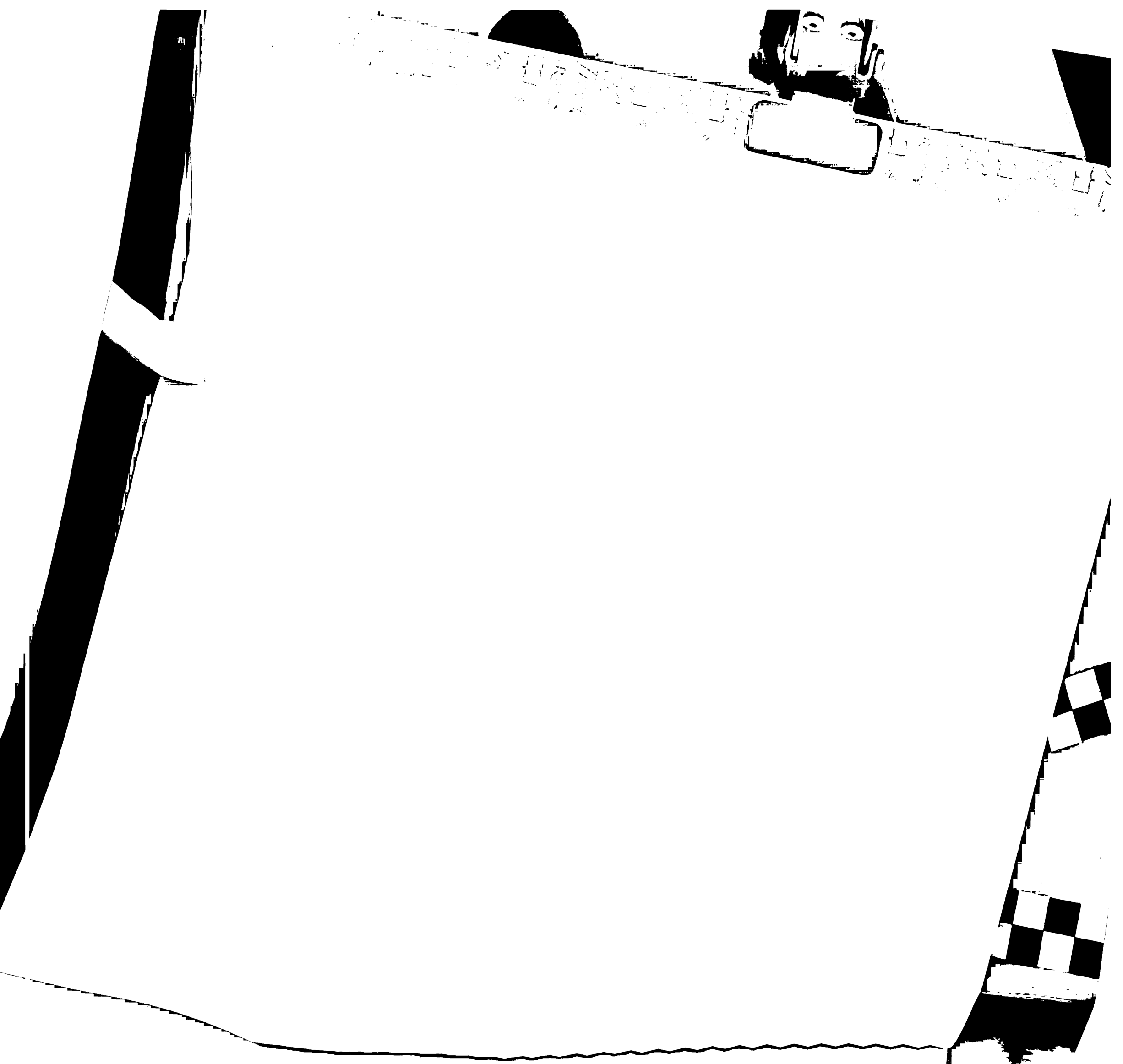


microscope equipped with a Nikon 60X NA1.4 objective and epifluorescence optics; confocal fluorescence microscopy was carried out using a Bio-Rad MRC 1000 and a Zeiss 100X NA1.3 objective. Images were collected using a 12-bit cooled charge-coupled device camera (Princeton Instruments) interfaced to a Macintosh computer.

Results

A variant vasopressin 2 receptor was created to facilitate the addition of amino acid sequences from the β_2AR. This resulted in the change of the serine at position 370 to a glycine (-DTSS-COOH to –DTGS-COOH). This serine is distinct from the ser/thr cluster (at positions from 345-364) associated with increased β-arrestin binding and slow recycling. As seen in figure 1, this mutation did not affect receptor internalization as previously described for the wild-type receptor (14,17). The receptor did not recycle rapidly back to the plasma membrane following ligand-induced internalization and agonist washout. Thus the behavior of SSF-V2R (S393G) appears identical to that previously described for wild-type vasopressin 2 receptor (13,14).

A Chimeric vasopressin 2 receptor containing the last ten residues of the β_2AR was constructed and assayed for agonist-induced internalization and rapid recycling. The fluorescence microscopy data shown in figure 1 shows that in contrast to results obtained with the δ opioid receptor, the β_2AR derived recycling sequence is insufficient to promote the rapid recycling of the V2R. This chimeric receptor internalized normally and exhibits behavior similar to the wild-type vasopressin 2 receptor, remaining in internal vesicles after [Arg^8] vasopressin treatment and up to 120 minutes of agonist washout. The further addition of an alanine residue to the carboxyl-terminal sequence did not alter receptor behavior following AVP application.

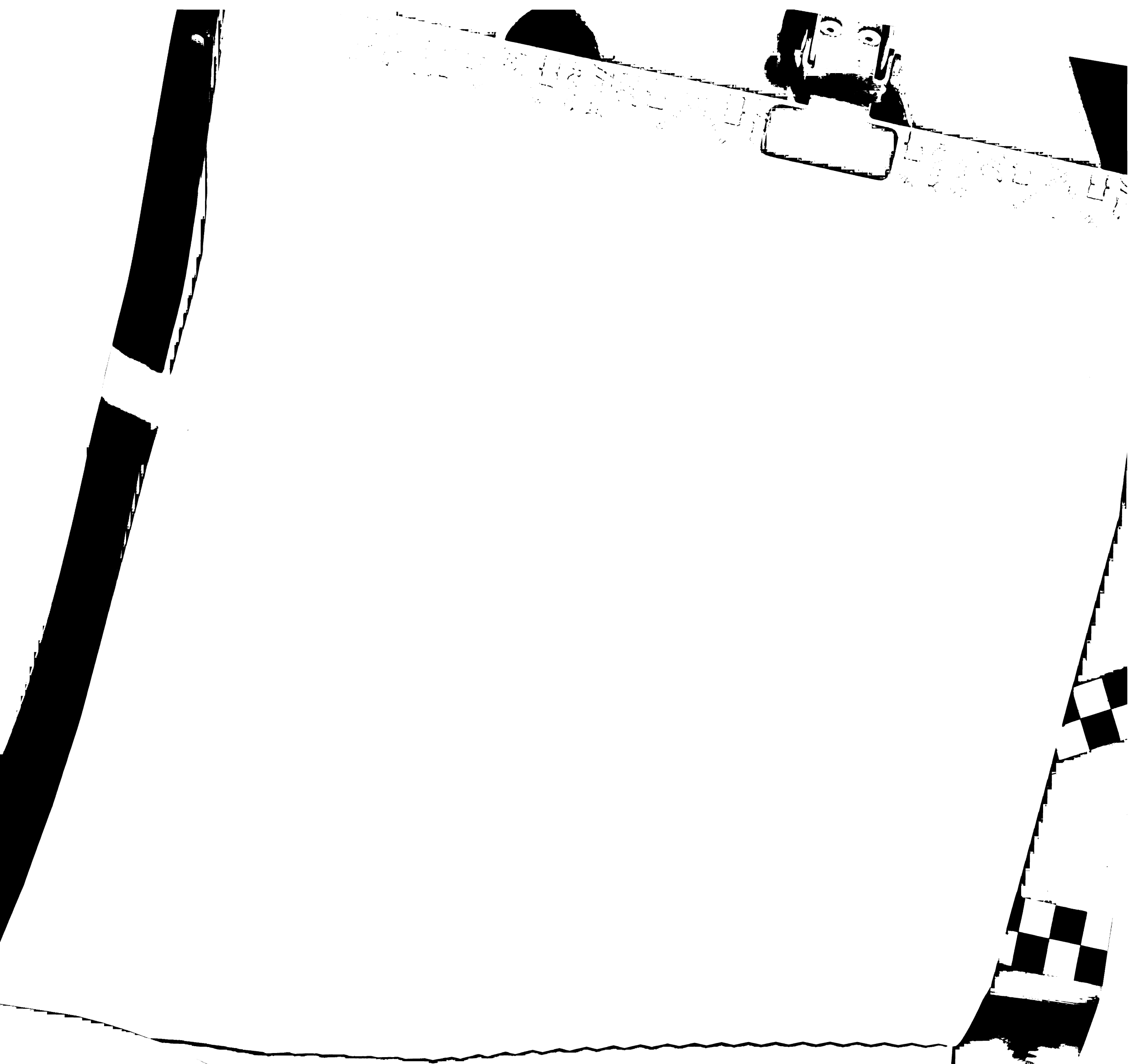


Discussion

It appears that while the recycling sequence taken from the β_2AR is capable of specifically increasing the recycling efficiency of the δ opioid receptor, it is incapable of enhancing the recycling of the V2R. Replacing the entire carboxyl-terminal tail of the V2R with the tail from the β_2AR results in a mutant vasopressin 2 receptor which now acts as a rapidly recycling class A receptor (15). The converse receptor, consisting of the β_2AR with the tail from the V2R, internalizes into vesicles which retain binding to β-arrestin and no longer recycle rapidly (15). So while it is possible to swap the arrestin binding and recycling characteristics of the two receptors by physically replacing the entire tail region, appending the rapid recycling sequence from the β_2AR to the end of the V2R tail is insufficient to alter the membrane trafficking phenotype of the V2R. The slow recycling phenotype of the wild-type vasopressin 2 receptors appears to be dominant to the rapid recycling sequence from the β_2AR. Perhaps in order for the PDZ-ligand mediated rapid recycling sequence to function correctly, it must have unimpeded access to the receptor tail. By enhancing binding to β-arrestin, the ser/thr cluster in the V2R tail may also preclude interaction with other proteins such as hNHERF / EBP50 after internalization. Thus the inability of the β_2 adrenergic receptor derived recycling sequence to function in the context of the full length vasopressin 2 receptor may be explained by a competition between β-arrestin and the PDZ domain containing protein responsible for the rapid recycling of the β_2AR.

References

1. von Zastrow, M., and Kobilka, B. K. (1994) *J Biol Chem* **269**, 18448-18452.
2. Goodman, O. B., Jr., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1998) *Advances in Pharmacology* **42**, 429-433
3. Ferguson, S. S., Zhang, J., Barak, L. S., and Caron, M. G. (1998) *Life Sci* **62**, 1561-1565
4. Lefkowitz, R. J., Pitcher, J., Krueger, K., and Daaka, Y. (1998) *Advances in Pharmacology* **42**, 416-420
5. Law, P.-Y., Hom, D. S., and Loh, H. H. (1984) *J. Biol. Chem.* **259**, 4096-4104
6. Law, P. Y., and Loh, H. H. (1999) *Journal of Pharmacology and Experimental Therapeutics* **289**, 607-624
7. Tsao, P., Cao, T., and von Zastrow, M. (2001) *Trends Pharmacol Sci* **22**, 91-96.
8. Innamorati, G., Sadeghi, H., and Birnbaumer, M. (1999) *Journal of Receptor and Signal Transduction Research* **19**, 315-326
9. Gage, R. M., Kim, K. A., Cao, T. T., and von Zastrow, M. (2001) *J Biol Chem* **276**, 44712-44720.
10. Gage, R. M., Matveeva, E. A., Whiteheart, S. W., and von Zastrow, M. (2005) *J. Biol. Chem.* **280**, 3305-3313
11. Cao, T. T., Deacon, H. W., Reczek, D., Bretscher, A., and von Zastrow, M. (1999) *Nature* **401**, 286-290.
12. Tanowitz, M., and von Zastrow, M. (2003) *J. Biol. Chem.* **278**, 45978-45986



13. Innamorati, G., Sadeghi, H. M., Tran, N. T., and Birnbaumer, M. (1998) *Proceedings of the National Academy of Sciences of the United States of America* **95**, 2222-2226

14. Innamorati, G., Le Gouill, C., Balamotis, M., and Birnbaumer, M. (2001) *Journal of Biological Chemistry* **276**, 13096-13103

15. Oakley, R. H., Laporte, S. A., Holt, J. A., Barak, L. S., and Caron, M. G. (1999) *Journal of Biological Chemistry* **274**, 32248-32257

16. Shenoy SK, L. R. (2003) *Biochem J.* **375(Pt 3)**, 503-515

17. Klein, U., Muller, C., Chu, P., Birnbaumer, M., and von Zastrow, M. (2001) *J Biol Chem* **276**, 17442-17447.

18. Okayama, H., and Berg, P. (1983) *Molecular and Cellular Biology* **3**, 280-289

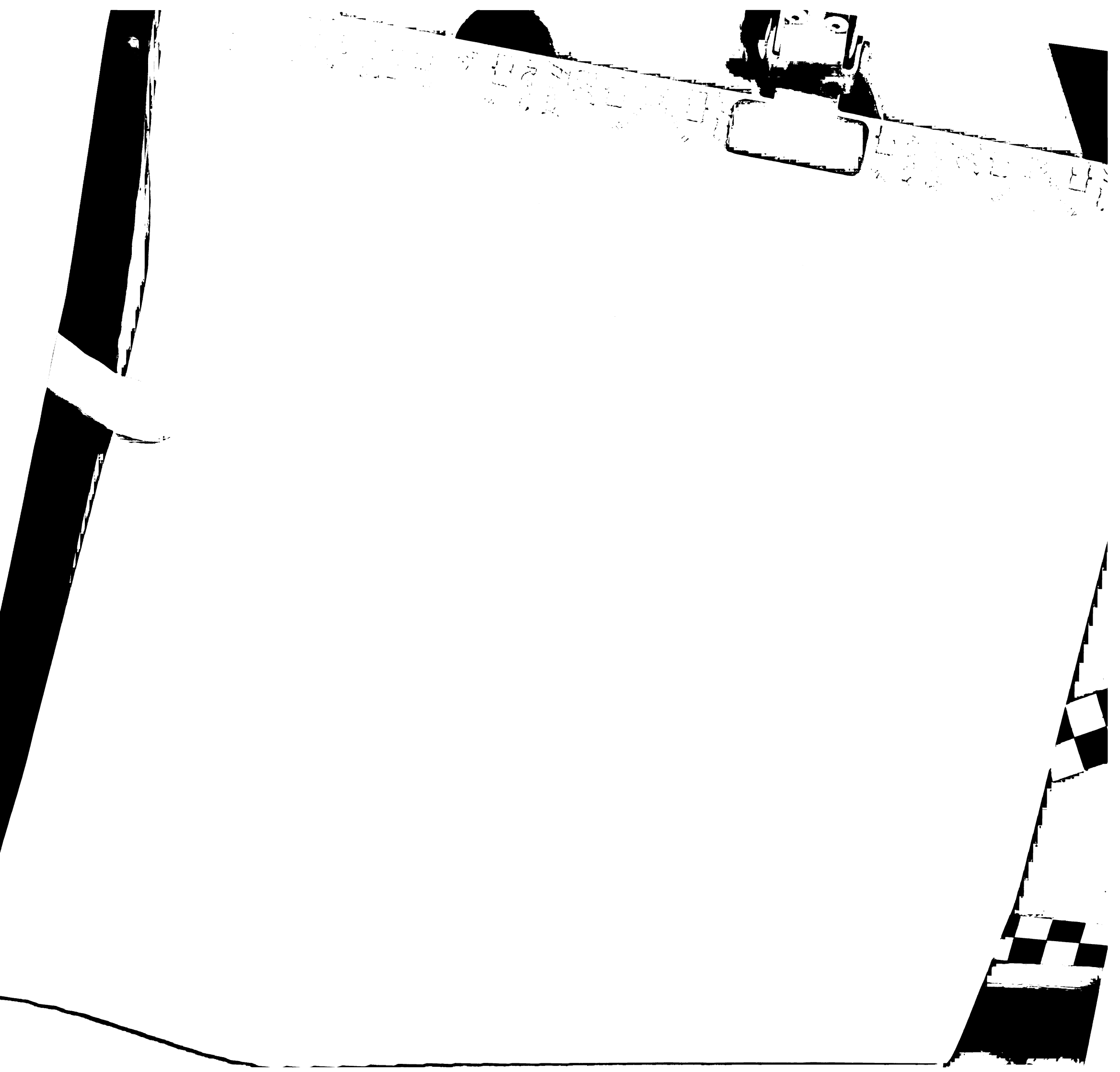
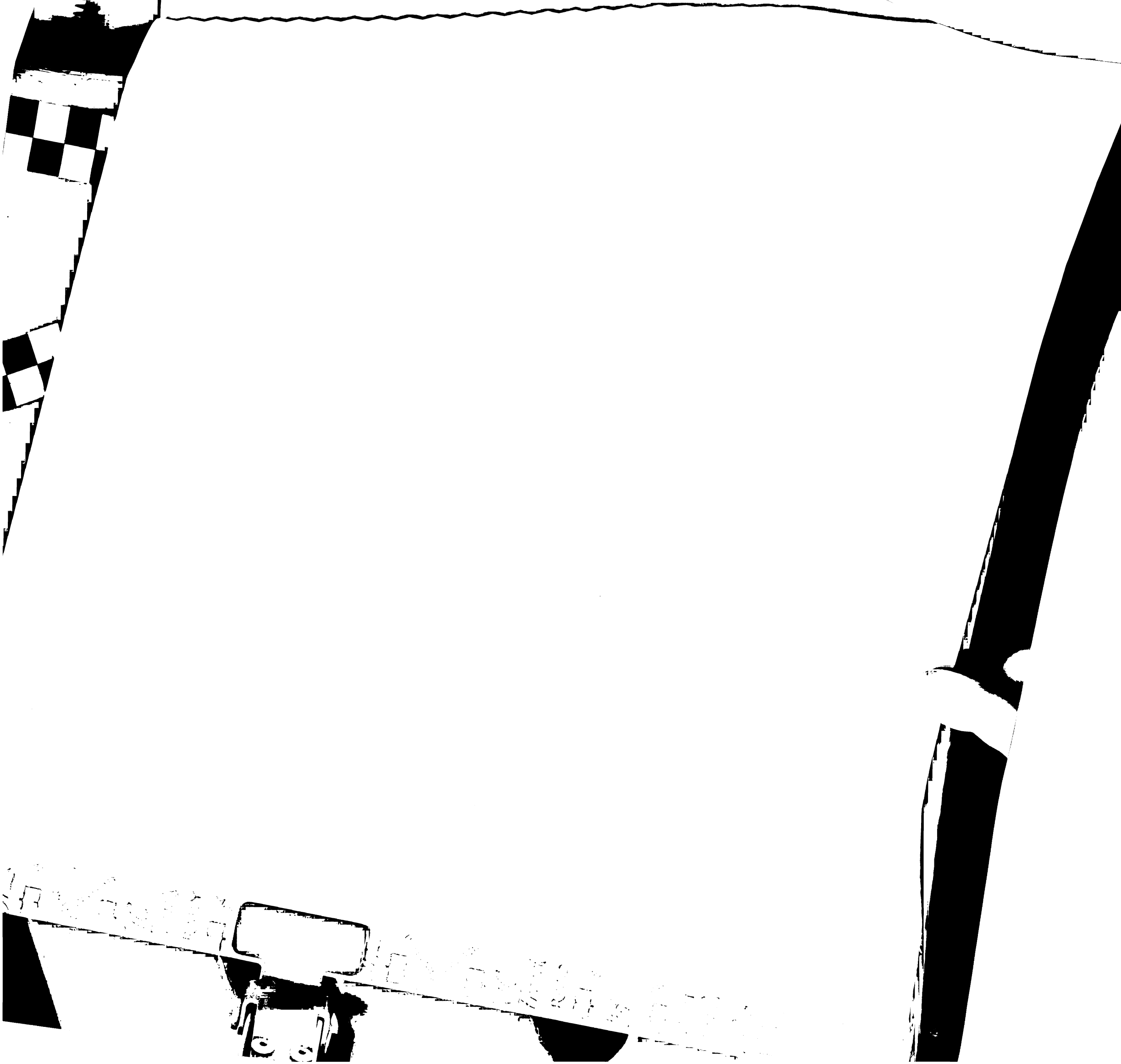


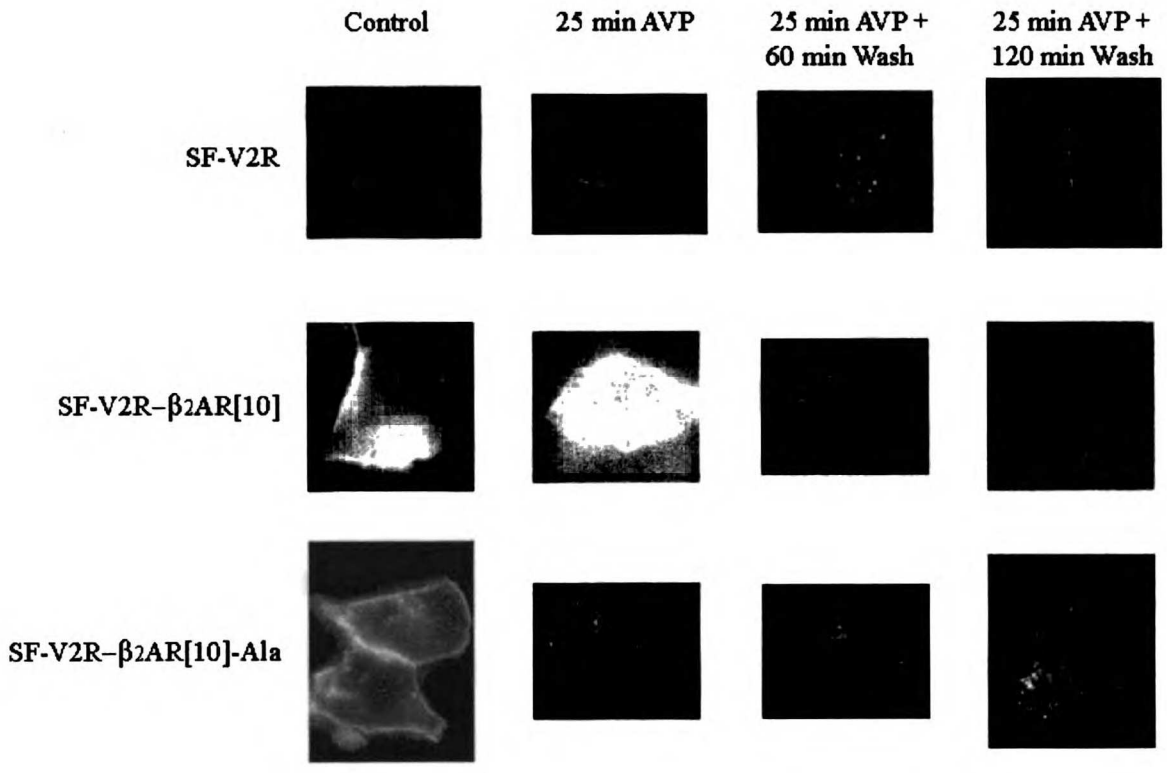
Figure Legends

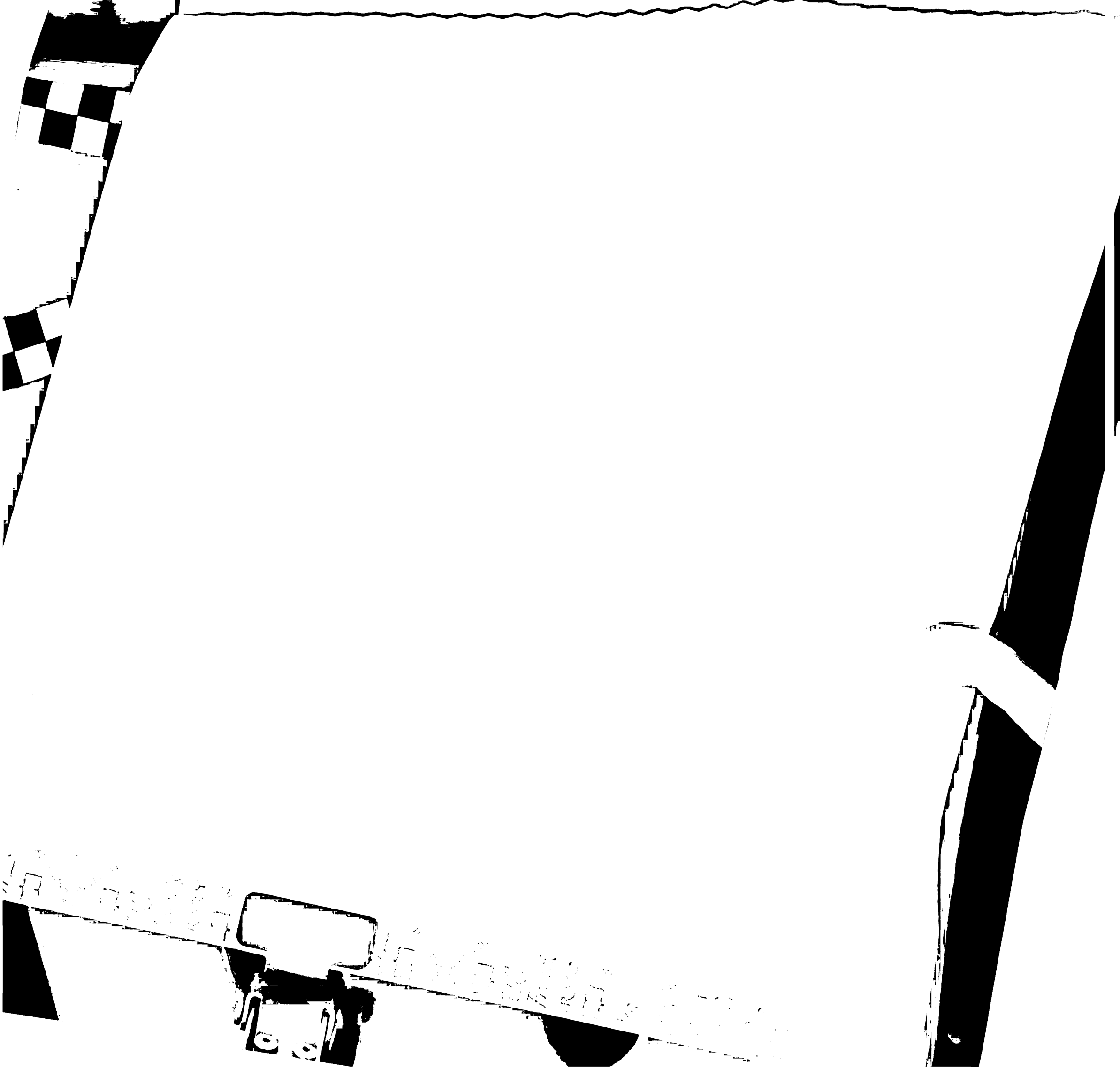
Figure 1: **The last ten residues of the β_2AR are not sufficient to enhance the recycling of the V2R.** HEK-293 cells stably transfected with the indicated constructs were incubated with [Arg^8] vasopressin for 25 minutes, washed in DMEM without agonist, and then allowed to recycle for 0, 60, or 120 minutes. Cells were fixed, stained for 45 minutes with M1 anti-FLAG antibody, and detected using fluorescein isothiocyanate–conjugated donkey anti-mouse antibodies. Following agonist washout, there was no significant increase in recycling efficiency accorded by the β_2AR derived sequence.



Figures

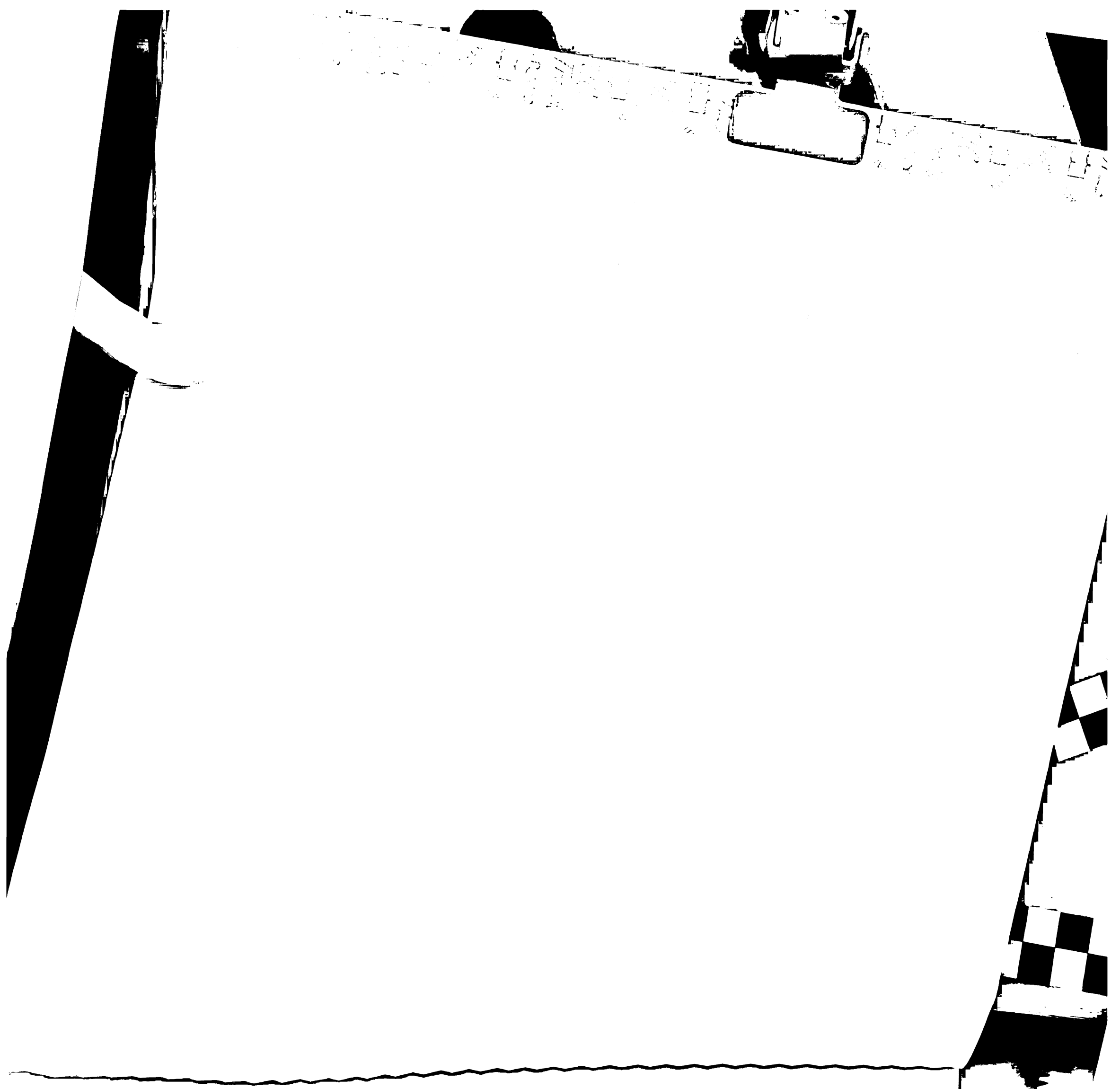
Figure 1:





Appendix 3: Motility Differences between endocytic vesicles containing δ OR or β_2AR

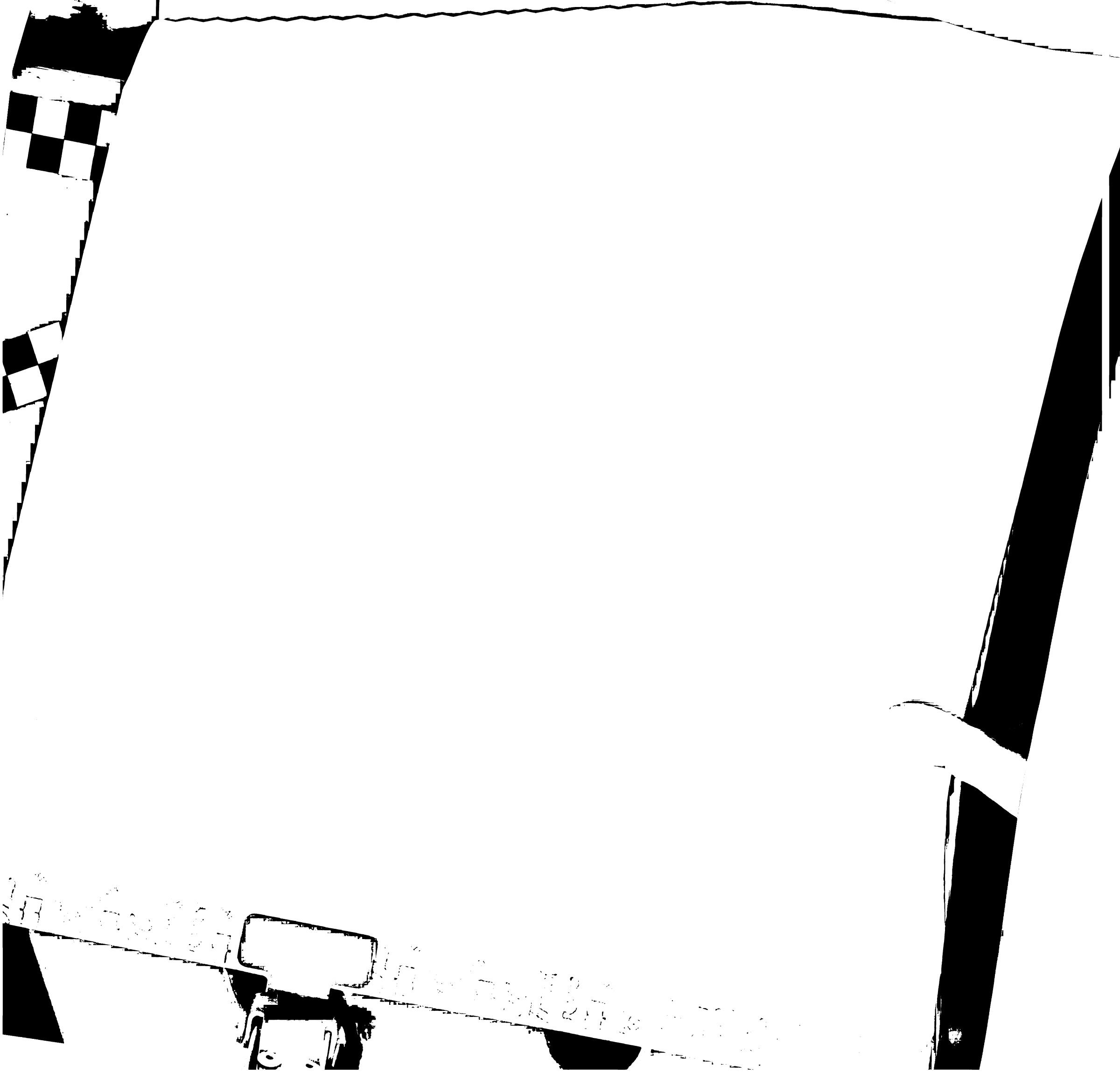
All work presented in this appendix was performed by Robert Michael Gage.



Introduction

Following agonist binding and coupling to G proteins, many GPCRs are rapidly phosphorylated by GRKs (G protein-coupled receptor kinases) (1-6). Phosphorylated receptors are then often bound by cytoplasmic proteins, arrestins, which squelch further G protein mediated signaling and promote the clustering of receptors in clathrin coated membrane structures (7-10). After internalization into clathrin coated vesicles, receptors can: rapidly recycle back to the plasma membrane, traffic to lysosomes where they are protealized, or remain within internal vesicles for prolonged periods of time with little to no degradation whereupon they eventually either recycle to the plasma membrane or degrade within lysosomes (11-17).

Many membrane proteins and bulk lipid traverse the endocytic pathway and rapidly recycle back to the plasma membrane without the need for a specific cytosolic signal or protein interaction (18-20). For example, the trasferrin receptor rapidly recycles to the plasma membrane even when stripped of all cytoplasmic amino acid residues (21). Certain fluorescently labeled lipids also recycle rapidly to the plasma membrane with similar kinetics to those exhibited by the transferring receptor (22). In contrast, several G protein-coupled receptors (GPCRs) including the β_2AR, β_1AR, and μ OR (beta 2 and 1 adrenergic receptors and the mu opioid receptor respectively), require a specific amino sequence in their cytoplasmic carboxyl terminus for their proper membrane trafficking following agonist induced endocytosis (23-26). Deletion of the recycling signal sequence or point mutation is sufficient to cause impaired receptor recycling (23-26).



Although the signals from the different receptors that are responsible for their enhanced recycling differ in sequence and in protein partner, there may be a common underlying mechanism. This study investigated the motility of receptor containing vesicles following agonist treatment in live transfected HEK-293 cells. The motion of β_2AR containing vesicles is slower on average than that of comparable vesicles from endocytosed δ opioid receptor (δ OR). The δ OR is degraded in lysosomes following agonist induced endocytosis (13,14,27,28) and often has very rapid vesicular motion.

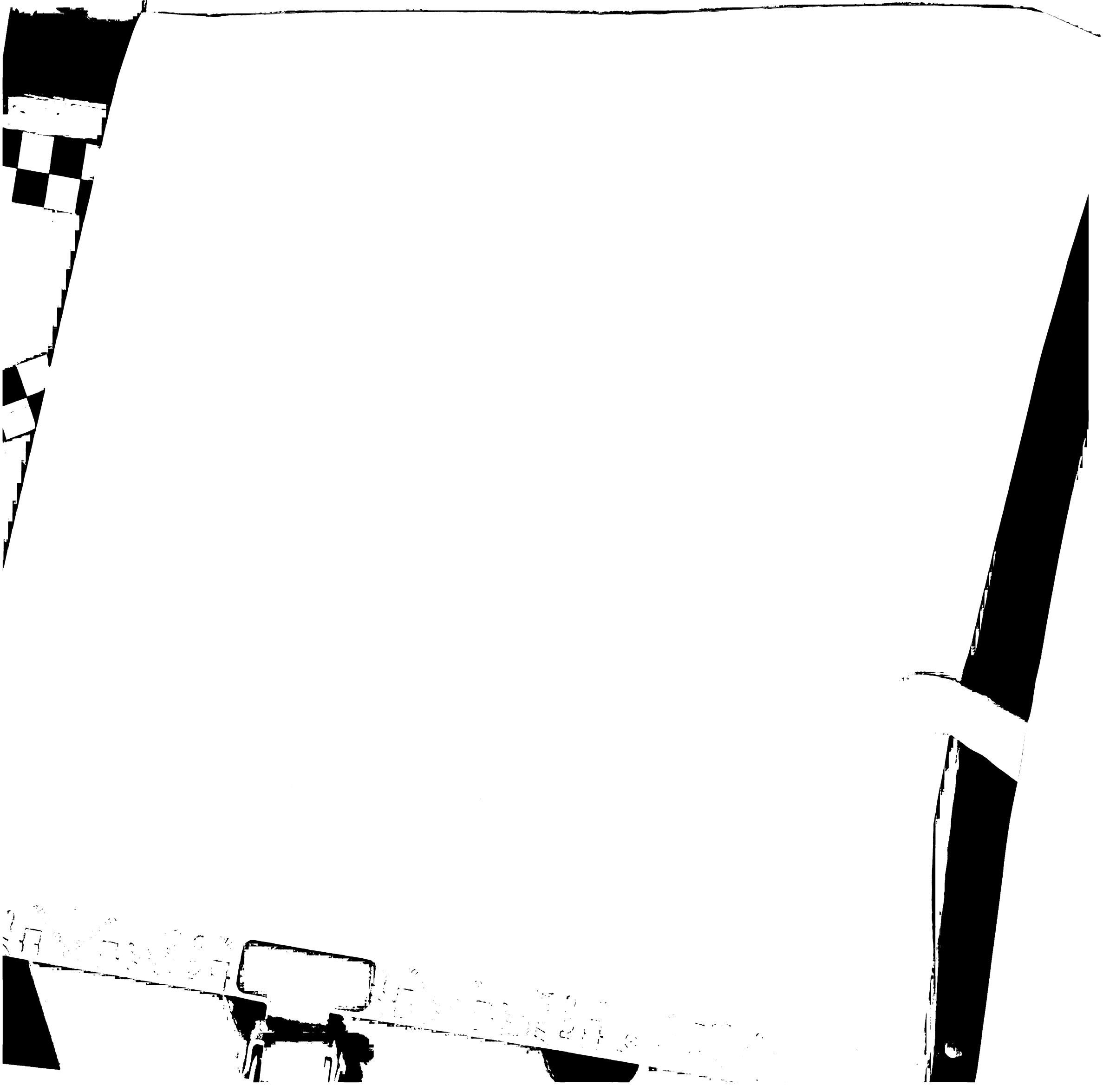
Experimental Procedures

cDNA Constructs and Mutagenesis

Several epitope-tagged versions of the cloned murine delta opioid receptor (δ OR (29)) and the human beta 2 adrenergic receptor (β_2AR (30)) were used in these studies: mutant receptors containing a FLAG epitope in the amino-terminal extracellular domain (SFδ OR and SFβ_2AR, respectively) were described previously and demonstrated to be functional (13,31,32). Mutant delta opioid receptors containing a FLAG epitope in the amino-terminal extracellular domain and the last six carboxyl-terminal cytoplasmic residues (NH_2-GGGAAA-COOH) deleted, replaced with either the ten carboxyl-terminal residues from the β_2AR (NH_2-RNCSTNDSLL-COOH) or the ten β_2AR residues plus an alanine (NH_2-RNCSTNDSLLA-COOH). This was accomplished by insertion of a synthetic linker-adapter (Operon Technologies) encoding the ten-residue or eleven-residue sequence followed by a stop codon into an Srf I site present near the 3' end of the sequence encoding the δ OR tail. Receptor cDNAs were cloned into pcDNA3 (Invitrogen) or pIRES (Clonetech) and all constructs were verified by dideoxynucleotide sequencing (UCSF Genetics Core Sequencing Facility).

Cell Culture and Transfection

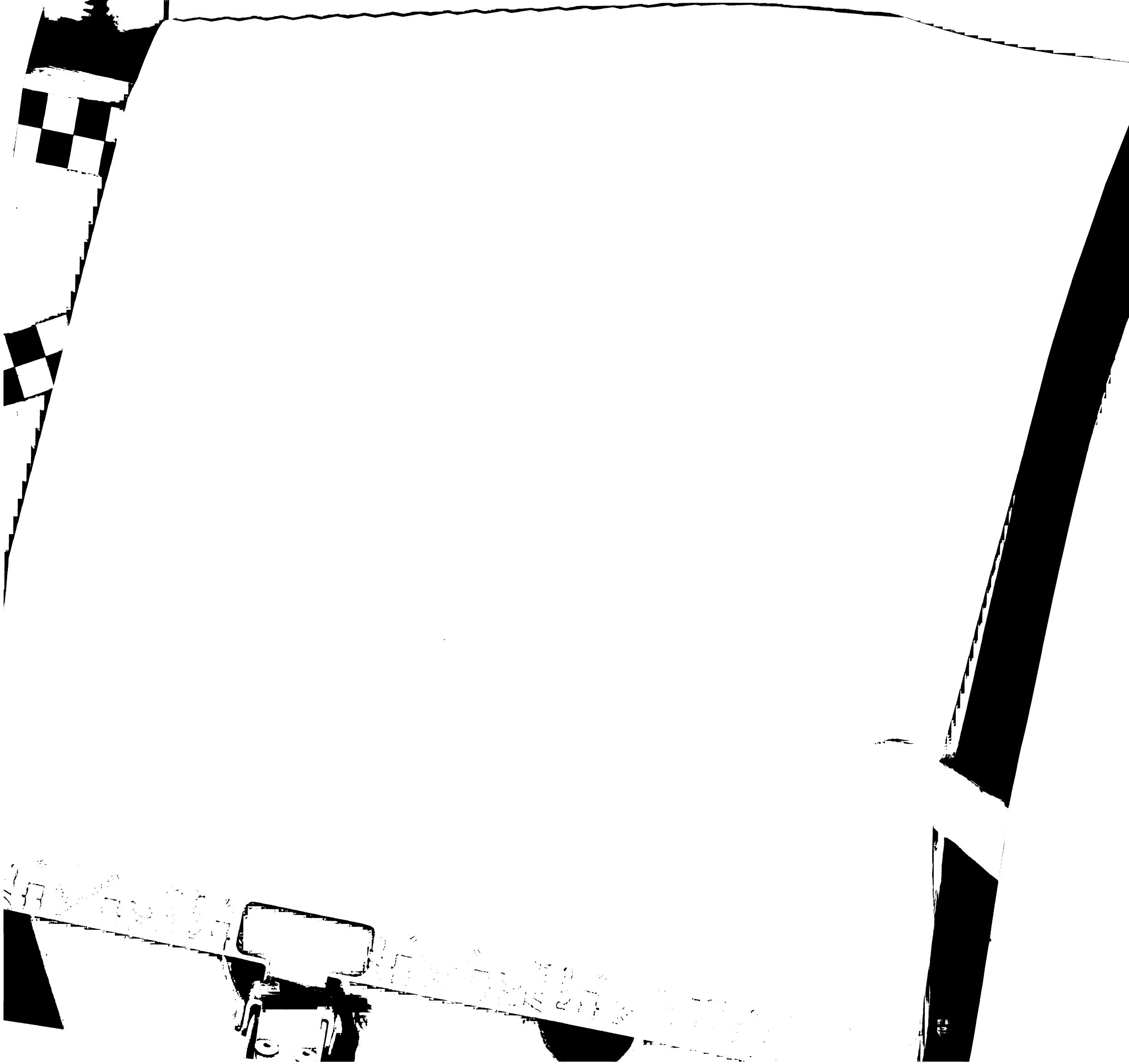
Human embryonic kidney 293 cells (ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (University of California San Francisco Cell Culture Facility). Cells grown in 6-cm dishes were transfected with ~5 ug of plasmid DNA containing the indicated receptor by calcium phosphate precipitation (15,33). For studies of receptor trafficking in transiently transfected cells,

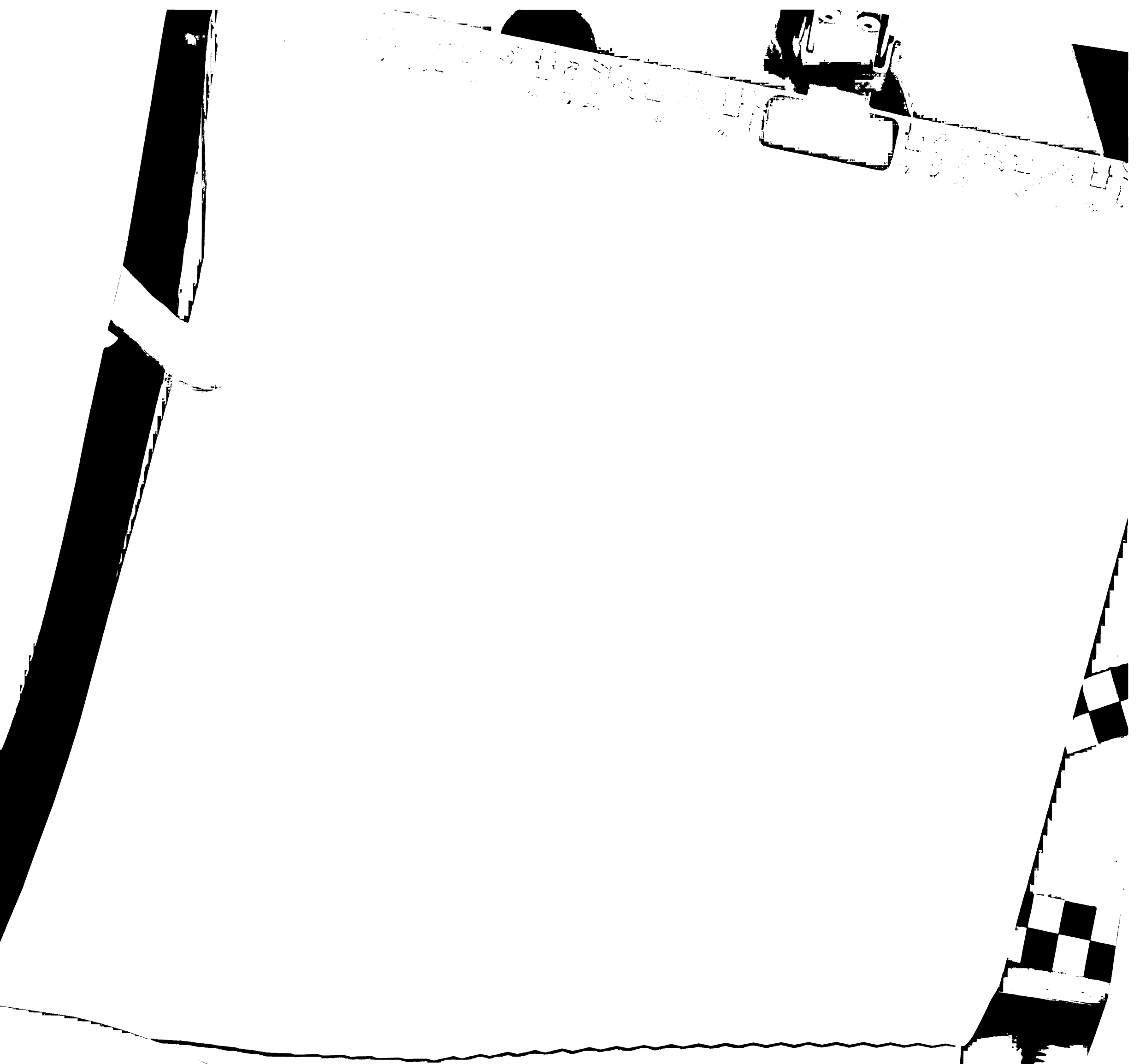


cells were transfected as above, plated onto coverslips 24 h post transfection and experiments were conducted 48 h post transfection. Stably transfected cells expressing epitope tagged receptors were generated by transfecting 293 cells in 6-cm dishes as above. Cell clones expressing transfected receptors were selected in 500 ug/ml Geneticin (Life Technologies, Inc.) and colonies were isolated and selected to have similar levels of receptor expression, as estimated by radioligand binding assay conducted as described previously (15). Receptor levels in stably transfected cell lines ranged from 0.7 to 4.2 pmol/mg of total protein.

Examination of Receptor Endocytosis and Recycling by Fluorescence Microscopy

Endocytic trafficking of receptors labeled initially in the plasma membrane was visualized by fluorescence microscopy using a minor modification of a previously described method (23). Briefly, stably or transiently transfected 293 cells expressing the indicated receptor were grown on glass coverslips (Corning) treated with M1 anti-FLAG antibody (2.5 ug/ml, Sigma) directly conjugated to Alexa-488 or Alexa-594 (Molecular Probes) at 37 °C for 25 min to label receptors. The cells were treated, at 37 °C on a peltier-unit stage with 10 mM isoproterenol (Research Biochemicals) or 10 mM DADLE ([$_{D}$-Ala^{2}, $_{D}$-Leu^{5} enkephalin], Research Biochemicals International) in PBS (phosphate buffered saline, cell culture facility, UCSF). Live cell imaging was performed using epifluorescence on an inverted Nikon Diaphot microscope equipped with a Nikon 60X NA1.4 objective and epifluorescence optics. Images were collected using a video-rate SIT camera (Hamamatsu) interfaced to a Macintosh computer. 100 or 200 imaged were collected per time point with 10 video-rate images per image integrated on an LG-3

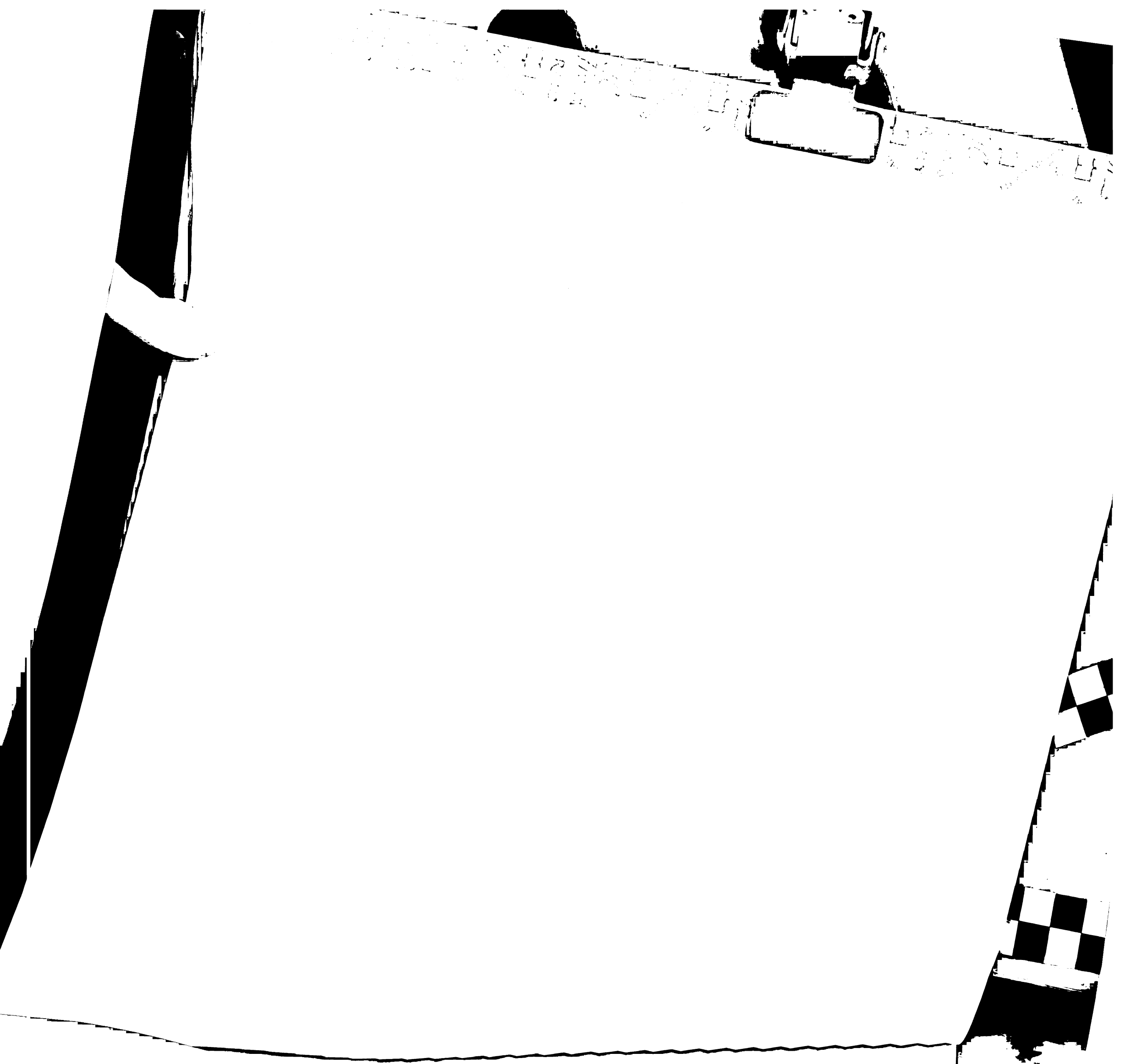




Results

HEK-293 cells expressing either β_2AR or δ OR were grown on coverslips for direct live visualization of receptor containing endosomes. Following addition of either 10 μM isoproterenol or DADLE, live cells were imaged on a heated stage and video images were taken. As seen in figure 1 (and supplemental material movie "beta movie 1"), vesicles containing the β_2AR move relatively slowly and often remain within a short distance from their starting position. In contrast, multiple vesicles filled with endocytosed δ OR move rapidly and processively within the HEK-293 cells (figure 2 and supplemental movie "delta movie 1"). Although not all vesicles moved rapidly and occasional experiments with the δ OR did not reveal any rapid motility, overall the motion of vesicles containing δ OR were much more processive and rapid than those containing the β_2AR.

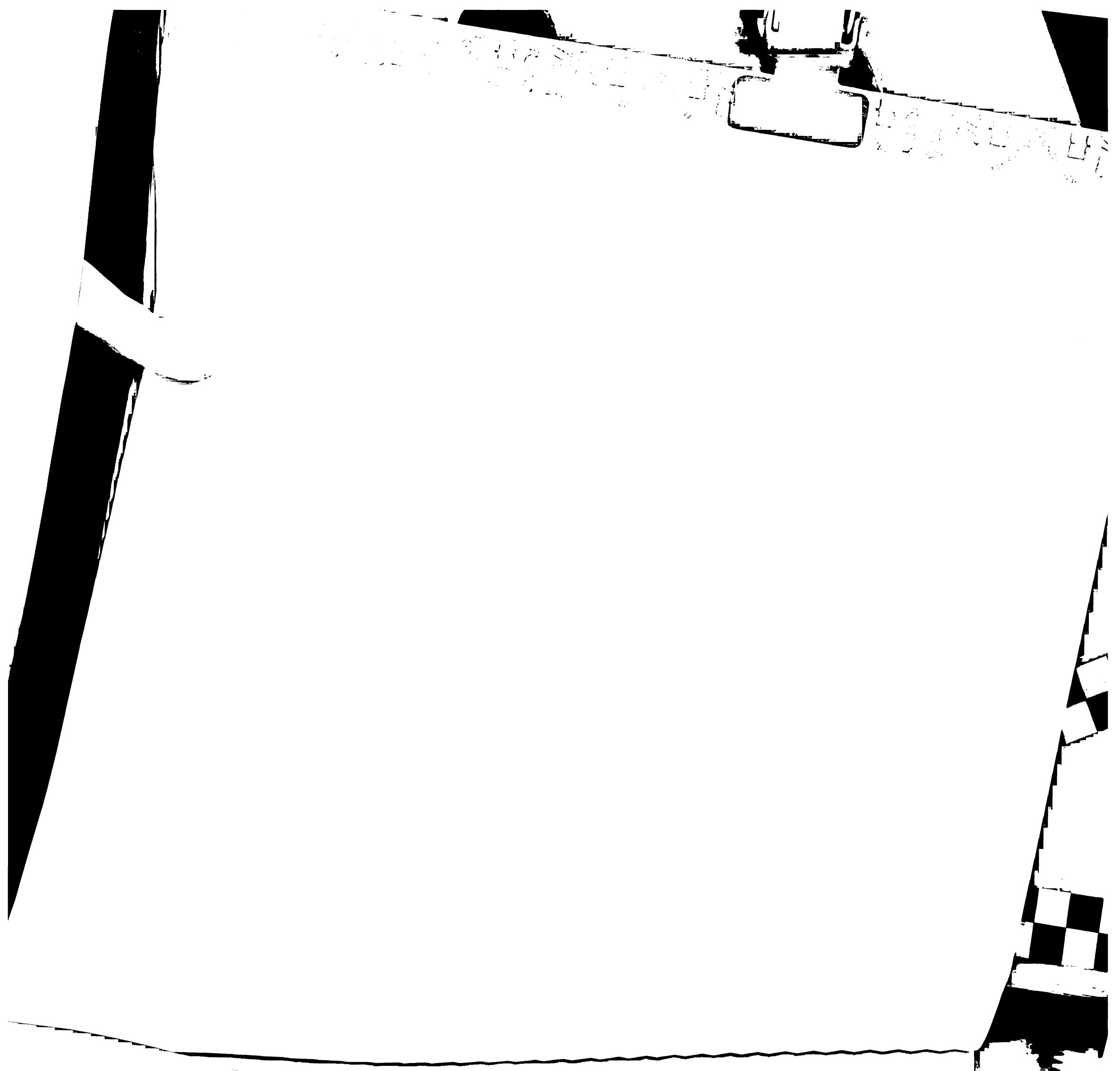
The last ten residues from the β_2AR are sufficient to decrease the motility of receptor containing endosomes when appended to the δ OR (figure 3 and supplemental movie "delta_beta movie 1"). It does so in a sequence specific manner as the addition of a single alanine to the β_2AR derived sequence produces a receptor which is capable of rapid motion in endocytosed vesicles (figure 4 and supplemental movie "delta_beta_ala movie 1"). Future experiments could be performed to investigate the requirement for maintaining the actin cortex by looking at vesicular movement in the presence of latrunculin or cytochalasin D which disrupt actin polymerization.



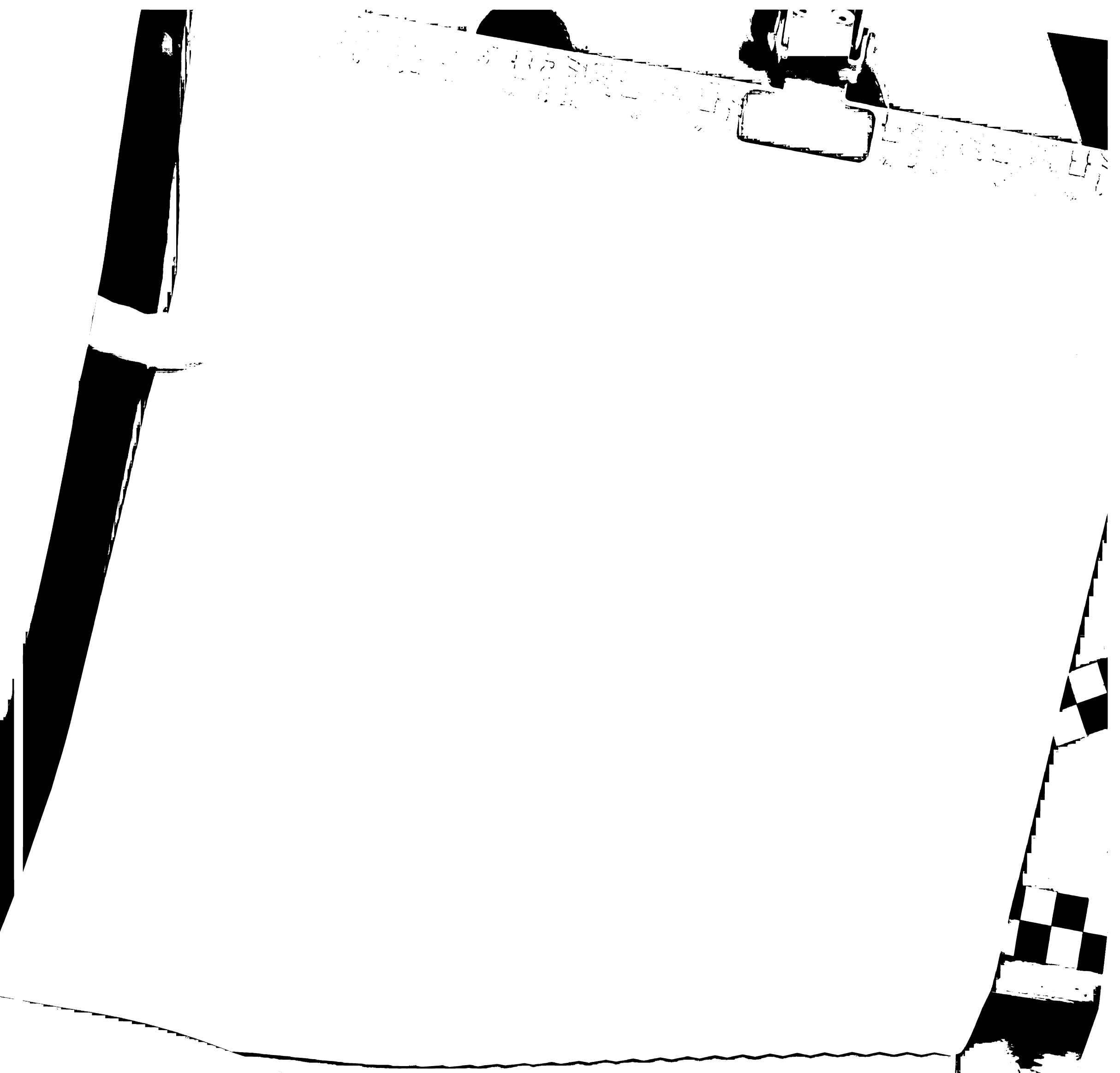
Discussion

Although the GPCRs with a signal mediated recycling sequence seem to differ in their direct protein partners, they may all be linked to a common underlying mechanism for promoting local recycling back to the plasma membrane. Both the β_2AR and β_1AR contain class I PDZ ligands that bind to PDZ domain containing proteins which are known to anchor membrane proteins in signaling complexes and also have been associated with the cortical actin cytoskeleton (34-42). The β_2AR binds to hNHERF1 / EBP50 which is bound to the actin network via its ERM (ezrin / radixin / moesin) binding domain and an ERM protein(s). The β_1AR interacts with both MAGI-2 and PSD-95, both PDZ domain containing proteins associated with the plasma membrane (34-36,43). Both recycling sequences thus link the receptor and potentially the receptor containing endosomes to the cortex of the cell.

One simple mechanism (figure 5) could explain the ability of the β_2AR to be rapidly recycled while the δ OR which internalizes into the same vesicles is not. If the adrenergic receptors retain binding to the actin cytoskeleton following endocytosis, they may be selectively retained in the cellular cortex. Fission of endosomes containing both the β_2AR and δ OR could lead to separation of the two receptors with cortical actin binding causing preferential retention of those vesicles containing the β_2AR. Simply binding to the cortical actin network may be sufficient to cause rapid recycling of a GPCR. If this is the case, it would be expected that an actin binding motif from an ERM protein or the ERM binding domain from hNHERF / EBP50 would be sufficient to re-route a GPCR that would normally degrade into a rapidly recycling pathway.

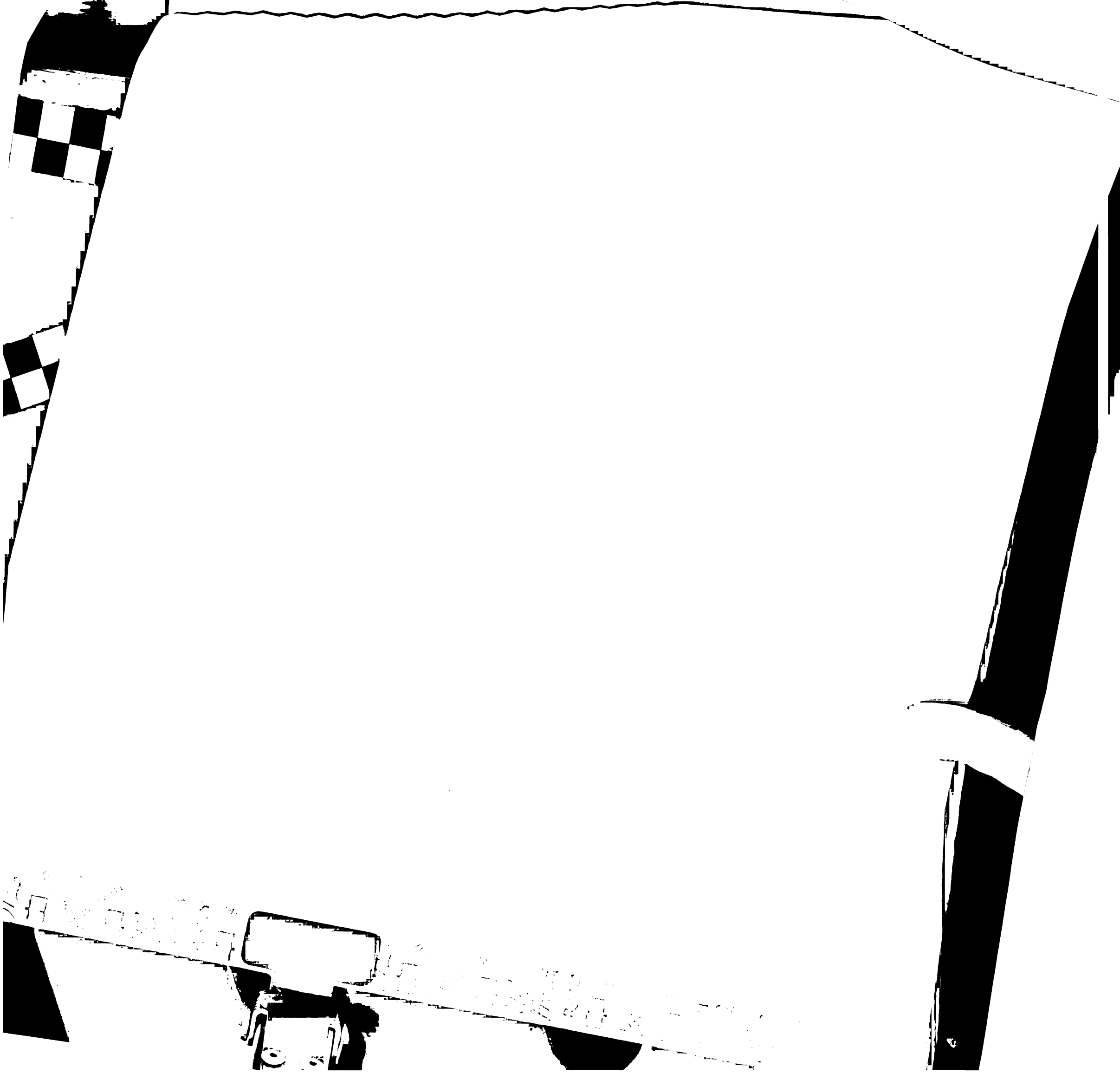


Preliminary results with a mutant δ OR with the actin binding motif (30 amino acid sequence) from ezrin show that it indeed increases the recycling efficiency of the δ OR (Gabriel Vargus, unpublished data). There may be other as yet unidentified receptors which link to the actin cytoskeleton through mechanisms different from PDZ domain mediated interactions. This may represent a general mechanism for membrane receptor recycling.



References

1. Sibley, D. R., Strasser, R. H., Caron, M. G., and Lefkowitz, R. J. (1985) *J Biol Chem* **260**, 3883-3886
2. Benovic, J. L., Kuhn, H., Weyand, I., Codina, J., Caron, M. G., and Lefkowitz, R. J. (1987) *Proc Natl Acad Sci U S A* **84**, 8879-8882
3. Benovic, J. L., Strasser, R. H., Caron, M. G., and Lefkowitz, R. J. (1986) *Proc Natl Acad Sci U S A* **83**, 2797-2801
4. Gurevich, V. V., Pals, R. R., Benovic, J. L., Hosey, M. M., and Onorato, J. J. (1997) *J Biol Chem* **272**, 28849-28852
5. Gurevich, V. V., and Benovic, J. L. (2000) *Methods Enzymol* **315**, 422-437
6. Gurevich, V. V., and Benovic, J. L. (1997) *Mol Pharmacol* **51**, 161-169
7. von Zastrow, M., and Kobilka, B. K. (1994) *J Biol Chem* **269**, 18448-18452.
8. Goodman, O. B., Jr., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1998) *Advances in Pharmacology* **42**, 429-433
9. Ferguson, S. S., Zhang, J., Barak, L. S., and Caron, M. G. (1998) *Life Sci* **62**, 1561-1565
10. Lefkowitz, R. J., Pitcher, J., Krueger, K., and Daaka, Y. (1998) *Advances in Pharmacology* **42**, 416-420
11. Gagnon, A. W., Kallal, L., and Benovic, J. L. (1998) *Journal of Biological Chemistry* **273**, 6976-6981
12. Tsao, P., and von Zastrow, M. (2000) *Curr Opin Neurobiol* **10**, 365-369.



13. Tsao, P. I., and von Zastrow, M. (2000) *J Biol Chem* **275**, 11130-11140.

14. Tsao, P., Cao, T., and von Zastrow, M. (2001) *Trends Pharmacol Sci* **22**, 91-96.

15. Tsao, P. I., and von Zastrow, M. (2001) *Pharmacol Ther* **89**, 139-147.

16. Innamorati, G., Sadeghi, H., and Birnbaumer, M. (1999) *Journal of Receptor and Signal Transduction Research* **19**, 315-326

17. Innamorati, G., Le Gouill, C., Balamotis, M., and Birnbaumer, M. (2001) *Journal of Biological Chemistry* **276**, 13096-13103

18. Gruenberg, J., and Maxfield, F. R. (1995) *Current Opinion in Cell Biology* **7**, 552-563

19. Gruenberg, J. (2001) *Nat Rev Mol Cell Biol.* **2**, 721-730

20. Trowbridge, I. S., Collawn, J. F., and Hopkins, C. R. (1993) *Annual Review of Cell Biology* **9**, 129-161

21. Mayor, S., Presley, J. F., and Maxfield, F. R. (1993) *Journal of Cell Biology* **121**, 1257-1269

22. Hao, M., and Maxfield, F. R. (2000) *Journal of Biological Chemistry* **275**, 15279-15286

23. Cao, T. T., Deacon, H. W., Reczek, D., Bretscher, A., and von Zastrow, M. (1999) *Nature* **401**, 286-290.

24. Gage, R. M., Kim, K. A., Cao, T. T., and von Zastrow, M. (2001) *J Biol Chem* **276**, 44712-44720.

25. Gage, R. M., Matveeva, E. A., Whiteheart, S. W., and von Zastrow, M. (2005) *J. Biol. Chem.* **280**, 3305-3313

26. Tanowitz, M., and von Zastrow, M. (2003) *J. Biol. Chem.* **278**, 45978-45986

27. Law, P.-Y., Hom, D. S., and Loh, H. H. (1984) *J. Biol. Chem.* **259**, 4096-4104

28. Law, P. Y., and Loh, H. H. (1999) *Journal of Pharmacology and Experimental Therapeutics* **289**, 607-624

29. Evans, C. J., Keith, D. J., Morrison, H., Magendzo, K., and Edwards, R. H. (1992) *Science* **258**, 1952-1955

30. Kobilka, B. K., Dixon, R. A., Frielle, T., Dohlman, H. G., Bolanowski, M. A., Sigal, I. S., Yang-Feng, T. L., Francke, U., Caron, M. G., and Lefkowitz, R. J. (1987) *Proceedings of the National Academy of Sciences of the United States of America* **84**, 46-50

31. von Zastrow, M., and Kobilka, B. K. (1992) *J Biol Chem* **267**, 3530-3538.

32. Keith, D. E., Murray, S. R., Zaki, P. A., Chu, P. C., Lissin, D. V., Kang, L., Evans, C. J., and von Zastrow, M. (1996) *J Biol Chem* **271**, 19021-19024.

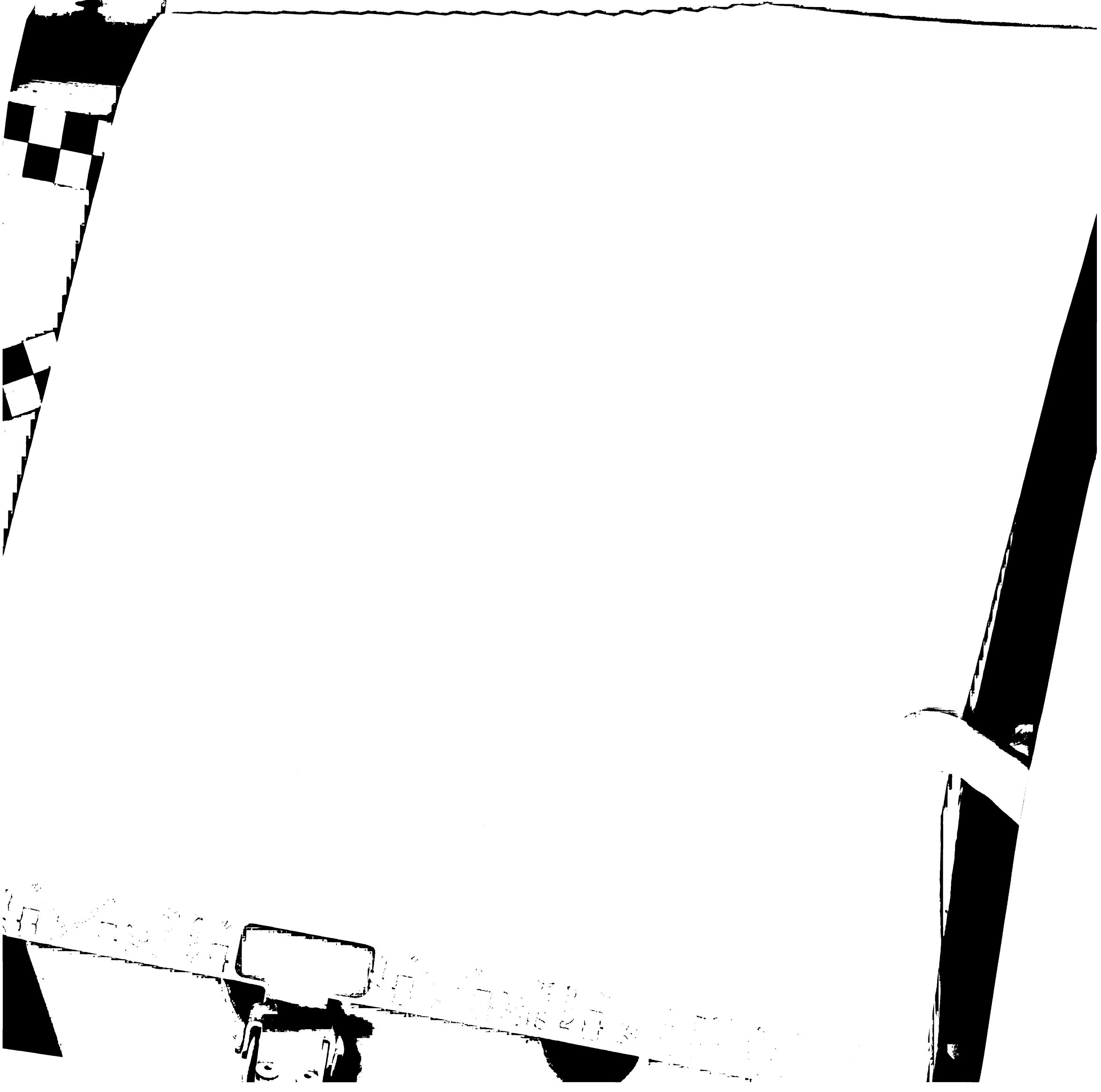
33. Okayama, H., and Berg, P. (1983) *Molecular and Cellular Biology* **3**, 280-289

34. Hall, R. A., Ostedgaard, L. S., Premont, R. T., Blitzer, J. T., Rahman, N., Welsh, M. J., and Lefkowitz, R. J. (1998) *Proceedings of the National Academy of Sciences of the United States of America* **95**, 8496-8501

35. Hall, R. A., Premont, R. T., Chow, C. W., Blitzer, J. T., Pitcher, J. A., Claing, A., Stoffel, R. H., Barak, L. S., Shenolikar, S., Weinman, E. J., Grinstein, S., and Lefkowitz, R. J. (1998) *Nature* **392**, 626-630

36. Xu, J., Paquet, M., Lau, A. G., Wood, J. D., Ross, C. A., and Hall, R. A. (2001) *J. Biol. Chem.* **276**, 41310-41317

37. Wang, S., Yue, H., Derin, R. B., Guggino, W. B., and Li, M. (2000) *Cell* **103**, 169-179.



38. Tiffany, A. M., Manganas, L. N., Kim, E., Hsueh, Y. P., Sheng, M., and Trimmer, J. S. (2000) *J Cell Biol* **148**, 147-158

39. Sheng, M., and Kim, E. (2000) *J Cell Sci* **113 (Pt 11)**, 1851-1856

40. Sheng, M., Sala, C. (2001) *Annu Rev Neurosci* **24**, 1-29

41. Naisbitt, S., Valtschanoff, J., Allison, D. W., Sala, C., Kim, E., Craig, A. M., Weinberg, R. J., and Sheng, M. (2000) *J Neurosci* **20**, 4524-4534

42. Harris, B. Z., and Lim, W. A. (2001) *J Cell Sci* **114**, 3219-3231

43. Hu, L. A., Tang, Y., Miller, W. E., Cong, M., Lau, A. G., Lefkowitz, R. J., and Hall, R. A. (2000) *J. Biol. Chem.* **275**, 38659-38666

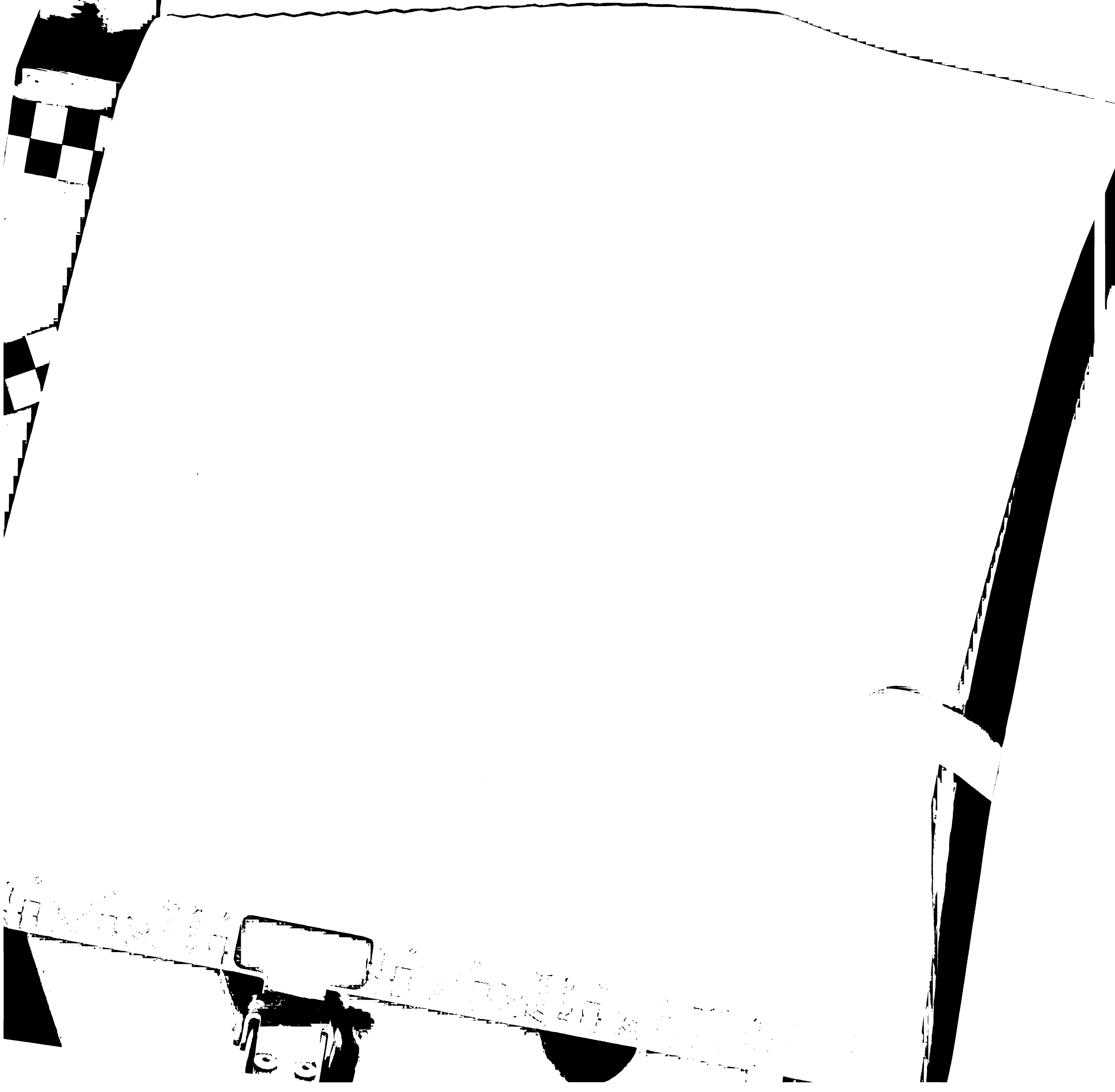
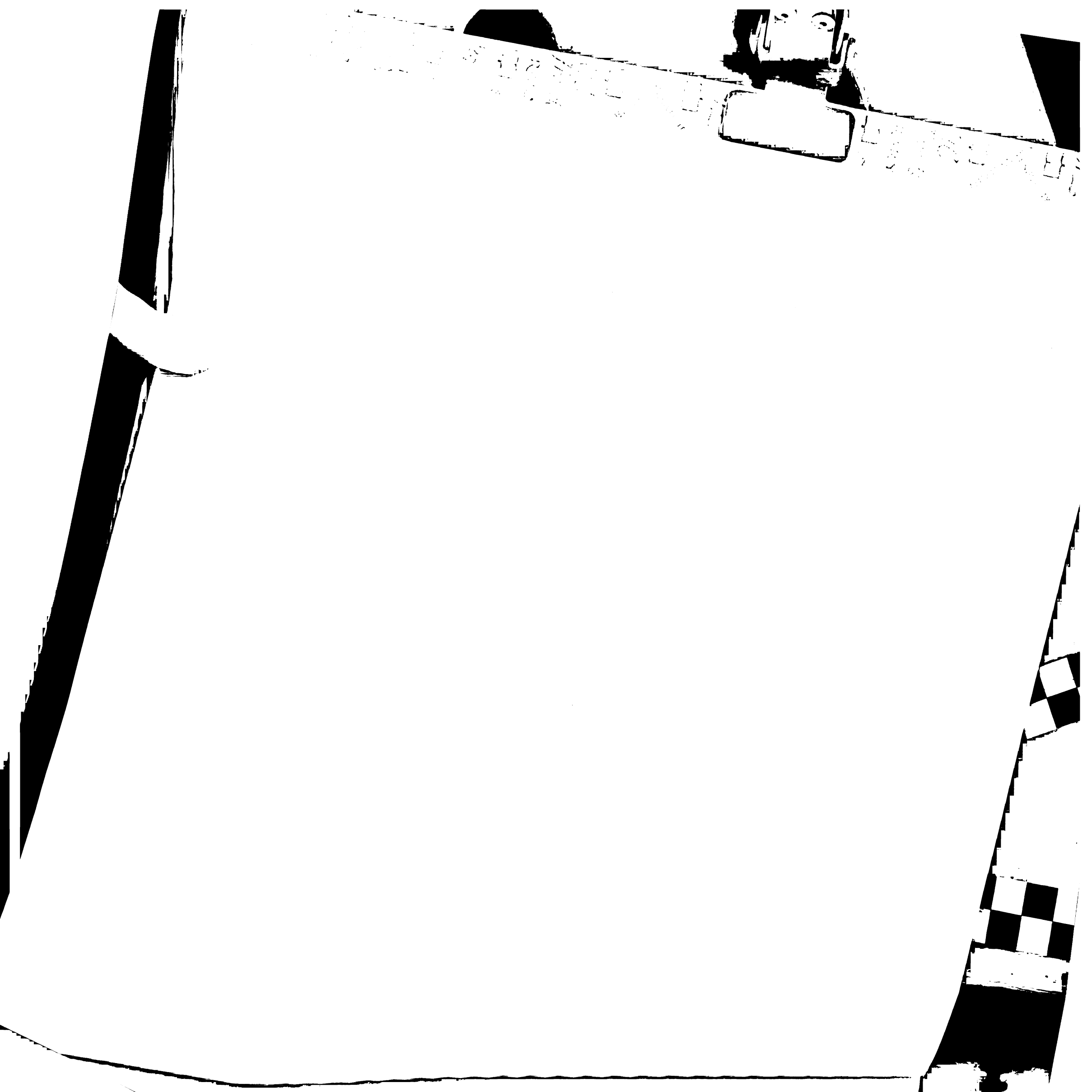


Figure Legends

Figure 1: Live imaging of cells expressing β_2AR shows little motility of vesicles containing the adrenergic receptor. HEK-293 cells were stained with M1 antiFLAG antibody directly conjugated to FITC for the β_2AR then stimulated with 10 μM isoproterenol. Receptor motion was tracked in live cells on-stage by fluorescent light microscopy. Times are as indicated in seconds after the first frame. An example vesicle is tracked with the white arrow. Little vesicle motility was seen for the β_2AR during multiple observations.

Figure 2: Live imaging of cells expressing δ OR illustrates the greater motility of delta opioid receptor containing endosomes as compared to those containing β_2AR. HEK-293 cells were stained with M1 antiFLAG antibody directly conjugated to FITC for the δ OR then stimulated with 10 μM DADLE. Receptor motion was tracked in live cells on-stage by fluorescent light microscopy. Times are as indicated in seconds after the first frame. An example vesicle is tracked with the white arrow. More vesicle motility was seen for the δ OR as compared to the β_2AR during multiple observations.

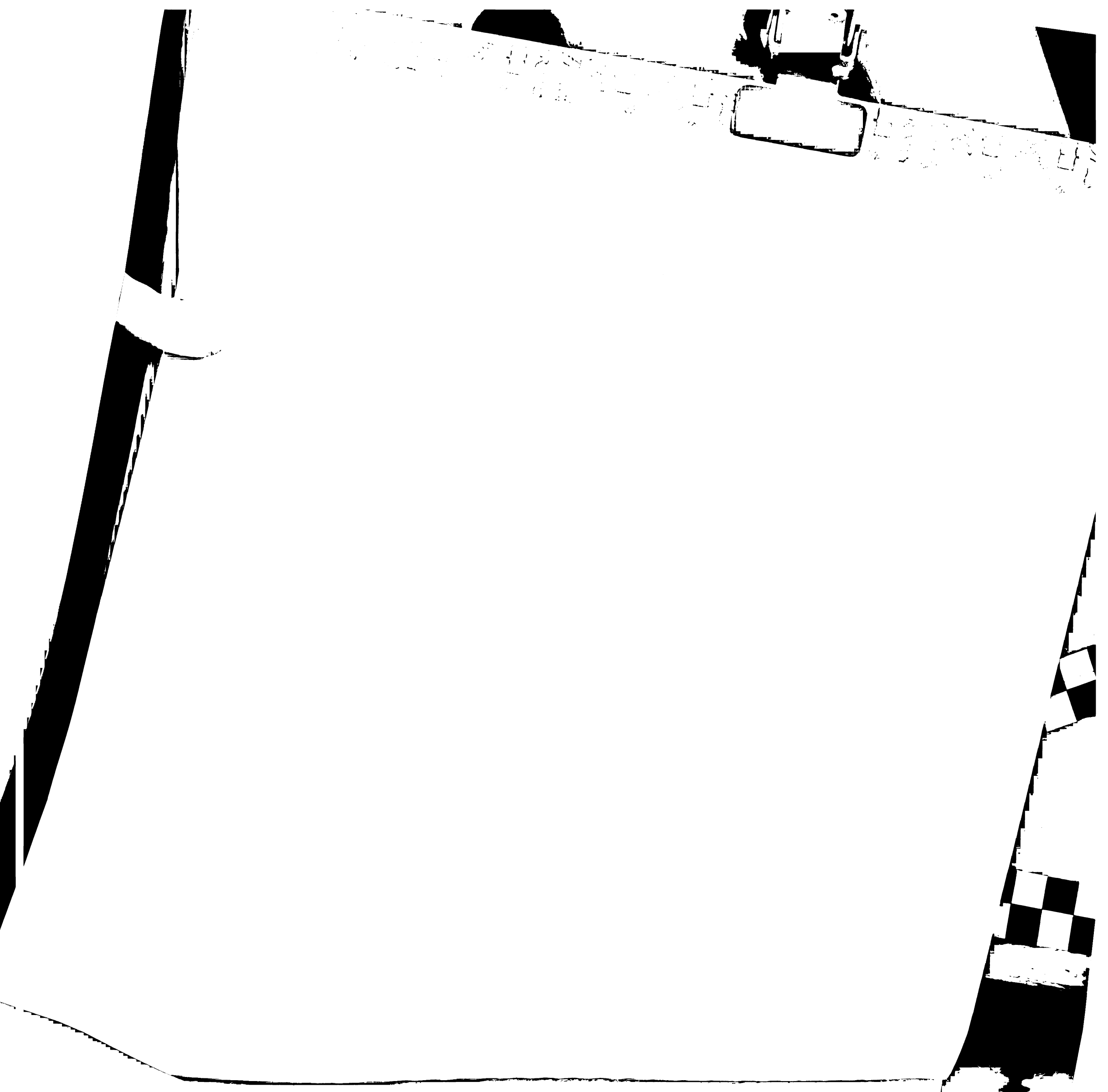
Figure 3: Live imaging of cells expressing δ/β_2AR chimeric receptors shows little motility of receptor containing endosomes. HEK-293 cells were stained with M1 antiFLAG antibody directly conjugated to FITC for the β_2AR then stimulated with 10 μM DADLE. Receptor motion was tracked in live cells on-stage by fluorescent light microscopy. Times are as indicated in seconds after the first frame. An example vesicle



is tracked with the white arrow. Little vesicle motility was seen for the δ/β_2AR and range of motility was similar to that of the wild type β_2AR during multiple observations.

Figure 4: Live imaging of cells expressing chimeric δ/β_2AR-Ala illustrates the greater motility of δ/β_2AR-Ala containing endosomes as compared to those containing δ/β_2AR or β_2AR. HEK-293 cells were stained with M1 antiFLAG antibody directly conjugated to FITC for the chimeric receptor then stimulated with 10 μM DADLE. Receptor motion was tracked in live cells on-stage by fluorescent light microscopy. Times are as indicated in seconds after the first frame. An example vesicle is tracked with the white arrow. More vesicle motility was seen for the δ/β_2AR-Ala chimera as compared to the δ/β_2AR or β_2AR during multiple observations.

Figure 5: Model showing receptor sorting and recycling via actin binding.
A Vesicles containing receptors such as the β_2AR (orange) which can be linked to the actin cytoskeleton may have a more limited mobility than other vesicles which lack such receptors (green). Following agonist induced endocytosis (**a** and **b**), vesicles may contain both receptors which can be linked to the actin cytoskeleton and those that cannot. Fission of these vesicles, facilitated by differential binding to the actin network, may lead to the sorting of receptors based upon their actin binding potential. The β_2AR (orange), which can bind to the PDZ domain containing protein EBP50 is linked through this protein to ERM proteins which directly bind to polymerized actin. This binding may restrict the motility of β_2AR containing vesicles and concentrate them in the peripheral actin cortex (**c**). This may effectively increase the recycling efficiency of these receptors

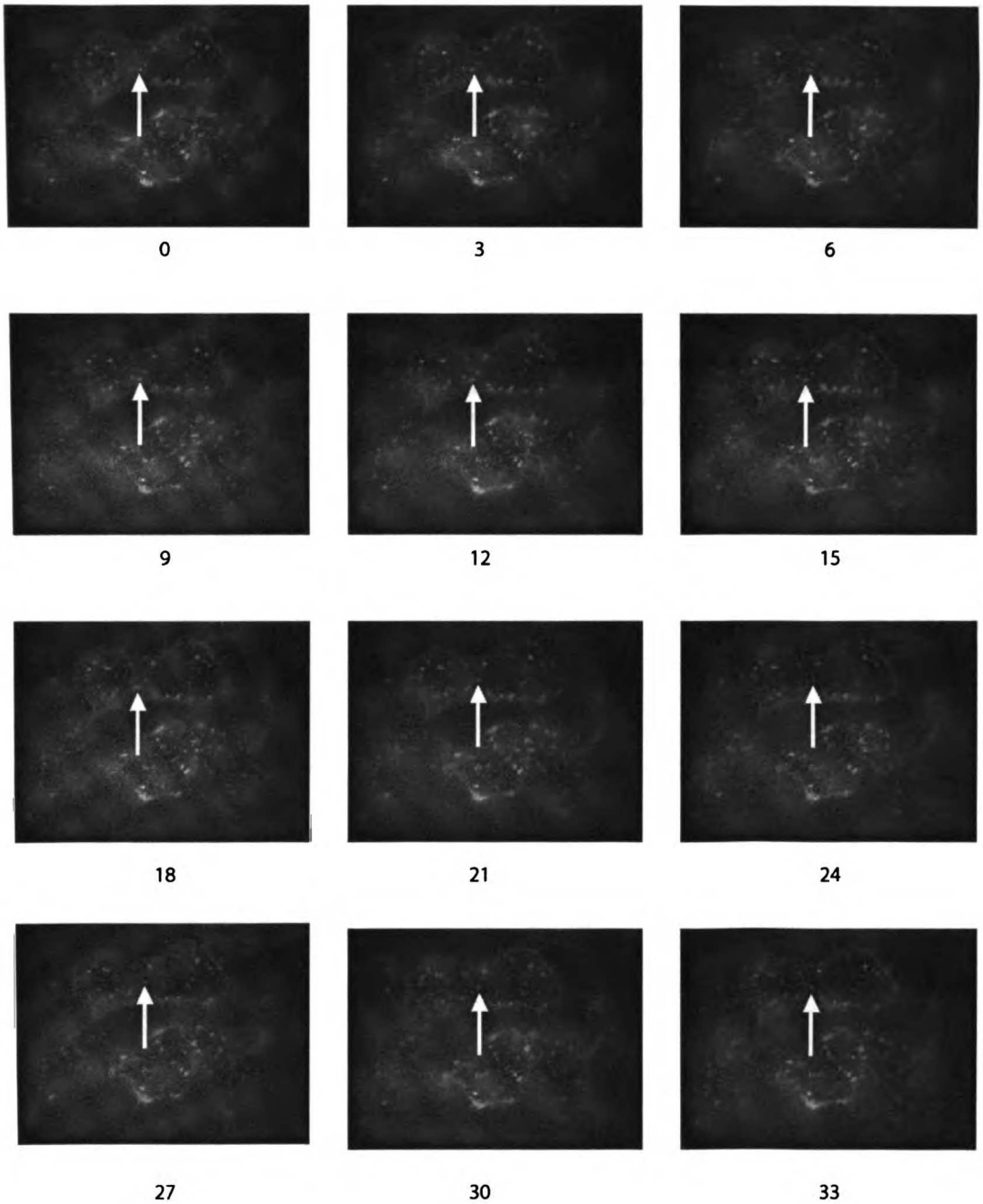


by maintaining the receptors in close proximity to the plasma membrane. Receptors lacking the actin linkage motif (green) are sorted away from those that do and can enter structures termed early endosomes (**d**). From early endosomes receptors can rapidly recycle back to the plasma membrane (**e**), traffic further to multi-vesicular bodies (MVB) or lysosomes whereupon they are degraded (**f**), or slowly recycle back to the plasma membrane from a peri-nuclear recycling compartment (**g**).

B Vesicles containing receptors differing in their actin binding potential undergoing fission (**a**). Actin binding acts as a separation mechanism (**b**); receptors which bind to the actin cytoskeleton are concentrated in the actin-rich cortex while those lacking an actin-binding motif are free of this constraint and may move rapidly via microtubuole or actin based mechanisms (**c**).

Figures

Figure 1:



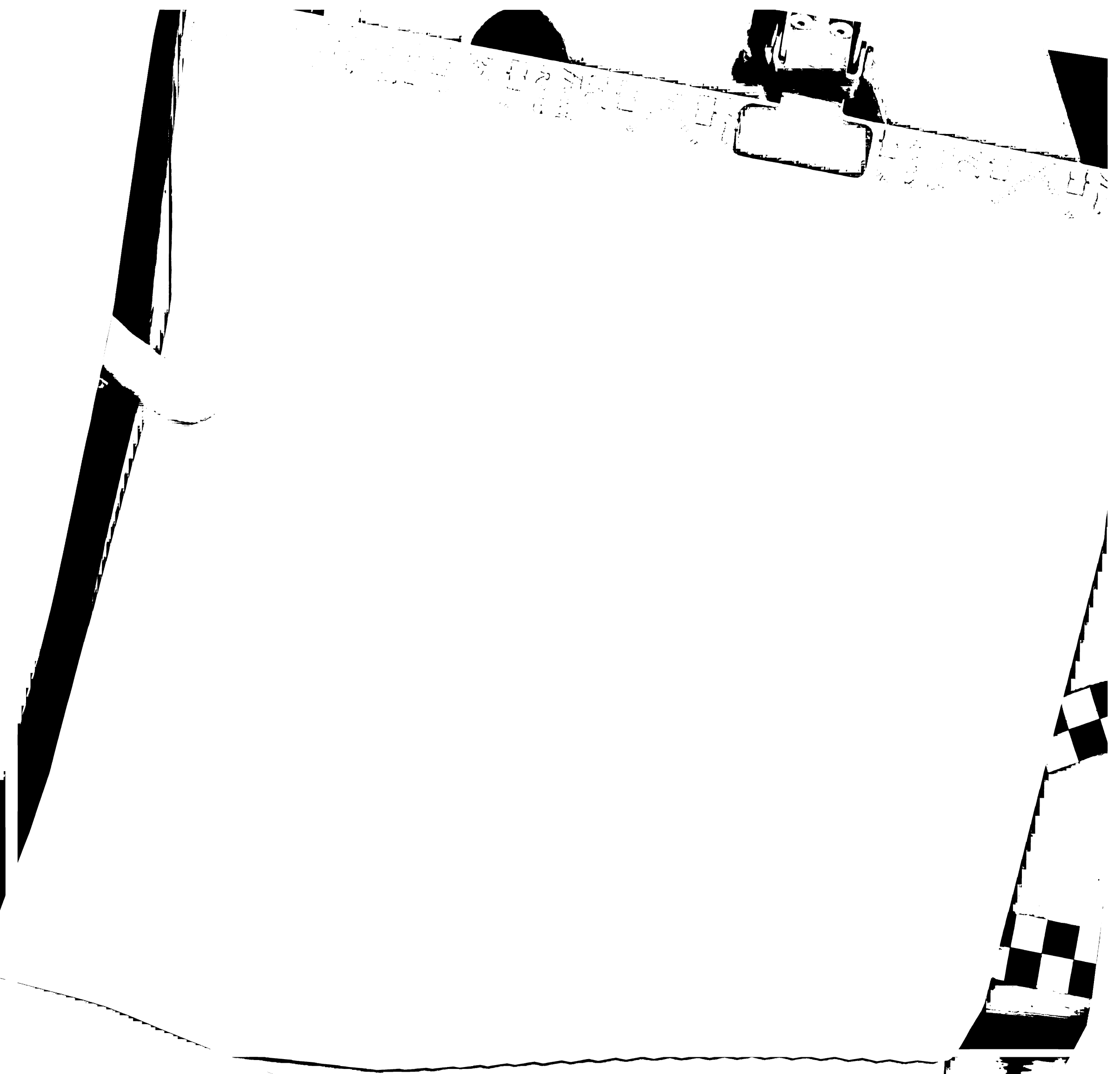
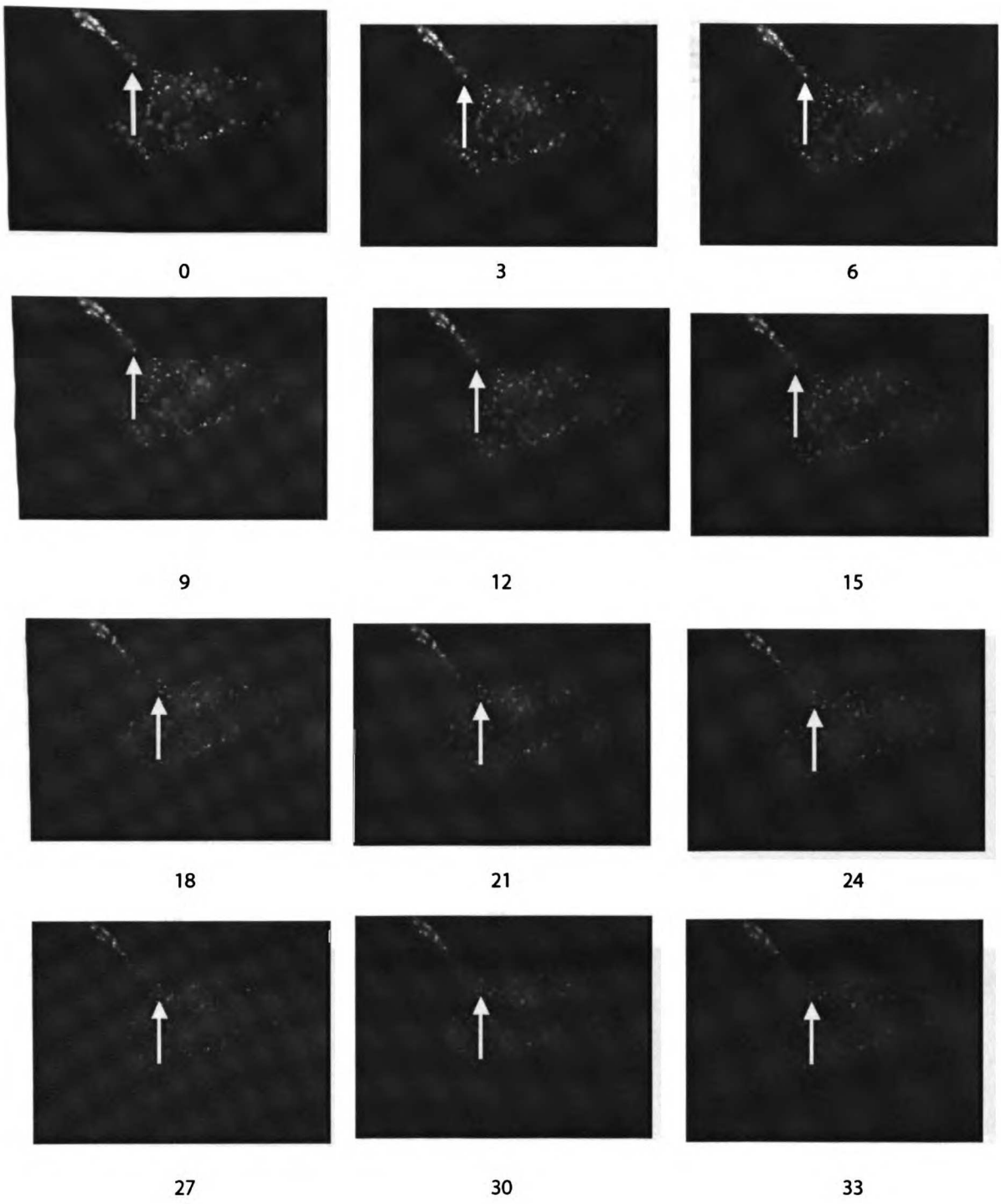


Figure 2:



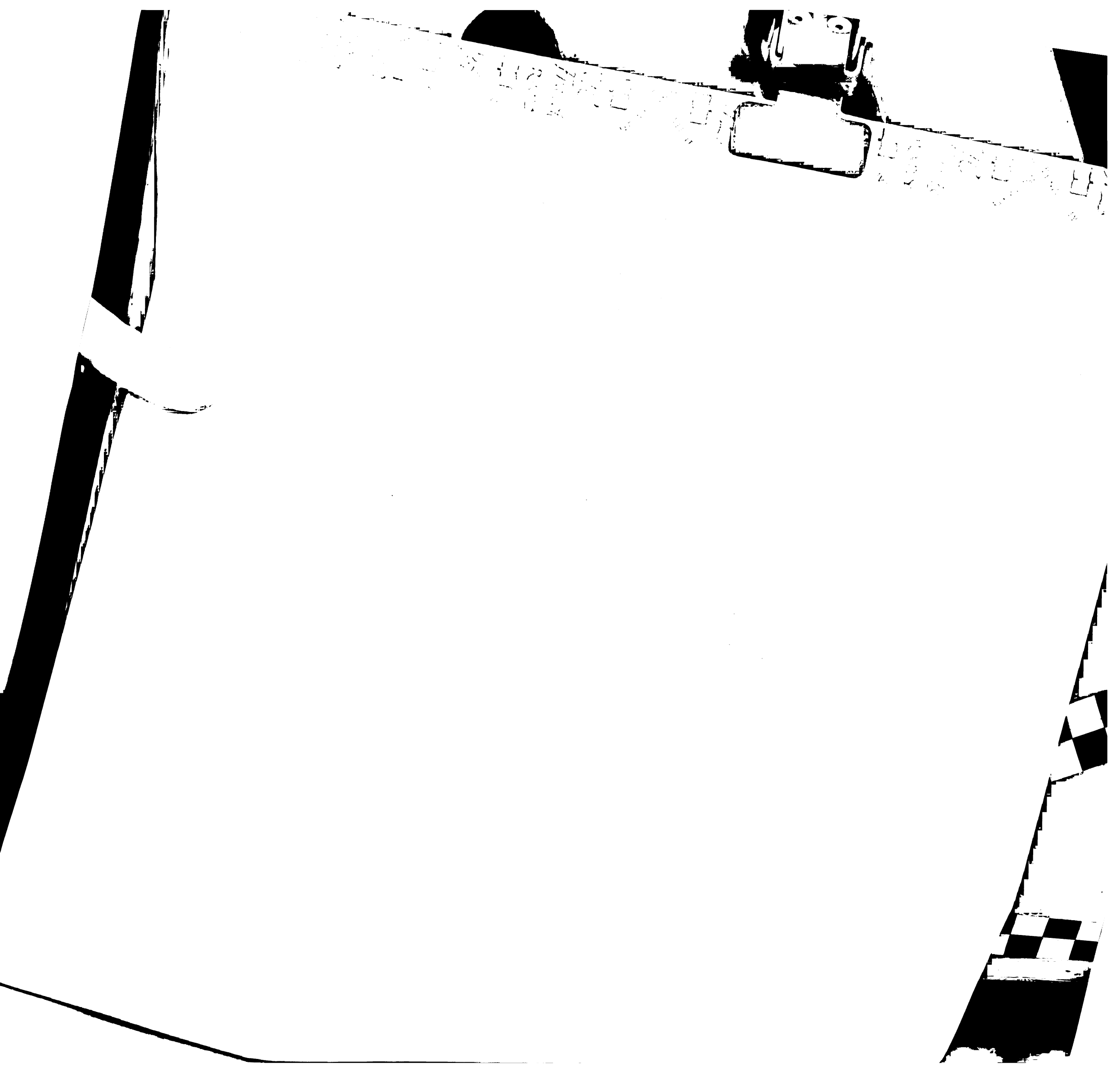
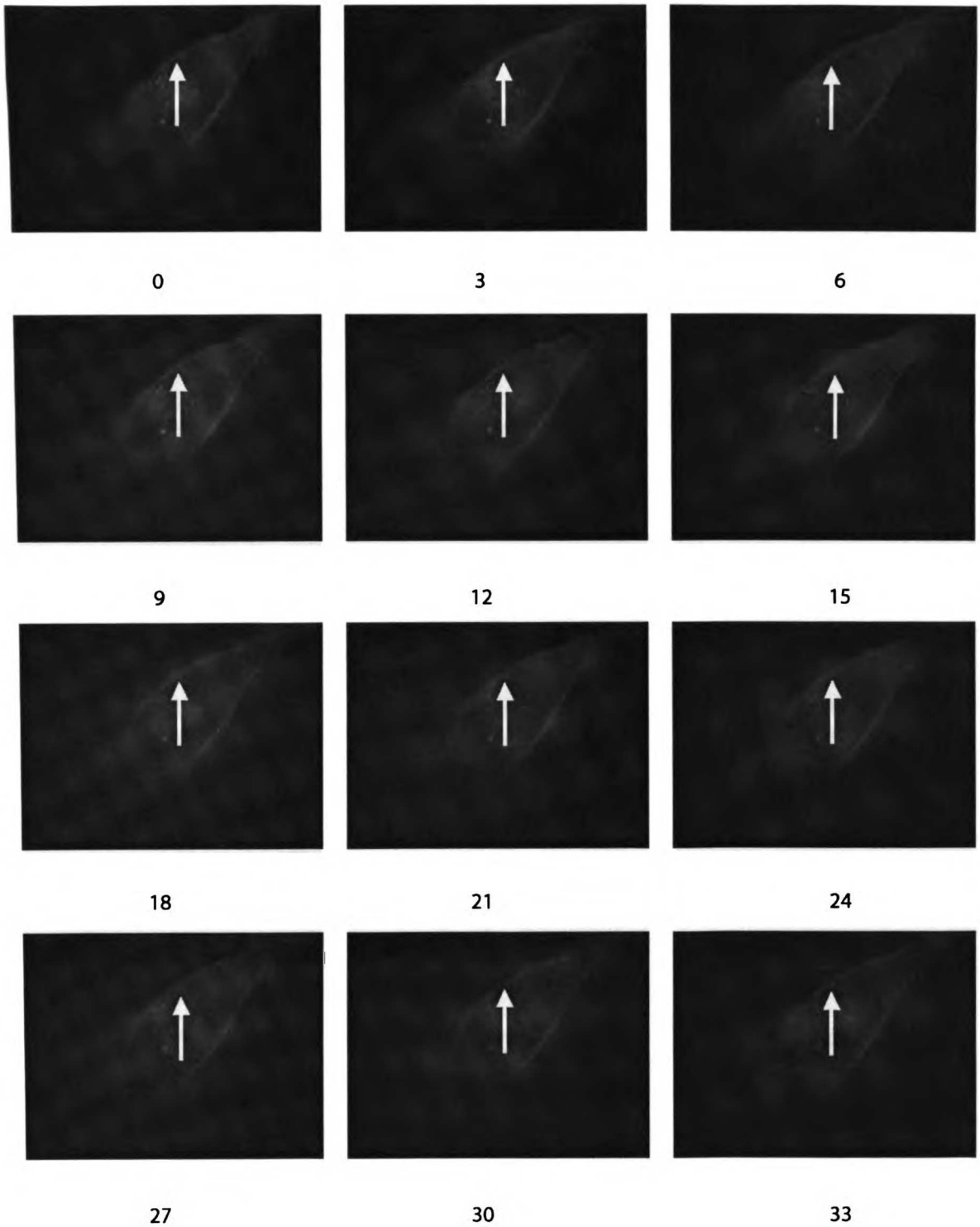


Figure 3:



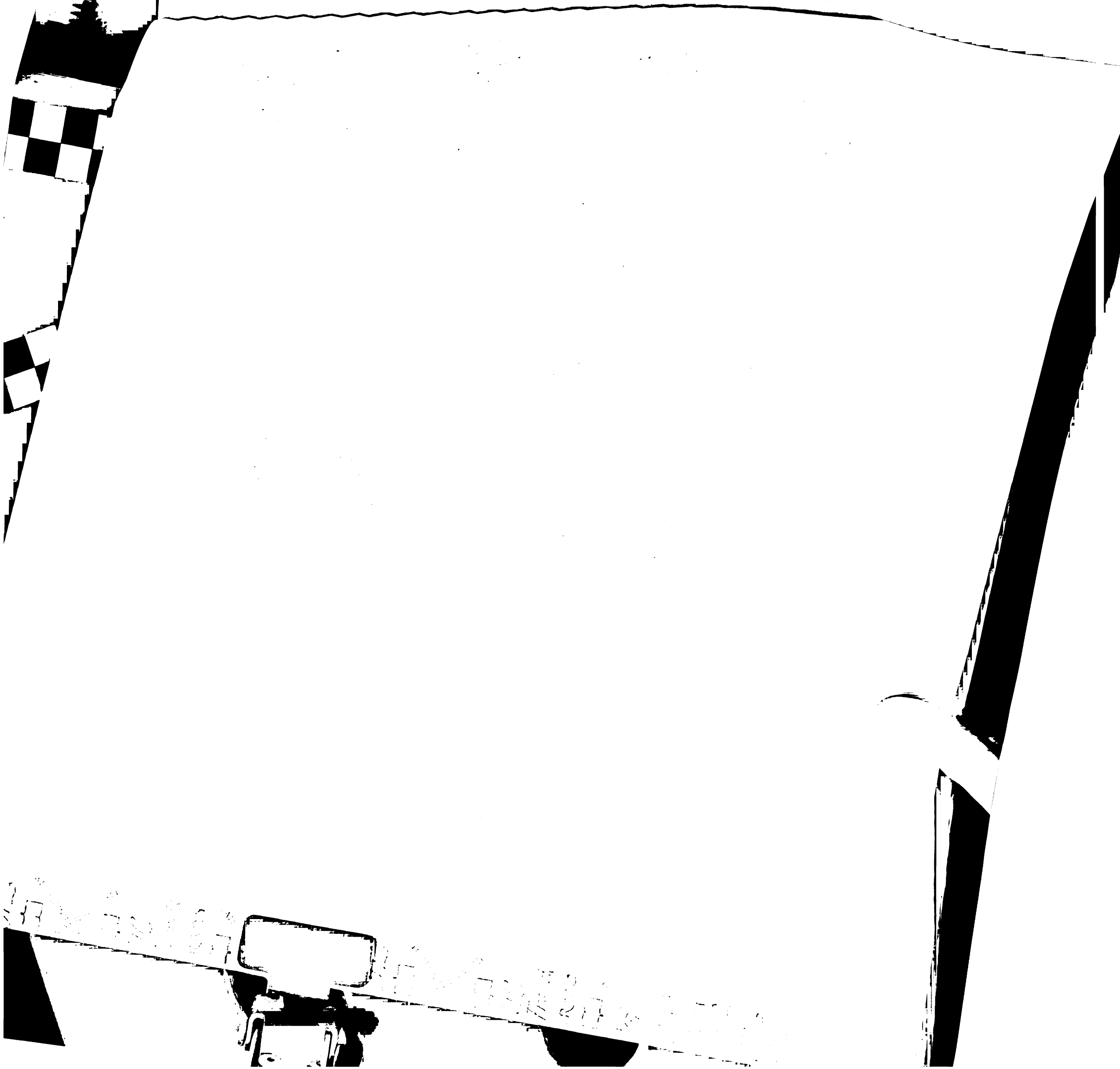


Figure 4: