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Protease-Activated Receptor-2 in the Intestinal Epithelium
&
Differential Effects of Beta Arrestins on the Internalization, Desensitization & ERK 1/2 Activation Downstream of Protease-Activated Receptor-2

A Dissertation submitted in partial satisfaction
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in

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by

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Committee Chairperson

University of California, Riverside

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ABSTRACT OF THE DISSERTATION

Protease-Activated Receptor-2 in the Intestinal Epithelium
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Doctor of Philosophy, Graduate Program in Biochemistry and Molecular Biology
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Dr. Kathryn A. DeFea, Chairperson

Research of G-protein coupled receptor signaling (GPCR) for therapeutic applications have historically been a hot topic of research. In the past, GPCR studies have been primarily focused on the molecular mechanism of its signaling pathway debunking the components of either traditional G-protein signaling or by other novel and more complex signaling pathways. However, recent GPCR studies have transitioned to examining distinct behaviors exhibited by and physiological responses that are triggered by cell- and localization-specific GPCRs. These responses include inflammatory, anti-inflammatory, cell migration and metastasis. Further, research of GPCR signaling have extended to the utilization of "biased agonists" where these modified agonists activates a specific GPCR and have the ability to trigger the activation of a specific pathway and inhibit other pathways that otherwise is propagated via activation by the non-modified agonist.

In the first study, we focus on the two distinct pools of protease-activated receptor-2 (PAR-2) present in intestinal epithelial cells: an apical pool facing the lumen, and a basolateral pool facing the bloodstream. Studies have demonstrated that introduction of PAR-2 agonists such as 2-furoyl-LIGRL-O-NH2 (2fAP) to the lumen can activate apical PAR-2 while it has been predicted mast cells and recruited leukocytes release proteases that can activate basolateral PAR-2. However, whether both pools of PAR-2 are equally capable of signaling, and the possible distinction between apical and basolateral PAR-2 induced responses have yet been addressed. Here we identified the expression of both apical and basolateral PAR-2 in cultured CACO2-BBe monolayers, and in mouse proximal jejunum. We reveal apical and basolateral PAR-2 in the intestinal epithelium are accessible by PAR-2 agonist 2fAP, and have both temporally and mechanistically signaling distinctions. Further, we demonstrate the apical and basolateral pools of PAR-2 can trigger distinct physiological responses as well.

Secondly, we illustrate that PAR-2 activation enhanced bacteria uptake in cultured colonocytes via a clathrin and phosphotidyl-inositol-3-kinase dependent manner. Moreover, we show that PAR-2 activation stimulated bacteria uptake in mice intestines. These results suggested that activation of the receptor plays a crucial role in gastrointestinal barrier dysfunction and is indeed a contributing factor to bacterial infection, further allowing for better understanding of PAR-2 and its physiological role in the gastrointestinal system.

On the whole these studies attempts to provide clarification for the characterization of PAR-2 in the gastrointestinal system that can be used further in the research for the usage of PAR-2 as a therapeutic target.

Lastly, findings for which I was a supporting author in a recent publication, we examined the differential effects of β-arrestins on internalization, desensitization and signaling of PAR-2. We demonstrate here that β-arrestin-1 and β-arrestins-2 have distinctive roles in PAR-2 signaling, including mechanistic and temporal distinctions in signaling, signal termination, cellular localization of signaling components and degradation.

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Chapter 1

Introduction

1.1 BACKGROUND

1.1.1 *Protease-Activated Receptor-2 (PAR-2)*

The seven transmembraned G-protein coupled receptor PAR-2 is the second member of a family of four protease activated receptors (PAR-1, 3 and 4) (1, 2, 4, 7, 8). Thrombin is the principle activator of PAR-1, 3 and 4 however it cannot activate PAR-2. Activated of PAR-2 is achieved upon exposure to trypsin-like proteases including trypsin, mast cell tryptase, kallikreins, and the tissue factor VIIa-Xa complex resulting in the proteolytic cleavage of its N-terminal domain exposing a tether ligand that folds back and activate the receptor itself (3, 4, 5, 6, 7, 8).

1.1.1.1 *PAR-2 discovery*

Cloning of PAR-2 came about after the cloning of PAR-1. Human isoform of PAR-2 was cloned following the cloning of mouse PAR-2 from a mouse genomic DNA library (9, 10, 11). The PAR-2 was found to be activated by the serine protease trypsin when the receptor was expressed in *Xenopus* and thrombin failed to activate the receptor. Previous work show the amino acid sequence of human PAR2 was found to be 83% & 35% identical to mouse PAR-2 and human PAR-1. Moreover, the human PAR-2 was found to be localized to chromosome 5q13 and consisting of double exons, similar to that of human PAR-1.

1.1.1.2 *Activators and activation of PAR-2*

PAR-2 is activated via cleavage of Arg 34 of its N-terminal domain by trypsin-like proteases including trypsin, mast cell tryptase, kallikreins, and the tissue factor VIIa-Xa complex (Fig. 1-1) (3,4, 5, 6, 7, 8, 12). Proteolytic cleavage of the N-terminal domain exposes a tethered ligand that consequently folds back and binds to the receptor itself. *In vitro,* there are synthetic peptides corresponds to the sequence of the tether ligand (SLIGKV for human PAR2 and SLIGRL for mouse PAR2) which can be used to activate PAR-2 without the presence of trypsin-like proteases (Fig. 1-2) (12). Several bacterial proteases have been shown to activate PAR-2 as well, including Gingipain R from *Porphyromonas gingivalis* (24) and certain cockroach proteases.

Inactivation of PAR-2 can occur as well via exposure to proteases. Elastase and Cathepsin G can cleave PAR-2 in cultured cells cell lines removing the N-terminal epitope for required for proteolytic cleavage, thus, PAR-2 is unresponsive upon exposure to trypsin-like proteases (1,2,3, 13,15).

1.1.1.3 *Structure and domains of PAR-2*

Examination of all four PAR's, examination of conservation between the members revealed three funcationally important domains (Shown in Fig. 1-4) (13). The N-terminal domain contains the site of protease binding and cleavage, extracellular loop-2 domain contains the ligand binding domain, and the C-

terminus contains phosphorylation sites responsible for mediating internalization and desensitization of PAR-2.

1.1.1.4 *Tissue and Cellular specific expression of PAR-2*

PAR-2 expression can be found in a variety of cell lines and tissues. It has been shown to be present in small intestines, colon, liver, pancrease, kidney, prostate, trachea, brain and lung (16, 17, 18, 19, 20, 21, 22). Cell lines expressing PAR-2 include MDA MB 468 (breast cancer), A549 (lung adenocarcinoma), epithelial cells of the gastrointestinal system and airway, vascular endothelial and smooth muscle cells, cells of the nervous system such as neurons, glia, astrocytes, SW480 (colon adenocarcinoma), PC-3 (prostatic adenocarcinoma) and skin cells (melanocytes and keratinocytes) (23).

1.1.1.5 *PAR-2 activators*

PAR-2 activation can be achieved without the presence of a proteolytic moiety via synthetic peptides (PAR-2 activating peptides, or PAR-2 AP's) that corresponds to the sequence of the tethered ligand, which became a stepping stone for the development of enhanced PAR-2 agonist and antagonists. Various studies have indicated modifications of PAR-2 AP generate enhanced selectivity and affinity for PAR-2. Some of these modifications include: (1) Replacement Ala at position two diminished the activity of the agonist, thus demonstrated the importance of the Leu^2 side chain (27). (2) Amidation of the C-terminus of PAR-

2 increased the potency of PAR-2 AP (26). (3) Substitutions at Ser^1, Ile^3, and Arg5 resulted in reduction of potency (27). (4) For maximal activation of PAR-2, AP must have at least five residues, truncation leads to decreased activity (25). (5) Replacement of Ser^1 with sterically hindering groups such as trans-cinnamoyl-and 2-furoyl improved the efficiency of PAR-2 AP (28, 29).

Studies using PAR-2 chimeras tremendously aided in distinguishing important domains of the receptor. Some of these studies include findings of residues in the N-terminus and extracellular loop-2 was crucial for the binding/interaction with the tethered ligand upon proteolytic leavage. The studies demonstrated replaced the two domains in the *Xenopus* receptor with the domains of human PAR-2 allowed activation of the receptor using ligands corresponding to the human tethered ligand (30). Moreover, mutations of phosphorylation sites on the C-terminus of PAR-2 induced changes in internalization and desensitization, where the mutants exhibited internalization that is dynamin dependent, however, not via the traditional clathrin/ β-arrestins approach (31).

1.1.1.6 *Intracellular signaling of PAR-2*

PAR-2 can activate ERK 1/2 via Gαq dependent pathway. Upon activation of the receptor, heterotrimeric G-proteins are recruited, resulting in activation of PLC and cleavage of phosphoinositol-4, 5-biphosphate (PIP_2) to generate inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (28-33). Presence of cytosolic IP_3 induces intracellular influx of calcium (Ca^{2+}), where DAG and Ca^{2+} activate

PKC. Subsequent activation of Mitogen Activated Kinase proteins (MAPK) by PKC leads to activation and nuclear translocalization of ERK 1/2 (Fig. 1-5) (33, 34, 35, 36, 37, 38, 39, 40). Further, activation of PAR-2 induced release of arachidonic acid and production of prostaglandins E_2 and $F_{1\alpha}$ in enterocytes and transfected epithelial cells, indication of activation of phospholipase A2 and cyclooxygenase-1 (41).

1.1.1.8 *Termination of PAR-2 signaling*

PAR-2 signaling can be terminated by variety of methods. Firstly, as mentioned before, PAR-2 signaling can be terminated by other proteases, cleaving trypsin-like protease recognition sites (14). PAR-2 signaling can be terminated by the binding of β-arrestins to the C-terminus of the receptor preventing the binding of G-proteins and β-arrestins also mediate the endocytosis of the receptor (42). The endocytosis of PAR-2 is performed in a clathrin dependent manner (39, 43). The internalized PAR-2 enters the early endosome and sorted into the lysosome. PAR-2 may also be recycled and brought back to the cell surface via Golgi trafficking (42). Previous studies suggest internalization of PAR-2 is also Rab-5 GTPase dependent while Rab-11 is involved in the Golgi-sorting and recycling (46). Further, dynamin has been suggested to be involved in the process as well (31). PAR-2 internalization has been suggested to be involved in β-arrestins scaffolding of proteins allowing for efficient activation of intracellular targets such as ERK 1/2 or other kinases (44, 45).

1.1.1.9 *Physiological functions of PAR-2*

PAR-2 is reported to trigger a wide variety of cellular responses (e.g. proliferation, chemotaxis, ion transport, epithelial barrier function, and tumor cell metastasis) in a cell type-specific manner (47, 48, 49, 50, 51, 52, 53, 54, 55). Further, Studies showed activation of PAR-2 resulted in both pro and anti-inflammatory in numerous systems including gastrointestinal and cardiovascular. Some of these pro and anti-inflammatory effects identified included vasodilation, smooth muscle relaxation, up-regulation of cytokines, immune cell recruitment, increased nociception and hyperalgesia (56, 57, 58, 59).

Even though tentative functions of PAR-2 have been extensively studied, the physiological effects and diseases caused by PAR-2 still yet to be completely understood.

Further, numerous obstacles stand in the elucidation of PAR-2's physiological effects. For example, the administration of PAR-2 AP or trypsin-like proteases for PAR-2 studies is generally at a high nano- to micro-molar range. Treatment of agonist at a high dosage can have non-specific effects. Further, reversed/scramble sequence of PAR-2 AP can still trigger slight activation of the receptor and provoke downstream signaling, an indication negative control may evoke non-specific, unwanted activation of the receptor. Moreover, this indicates high precision agonists and antagonists of PAR-2 are not widely available.

1.1.1.9.1 *PAR-2 in the gastrointestinal system*

PAR-2 is expressed heavily in the gastrointestinal system in several cell types, including enterocytes, mast cells, smooth muscle cells, myenteric neurons and endothelial cells, studies suggest gastrointestinal PAR-2 activation may be both protective and damaging during inflammatory response depending on the disease model, the administration of PAR-2 agonist and the physiological readout (60). A 2, 4, 6-trinitrobenzene sulfonic acid (TNBS) induced mouse colitis study showed PAR-2 activation prevented up-regulation of proinflammatory cytokine, cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) while down-regulating CD44 (61). Further, other gastrointestinal studies demonstrated that PAR-2 activation increased mucus and decreased gastric acid secretion (61). Contrarily, in a mouse colonic model, treatment with PAR-2 agonists 2fAP and tryptase caused increased colonic paracellular intestinal permeability, erythema, hemorrhage, presence of fecal blood and mucus, edema and diarrhea (60). Other studies have also shown PAR-2 activation stimulated colonocyte cytoskeletal reorganization leading to decreased transepithelial resistance (TER) due to recruitment of a family of proteins known as β-Arrestins (49).

1.1.2 *β-arrestins*

β-arrestins are adapter proteins that bind and "arrest" most GPCR signaling post agonist binding and phophorylation by GPCR Kinases (GRK's). Upon binding β-arrestins are involved in internalization, desensitization and non-G-protein mediated signaling events.

1.1.2.1 *Regulation of β-arrestins for endocytotic processes*

Activation of β-arrestin-1 and β-arrestin-2 are regulated via post-translational modification of the proteins. Dephosphorylation of S412 amino acid, located on the C-terminal regulatory domain, is crucial for β-arrestins association and recruitment of endocytotic components (66, 67). Moreover, β-arrestin-2 is regulated by ubiquitination, process catalyzed by the E3 ubiquitin ligase Mdm2 that binds directly to β-arrestin-2 (68, 69).

On the other hand, the endocytic function of β-arrestin-2 has been shown to be regulated by ubiquitination which is catalyzed by the E3 ubiquitin ligase Mdm2, which binds directly to the β-arrestin (78).

1.1.2.2 *Structural domains of β-arrestins*

β-arrestins have been shown to be comprised of two structural domains (N and C), that are in a seven-stranded-β-sheet conformation (Fig. 1-6) (67, 70). There are also regulatory motifs within the N and C-termini. The N-terminal domain has been found to be responsible for the recognition of activated GPCR's and the C-terminal domain is responsible for secondary receptor recognition (71).

1.1.2.3 *β-arrestins in GPCR signaling*

β-arrestins upon recruitment and binding to phosphorylated GPCR can induce desensitization of the receptor/ termination of G-protein signaling, internalization of the receptor, degredation of the receptor and non-classical GPCR/β-arrestins mediated signaling.

1.1.2.3.1 *β-arrestins and termination of G-protein signaling*

Desensitization of GPCRs and termination of G-protein signaling is achieved where upon agonist exposure, β-arrestins recruitment and binding to the phosphorylated C-terminal tail of GPCR triggers the uncoupling of G-protein from GPCR. This ceases the signaling that is induced by G-proteins. Phophorylation of the C-terminus, important for desensitization of GPCR's, is achieved by GRK's and other kinases, where mutations to the phophorylation sites have partially diminished β-arrestins mediated desensitization/termination (31). Although desensitization and termination of G-protein induced signaling may occur without the presence of β-arrestins, β-arrestins to phosphorylation C-terminal tail of GPCRs enhances the potency and specificity of the process (62, 63).

1.1.2.3.2 *β-arrestins mediated internalization of GPCR's*

Internalization of many GPCR's is mediated by β-arrestins are achieved via induction of clathrin mediated endocytosis (Fig. 1-7) (64). These GPCR's include β2 adrenergic, angiotensin II type 1a, m2-m5 muscarinic cholinergic, endothelin A, D2 dopamine, follitropin, monocyte chemoattractant protein-1, CCR-5 and CXCR-1 receptors (64, 65). The C-terminal tail appears to be the domain which interacts with the endocytotic machinery. It is this domain for which interacts with clathrin, small G-protein ARF6 and its GEF (Guanine nucleotide exchange factor), NSF and clathrin adaptor protein AP2 (72, 73, 74). Whether β-arrestins are involved in the GPCR endocytotic process are cell, receptor and agonist specific. Further, phosphorylation of GPCRs via GRKs and conformation of the receptors upon agonist binding determines whether interaction with β-arrestins and internalization are induced.

1.1.2.3.3 *β-arrestins as a scaffold for signaling components*

There are increasing studies that suggest β-arrestins are involved in more than just desensitization, termination, and internalization of GPCR's. In these studies, β-arrestins were shown to be involved in signaling by acting as a scaffold for signaling complexes, and shuttling the components to the receptors. This agonist-dependent process introduces a new role for β-arrestins that do not follow the classic paradigm for GPCR signal transduction (64, 75).

One example as previously mentioned, β-arrestins serves as a scaffold for MAPK components upon PAR-2 activation. β-arrestins bind to phosphorylated C-terminal tail of PAR-2 ceasing G-protein mediated signaling and acts as a scaffold for RAF-1, MEK and ERK 1/2. This leads to ERK 1/2 activation and inhibition of its nuclear localization, retaining the kinases in the cytosol (Fig 1-8) (64).

1.1.3 *Cofilin*

Cell migration is a process mediated by the extension of pseudopodia at the leading edge and dissolving stress fibers while inhibiting extension at the trailing edge (78, 79, 80). This process is mediated by cofilin, an actin severing protein which allows for rapid changes in the cytoskeletal architecture. The actin severing triggers the formation of new barbed ends for elongation and recycling of ATP monomers. (78, 80, 81, 82, 83).

1.1.3.1 *PAR-2 and activation of cofilin*

Cofilin activation is controlled by contradicting actions of LIM Kinase (LIMK) (inactivation of cofilin by phophorylation on Ser^3) and phosphatases chronophin (CIN) and slingshot (activation via dephosphorylation) (83, 84, 85, 86). Moreover, intracellular pH and phosphoinositol 4,5 biphosphate levels can also regulate cofilin activity (87). Our studies showed activation of PAR-2 activates cofilin in a β-arrestins mediated fashion. Activation PAR-2 led to dephosphorylation of cofilin via inhibition of LIMK and activation/sequestration

of CIN and induced severing activity in a breast cancer cell line MDA-MB 468 (44). Predicted pathway of PAR-2 induced cofilin activation is shown in Fig. 1-9 (44).

1.1.4 *Phosphoinositide-3-kinase (PI3K)*

PI3K are a family of enzymes that are involved in numerous biological processes, including cell growth, differentiation, survival, proliferation, migration and metabolism (88, 89). PI3K phosphorylates the 3' hydroxyl group of the inositol ring of phosphatidylinositol (PtdIns) (90).

There are three classes of PI3K (I-III) that is categorized via structure, lipid specificity and regulations. Class I PI3K's are responsible for the production of phosphatidylinositol-3-phosphate (PI_3P) and phsphatidylinositol (3, 4, 5)- triphosphate (PIP_3)(91). Class I PI3K's are activated by signaling induced by GPCR's and receptor-tyrosine kinases (RTK's). They contain five regulatory domains (P85α, P55α, P50α, P85β and P55γ) and three catalytic domains (P110α, β and δ) (92). These PI3K's activate Protein Kinase B (known as Akt) and studies show mutations to class I PI3K's are cancer inducing (91, 92).

1.1.4.1 *PAR-2, β-arrestins and PI3K*

In our previous studies, we have shown PAR-2 activation can induce activation of both p110α and p110β catalytic subunits of PI3K via G-protein signaling, hence only in cell lines which with low endogenous expression of β-arrestins. (90, 91)

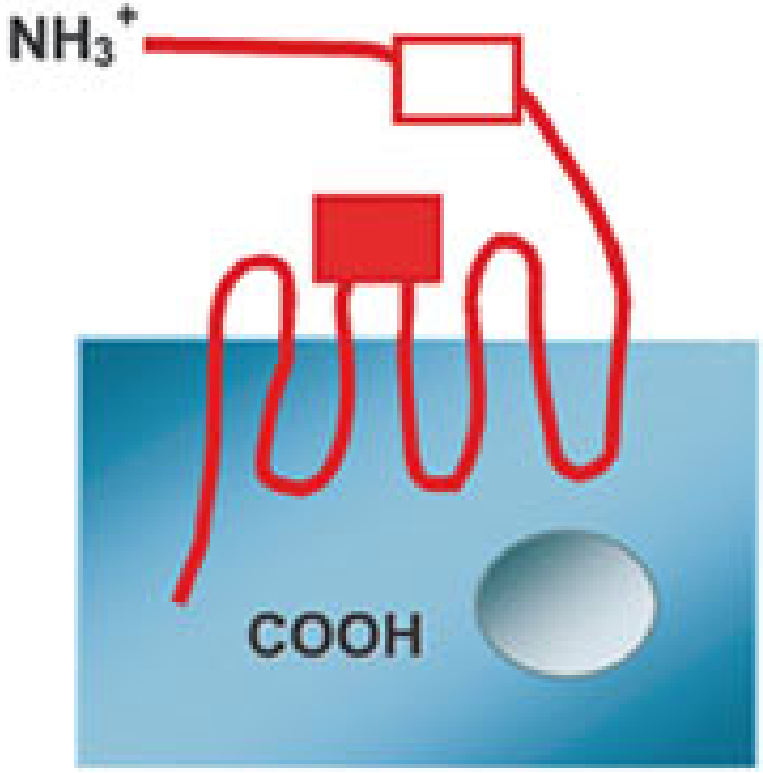
We showed that β-arrestins can regulate PI3K distinctively, with β-arrestin 1 inhibiting on p110β subunit and β-arrestin 2 can inhibit both p110 α and β (91).

1.1.4.2 PI3K and microbial internalization

More recent studies suggest PI3K pathway is important and required for the endocytosis of bacterial *Pseudomonas Aeruginosa* (P. Aeruginosa) into epithelial cells (93). Moreover, the same group showed in response to *P. Aeruginosa* attachment to the apical surface of the epithelial cells, many of the basolateral proteins including PI3K and actin translocated and recruited to the site of bacterial adhesion (93, 94). The proteins at the apical surface were removed as well from the site of bacterial attachment. Furthermore, these studies demonstrated the basolateral proteins that are found at the protrusion site of bacterial adhesion were shuttled from the basolateral membrane and directed via a dynamin and PI3K dependent transport mechanism (94).

1.2 FIGURES AND LEGENDS

Fig. 1-1: Activation of PAR's. This figure shows PAR's upon exposure to proteases that cleaves the N-terminus of the receptors exposing and tether ligand which binds to the activation domain inducing downstream signaling.



B

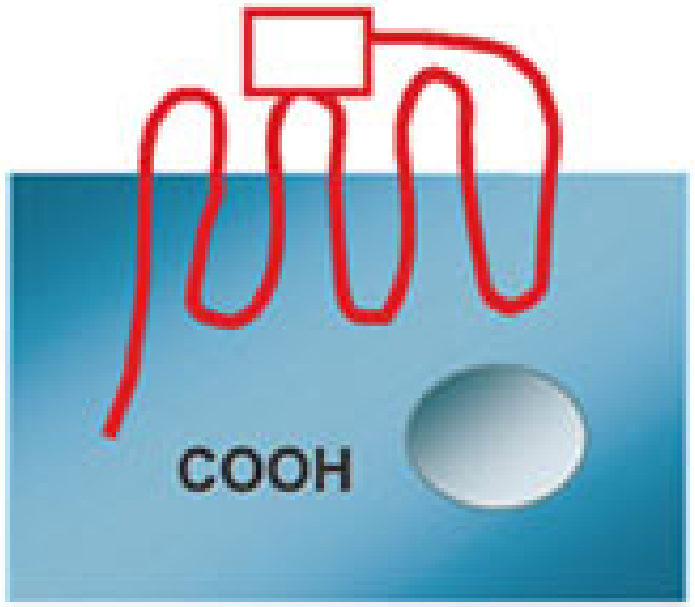


Fig. 1-2: Activation of PAR's via synthetic peptide. This figure shows PAR's can be activated by synthetic peptides without exposure to proteases which corresponds to the sequence of the tethered ligand upon binding to the receptor's activation domain. Binding of the synthetic peptide which corresponds to the sequence of the tether ligand of a specific PAR can induce downstream signaling.

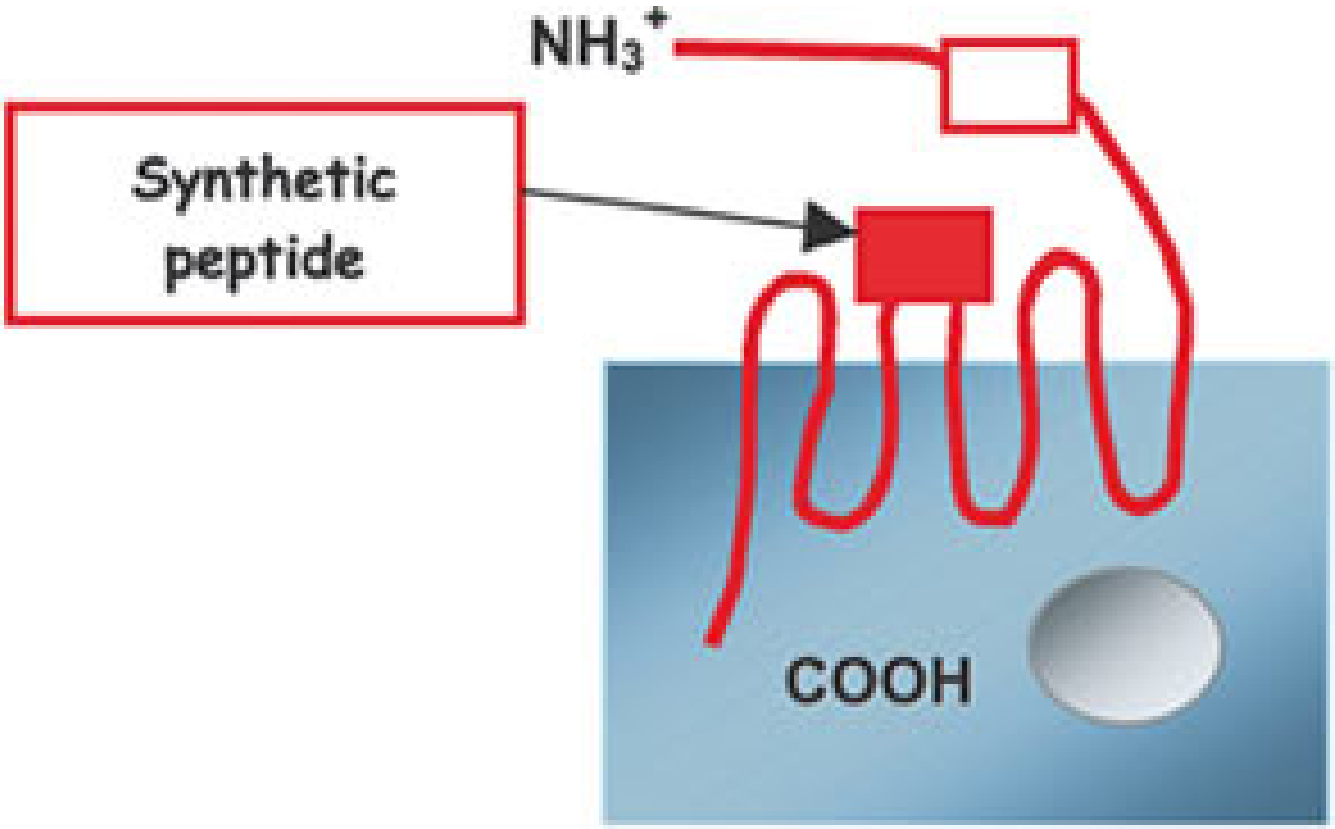

NH3+
Synthetic peptide
COOH

Fig. 1-3: Inactivation of PAR's. This figure shows PAR's are activated via proteolytic cleavage to the N-terminus, resulting in tether ligand formation that binds to the activation domain. Moreover, it shows PAR's can be inactivated via cleavage of the N-terminus via proteases such as cathepsin G which removes the tethered ligand.

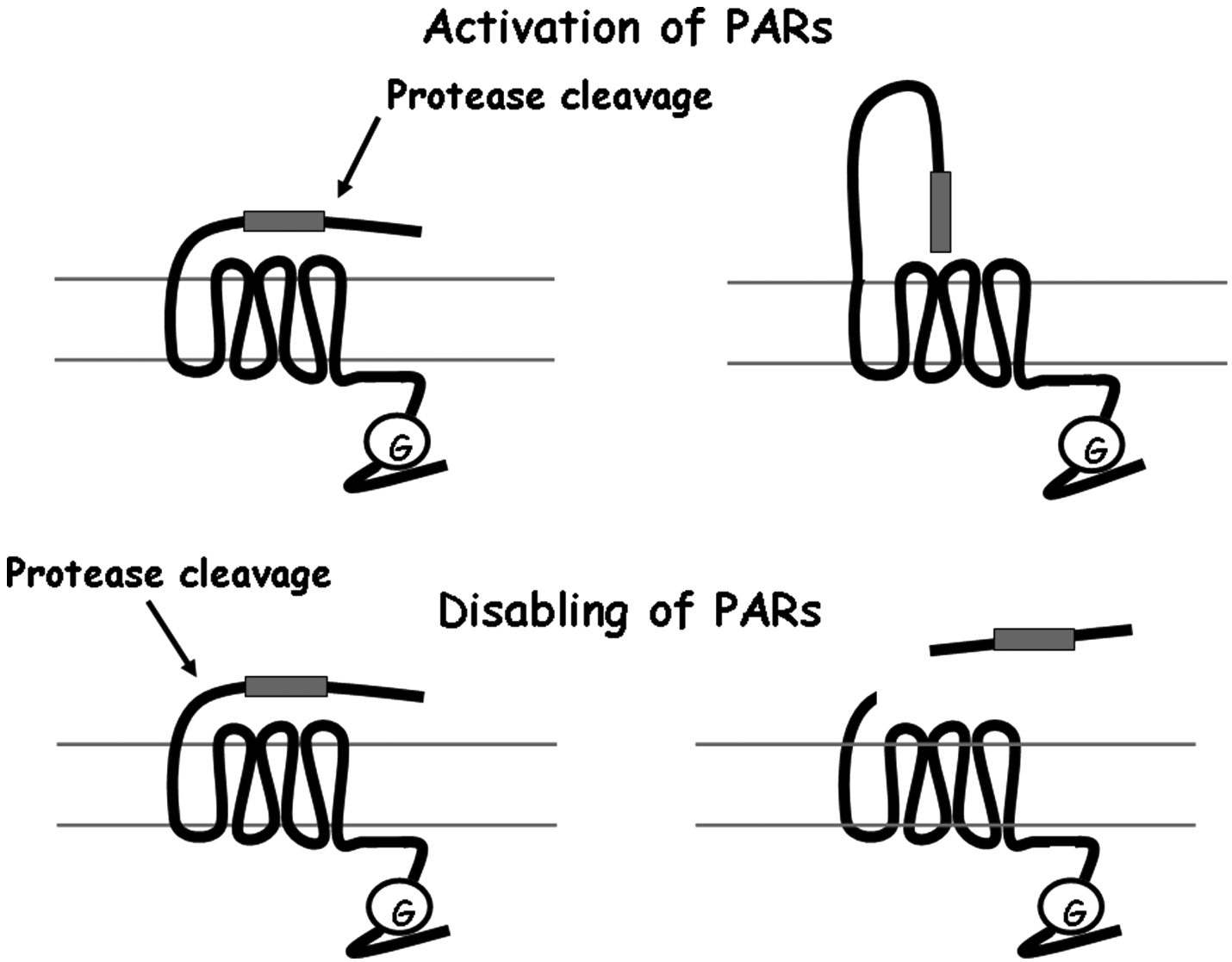

Activation of PARs
Protease cleavage
G
G
Protease cleavage
Disabling of PARs
G
G

Fig. 1-4: Functional domains of PAR's. The figures demonstrate functional domain in human family of PAR's (1-4). The pink highlights the tethered ligand sequence and green highlights the hirudin-like binding site. The bold represents conserved residues in the extracellular loop II between the families of PAR's.

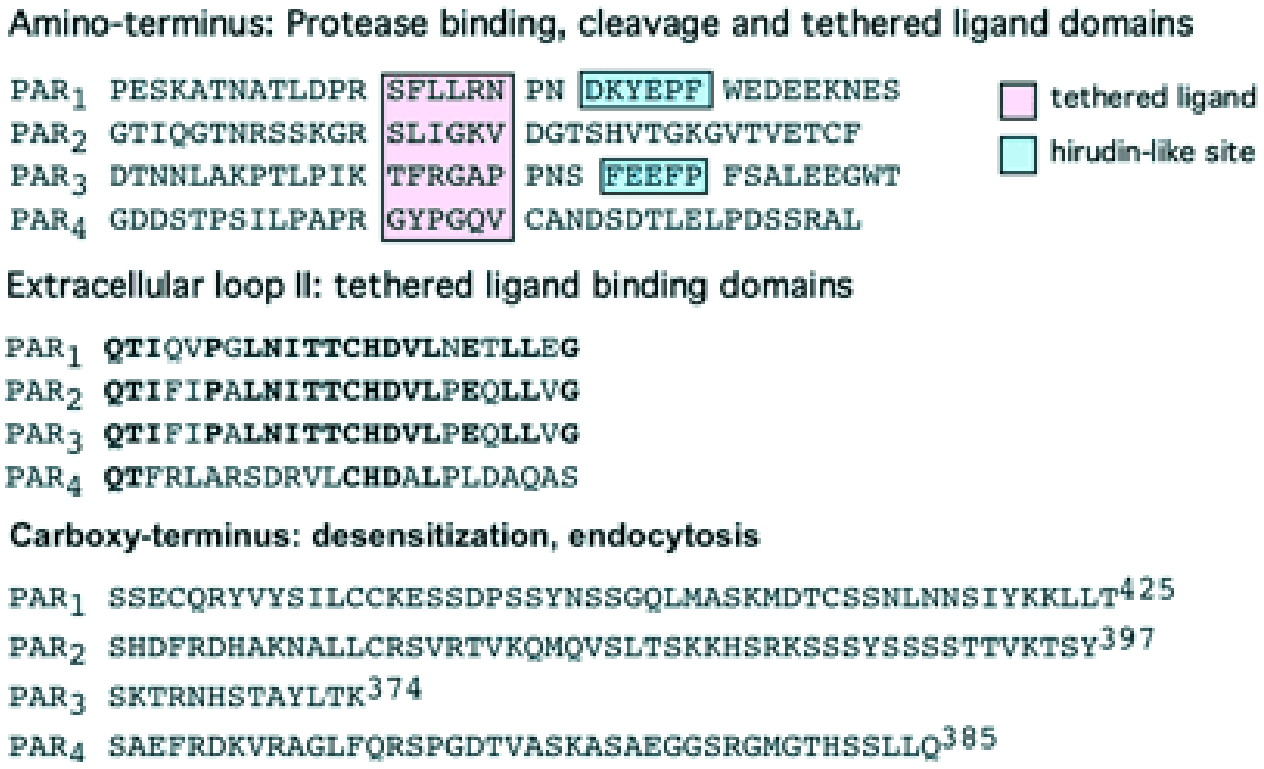

Amino-terminus: Protease binding, cleavage and tethered ligand domains
PAR1 PESKATNATLDPR SFLLRN PN DKYEPF WEDEEKNES
PAR2 GTIQGTNRSSKGR SLIGKV DGTSHVTGKGVTVETCF
PAR3 DTNNLAKPTLPIK TFRGAP PNS FEEFP FSALEEGWT
PAR4 GDDSTPSILPAPR GYPGQV CANDSDTLELPDSSRAL
tethered ligand
hirudin-like site
Extracellular loop II: tethered ligand binding domains
PAR1 QTIQVPGLNITTCHDVLNETLLEG
PAR2 QTIFIPALNITTCHDVLPEQLLVG
PAR3 QTIFIPALNITTCHDVLPEQLLVG
PAR4 QTFRLARSDRVLCHDALPLDAQAS
Carboxy-terminus: desensitization, endocytosis
PAR1 SSECQRYVYSILCCKESSDPSSYNSSGQLMASKMDTCSSNLNNSIYKKLLT425
PAR2 SHDFRDHAKNALLCRSVRTVKQMQVSLTSKKHSRKSSSYSSSSTTVKTSY397
PAR3 SKTRNHSTAYLTK374
PAR4 SAEFRDKVRAGLFQRSPGDTVASKASAEGGSRGMGTHSSLLQ385

Fig. 1-5: PAR-2 elicits dual and competitive modes of signaling. The figure illustrates PAR-2 signaling. PAR-2 activation can provoke G-protein dependent activation of ERK 1/2 and consequent nuclear localization of the kinases. However, PAR-2 activation can also elicit β-arrestins dependent ERK 1/2 activation resulting in cytosolic sequestration. PAR-2 activation leads to recruitment of heterotrimeric G-proteins resulting in cleavage of PIP2 into IP3 and DAG. Presence of IP3 induces intracellular calcium mobilization and with presence of DAG, leads to activation of PKC. Consequent activation of the MAPK cascade leads to ERK 1/2 activation and nuclear localization. Moreover, PAR-2 activation can lead to the recruitment of β-arrestins. Recruitment of the adapter proteins elicits recruitment of clathrin and internalization of PAR-2. Further, β-arrestins act as a scaffold for the MAPK cascade components sequestering ERK 1/2 in the cytosol inhibiting nuclear translocation, and triggering phosphorylation of cytosolic substrates.

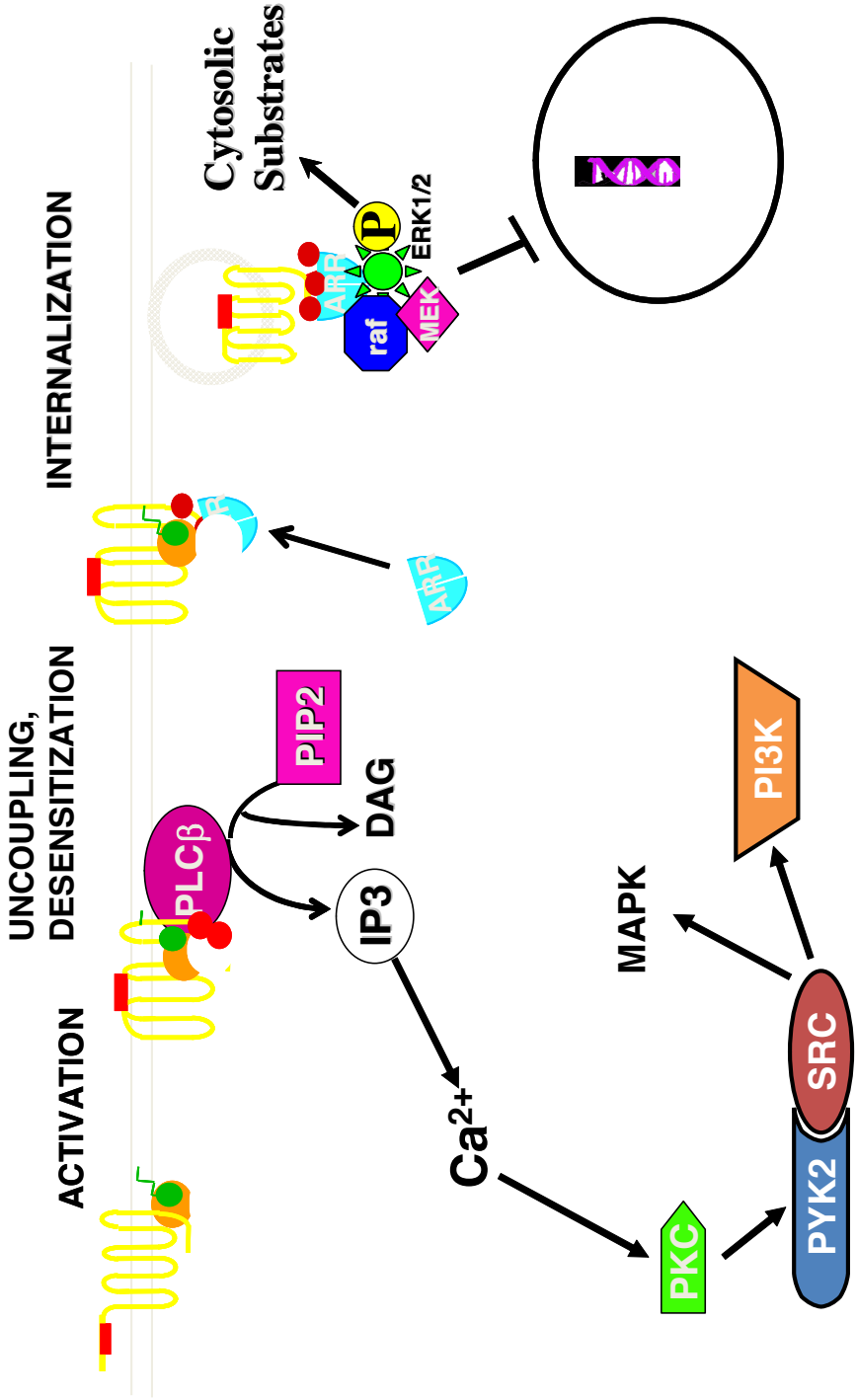

ACTIVATION
UNCOUPLING,
DESENSITIZATION
INTERNALIZATION
PLCβ
PIP2
DAG
IP3
Ca2+
PKC
PYK2
SRC
PI3K
MAPK
ARF
ARF
raf
MEK
ERK1/2
P
Cytosolic
Substrates

Fig. 1-6: Functional domains of β-arrestins. The figure shows β-arrestins contain two structural domains (N and C). There are also regulatory motifs within the N and C-termini. The N-terminal domain has been found to be responsible for the recognition of activated GPCR's and the C-terminal domain is responsible for secondary receptor recognition.

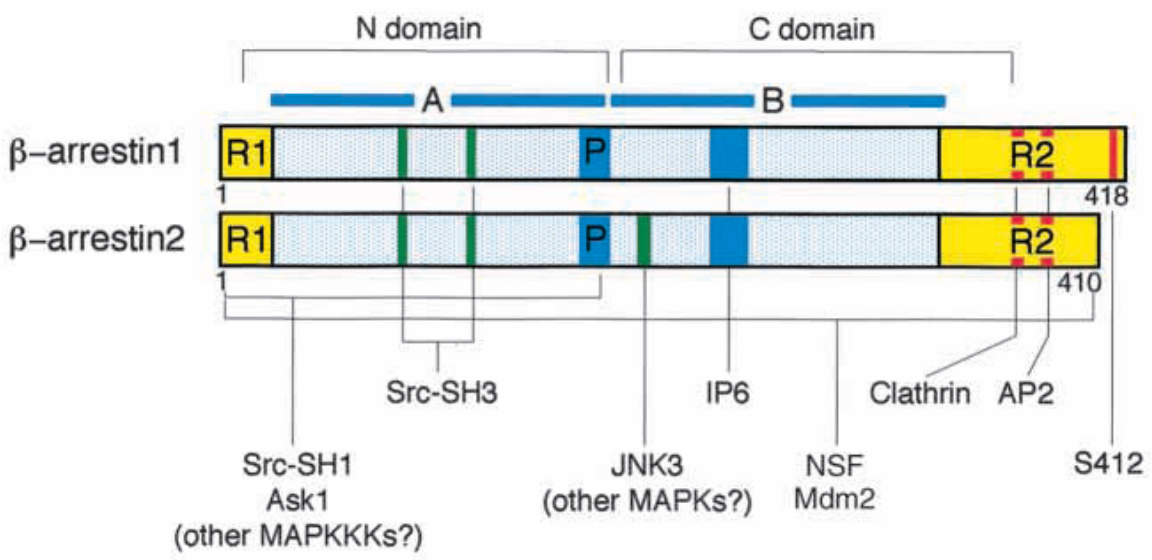

N domain
C domain
A
B
β–arrestin1
R1
P
R2
1
418
β–arrestin2
R1
P
R2
1
410
Src-SH3
IP6
Clathrin
AP2
Src-SH1
Ask1
(other MAPKKKs?)
JNK3
(other MAPKs?)
NSF
Mdm2
S412

Fig. 1-7: PAR-2 activation results receptor internalization in β-arrestins/clathrin dependent fashion. This figure shows internalization of PAR-2. Internalization of PAR-2 is mediated by β-arrestins are achieved via induction of clathrin mediated endocytosis. The C-terminal tail appears to be the domain which interacts with the endocytotic machinery. It is this domain for which interacts with clathrin, small G-protein ARF6 and its GEF, NSF and clathrin adaptor protein AP2. Phosphorylation of PAR-2 at the C-terminus and conformation of the receptors upon agonist binding determines whether interaction with β-arrestins and internalization are induced.

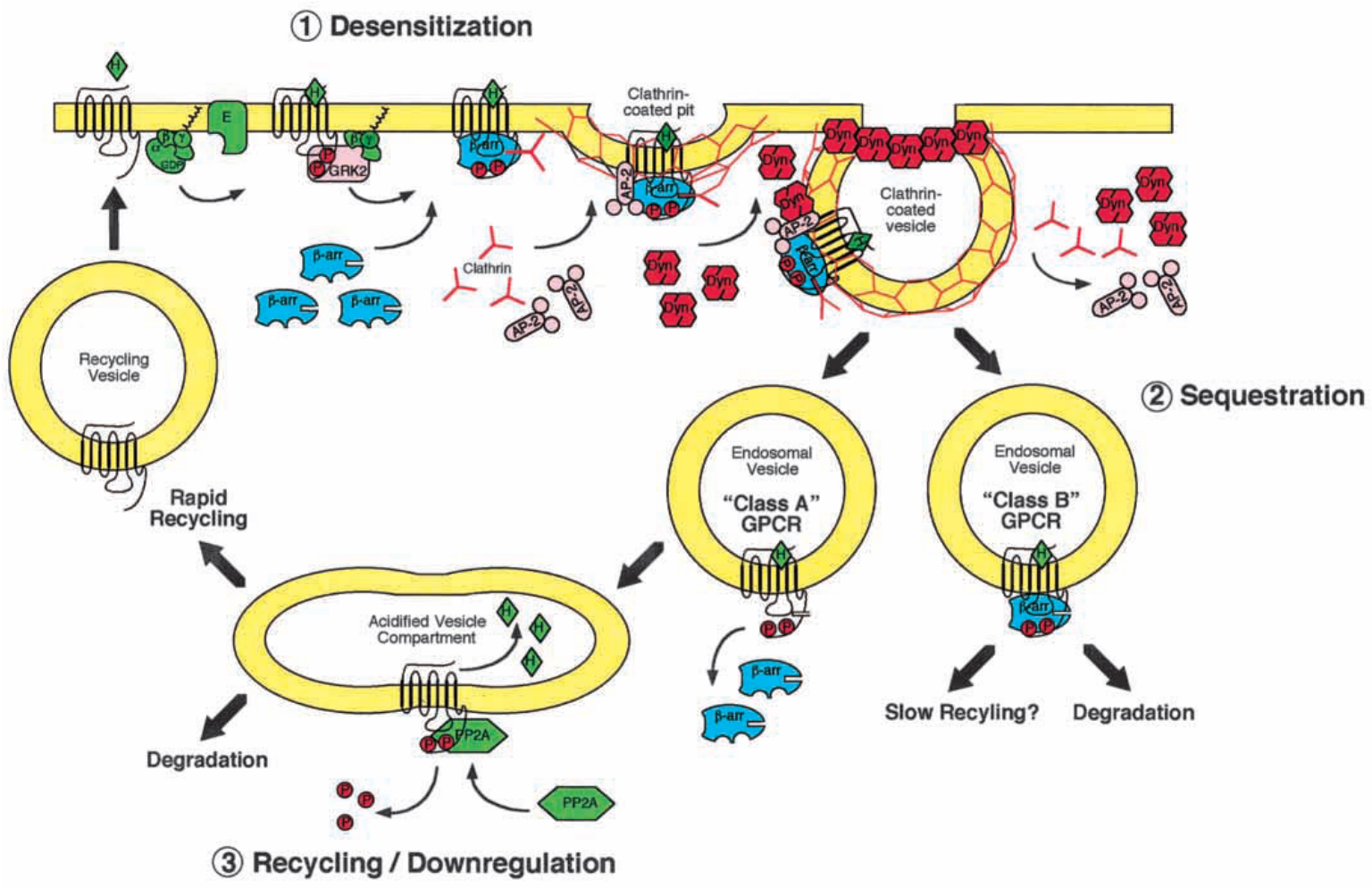

① Desensitization
Clathrin-coated pit
Clathrin-coated vesicle
Recycling Vesicle
Rapid Recycling
② Sequestration
Endosomal Vesicle
"Class A" GPCR
Endosomal Vesicle
"Class B" GPCR
Acidified Vesicle Compartment
Degradation
Slow Recyling?
Degradation
③ Recycling / Downregulation
β-arr
GRK2
Clathrin
AP-2
Dyn
PP2A

Fig. 1-8: PAR-2 activation leads to β-arrestins scaffolding of MAPK complex. The figure shows PAR-2 activation leads to recruitment of β-arrestins and clathrin. Consequent scaffolding of MAPK cascade resulting in cytosolic sequestration of ERK 1/2 follows.

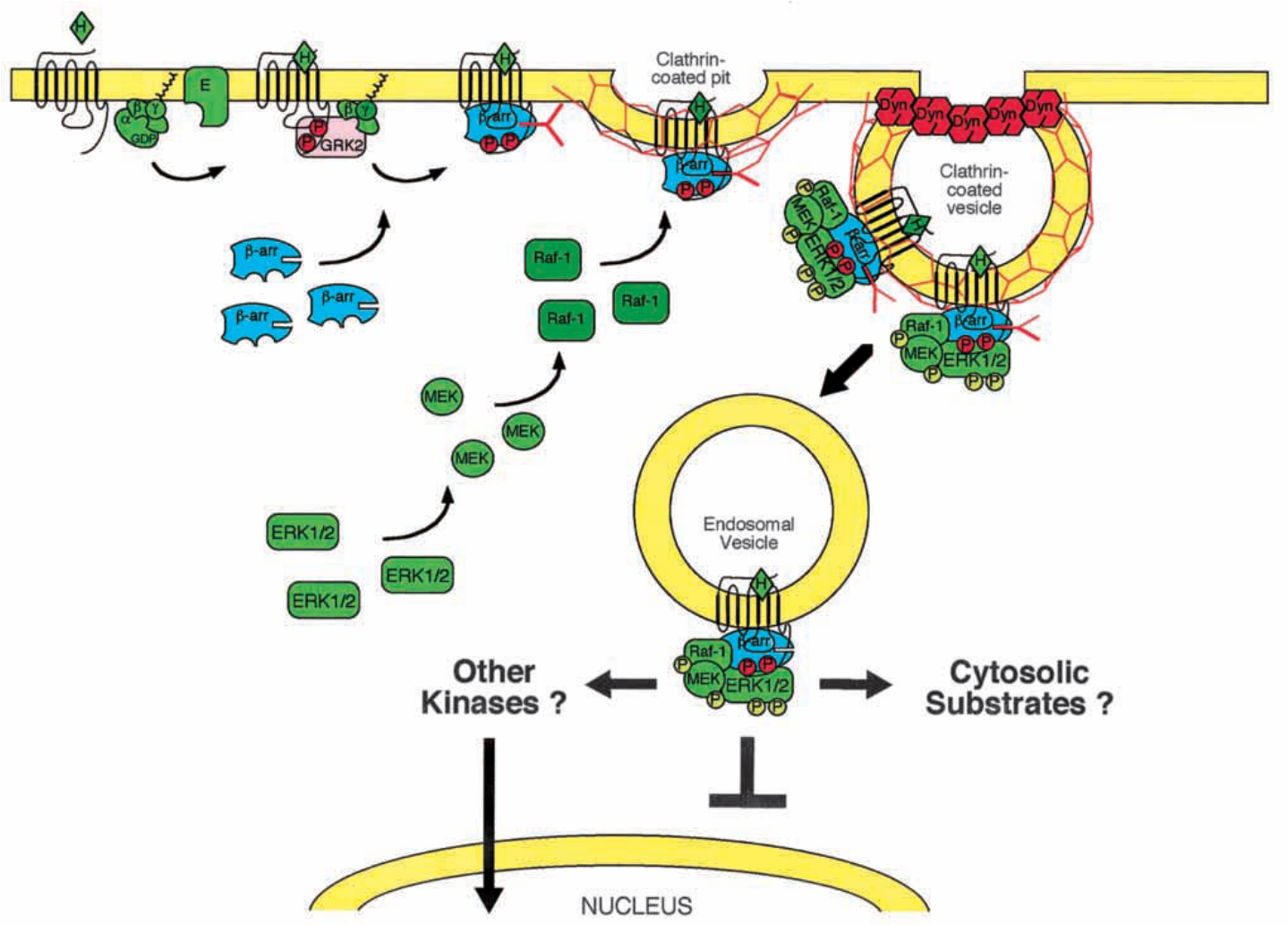
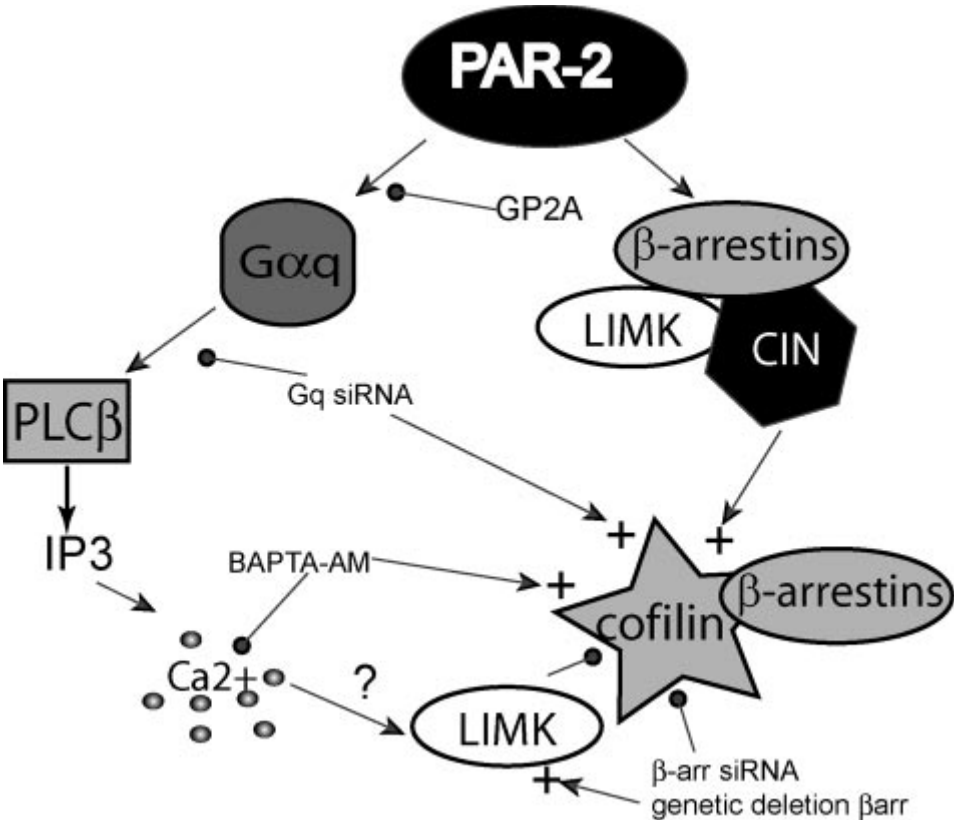

Clathrin-
coated pit
Clathrin-
coated
vesicle
Endosomal
Vesicle
β-arr
GRK2
Raf-1
MEK
ERK1/2
Dyn
Other
Kinases ?
Cytosolic
Substrates ?
NUCLEUS

Fig. 1-9: PAR-2 activation induces cofilin dephosphorylation and activation. The figure shows PAR-2 activates cofilin in a β-arrestins dependent fashion, and induces inhibition of LIMK (phosphorylates and inhibits cofilin) and recruitment of CIN (phosphatase that activates cofilin). Further, inhibition of components the hetrotrimeric G-protein signaling pathway revealed activation of the pathway inhibits activation of cofilin.


PAR-2
GP2A
Gαq
β-arrestins
LIMK
CIN
PLCβ
Gq siRNA
IP3
BAPTA-AM
cofilin
β-arrestins
Ca2+
?
LIMK
β-arr siRNA
genetic deletion βarr

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Chapter 2

Apical and basolateral pools of protease-activated receptor-2 direct distinct signaling events in the intestinal epithelium

2.1 ABSTRACT

Studies suggest there are two distinct pools of protease-activated receptor-2 (PAR-2) present in intestinal epithelial cells: an apical pool facing the lumen, and a basolateral pool facing the bloodstream. Further, studies have demonstrated that introduction of PAR-2 agonists such as 2-furoyl-LIGRL-O-NH2 (2fAP) to the lumen can activate apical PAR-2, while it has been predicted mast cells and recruited leukocytes release proteases that can activate basolateral PAR-2. However, whether both pools of PAR-2 are equally capable of signaling, and the possible distinction between apical and basolateral PAR-2 induced responses have yet been addressed. Here we identified the expression of both apical and basolateral PAR-2 in cultured CACO2-BBe monolayers, and in mouse proximal jejunum. We revealed both receptor pools can signal but these signals differed both temporally and mechanistically. Activation of basolateral PAR-2 activation increased short circuit current via induction of chloride secretion (ΔI_{sc}) decreased transepithelial resistance (TER) and induced cofilin activation, while activation of apical PAR-2 did not. In contrast, both pools of PAR-2 activated Extracellular Signal-Regulated Kinase 1/2 (ERK1/2), but the pattern of activation was different. Apical PAR-2 promoted a rapid, biphasic PKC-dependent activation while activation of basolateral PAR-2 promoted delayed ERK1/2 activation and required in put from both Gαq and β-arrestin-dependent pathways. These results suggested that the outcome of PAR-2 activation is dependent upon the specific receptor pool that is accessible to a given agonist, allowing for fine-tuning of the physiological response to different serine proteases.

2.2 INTRODUCTION

PAR-2 is a G-protein coupled receptor (GPCR) that is activated via cleavage of its N-terminus by trypsin-like proteases including trypsin, mast cell tryptase, kallikreins, and the tissue factor VIIa-Xa complex (1-3). Proteolytic cleavage of the N-terminal domain exposes a tethered ligand that consequently binds and activates the receptor itself. PAR-2 is reported to trigger a wide variety of cellular responses (e.g. proliferation, chemotaxis, ion transport, epithelial barrier function, and tumor cell metastasis) in a cell type-specific manner. (4-12). *In vitro*, there are synthetic activating peptides such as SLIGRL and 2-furoyl-LIGRL-O-NH2 (AP and 2fAP) that mimic the tethered ligand. Binding of AP and 2fAP can activate PAR-2 and trigger downstream signals that can lead to physiological processes in the absence of proteolytic cleavage. Studies showed activation of PAR-2 resulted in both pro and anti-inflammatory in numerous systems including gastrointestinal and cardiovascular. Some of these pro and anti-inflammatory effects identified included vasodilation, smooth muscle relaxation, up-regulation of cytokines, immune cell recruitment, increased nociception and hyperalgesia (13-16).

Here we examined and distinguished the role of apical and basolateral PAR-2 of intestinal epithelium. Epithelial cells act as the first line of defense for the gastrointestinal system that prohibits invasive pathogens from entering the bloodstream from the lumen (17, 18). Studies that used PAR-2 specific antibodies or conducted intracolonic administration of fluorescently-conjugated AP suggested that intestinal

epithelial cells (both in vivo and in various cell culture lines) expressed PAR-2 on both the apical and basolateral surfaces, although the accessibility of each receptor pool to agonists, the corresponding downstream signaling and physiological response to either basolateral or apical receptor activation have not yet been clarified (19). It is predicted that basolateral PAR-2 would be activated by mast cell tryptase or proteases released by recruited leukocytes, resulting in inflammatory responses such as gastrointestinal barrier dysfunction (20, 21). Further, apical receptor can be activated by lumenal proteases released by invading pathogens or secreted by the epithelial cells themselves (22). Moreover, studies showed inflammatory responses in the colon via lumenal introduction of 2fAP and serosal exposure to mast cell tryptase (21, 23). However, possible distinctions in signaling regulation and physiological responses between the two populations have not yet been studied. Thus, we examine the following: 1) whether apical and basolateral PAR-2 are accessible to labeled 2fAP in polarize colonic epithelial monolayers (CACO2-BBe), 2) whether there are temporal and mechanistic distinctions in apical and basolateral PAR-2 signaling in CACO2-BBe monolayers, 3) whether apical and basolateral PAR-2 are expressed in mice proximal jejunum, and 4) whether apical or basolateral PAR-2 activation in CACO2-BBe monolayers secrete Cl^- and induce increase in I_{sc}.

For the intestinal digestive process, extensive electrolytes and fluid secretion aid digestion of food particles and nutrient uptake. Further, relationship between hormonal processes (endocrine, neocrine, paracrine) and inflammatory stimuli provide favorable

lumenal fluidity, mixing and flushing in defense against pathogenic intruders (40). There are various methods of Cl^- secretion mechanisms which are distinguished by the length of the secretion, and also the form of stimuli that trigger the secretion. These methods include signals inducing cAMP and cGMP elevation which evoke sustained Cl^- secretion and cholergic Cl^- secretory response induced by agonists that elevate intracellular Ca^{2+} levels (41-43).

2.3 MATERIALS AND METHODS

All chemicals were from Sigma unless otherwise stated. Antibodies and final dilutions for Western blot (WB) and immunofluorescence (IF) were as follows: from BD Biosciences, rabbit anti-phospho (Thr 202/ Tyr 204)-ERK (1:1000 for WB); from Santa Cruz Biotechnology mouse anti-ERK-2 (1:1000 for WB); from Chemicon, rabbit anti-phospho (Ser3)-cofilin (1:1000 for WB) and mouse monoclonal anti-cofilin (1:1000 for WB); rabbit anti-PAR-2 9717 (1:300 for IF) were obtain as a gift from Dr. Gordon Ohning from the Center for Ulcer Research and Education (CURE) at UCLA. Activating peptides 2fAP (2-furoyl-LIGRL-ornithine-NH2) were biosynthesized by Genemed Inc. (South San Francisco, CA). Phalloidin Alexa 488 (1:20 IF) from Invitrogen; Rhodamine labeled 2fAP and reversed 2fAP (2-furyol-LRGIL-ornithine-NH2) were biosynthesized by Genemed. Pharmacological inhibitors were used as follows: Ca^{2+} chelator BAPTA-AM (Sigma) was prepared in dimethyl sulfoxide (DMSO) and used at 10 uM final concentration; PLC inhibitor U73122 (Tocris) was solubilized in chloroform and reconstituded to 100 mM in DMSO immediately before use at 10 uM final concentration; PKC inhibitor GF109203X (Tocris) was solubilized to 25 mM in DMSO and used at 10 uM final concentration.

Transfection and cell lines: Human intestinal cell line CACO2-BBe were gifts from Dr. David Lo (UC Riverside Biomedical Sciences) and grown in Advanced Dulbecco's modified Eagle's medium, 15 mM Hepes, 10% fetal calf serum. Transient transfections

were performed on 95% confluent cells using FuGENE6 (Roche) and experiments were performed between 48 and 72 hours after transfection.

PAR-2 labeling with rhodamine 2fAP: CACO2-BBe seeded at $2x10^4$ cells per well on 24 well transwell filters with .4 um pores (Corning) grown to confluent monolayer were treated with 1 um of rhodamine labeled 2fAP or RAP when monolayer resistance exceeded 750 ohms/cm^2 at 4^oC for 1 hour or at 4^oC for 1 hour then 37^oC for 1 hour. Cells are washed 3X with sterile phosphate buffered saline solution (PBS) and fixed in normal buffered formalin. Cells are then labeled with phalloidin alexa 488 and imaged with microscopy techniques described below.

Protein analysis and western blotting: CACO2-BBe seeded at $5x10^4$ cells per well to 6 well transwell filters with .4 um pores (Corning) grown to confluency and experiments were conducted after 10–14 days when monolayer resistance exceeded 750 ohms/cm^2, serum starved for 24 hours, treated with 1 uM 2fAP on the apical or basolateral surface for 0-60 minutes at 37^oC lysed in Laemmli Sample Buffer (LSB) (62.5 mM Tris-HCL (pH6.8), 2% (wt/vol) sodium dodecyl sulfate, 50 mM dithiothreitol, 0.01% (wt/vol) bromophenol blue. If inhibitors are used, after 24 hours of serum starvation, 10 uM of inhibitors are added to both apical and basolateral surfaces for 10 minutes, followed by 2fAP treatment. Lysate protein was analyzed by SDS-PAGE (15% for cofilin, 10% for ERK1/2) followed by WB. Blots were imaged using the LICOR Odyssey imaging system, and LICOR software was used to calculate integrated intensities of bands.

Images of WB were assembled using Adobe Photoshop 5.0 and Adobe Illustrator CS4. Some gels were spliced to eliminate blank lanes or lanes containing samples unrelated to the figure.

Electrical measurements: CACO2-BBe seeded at $1x10^4$ cells per well to 24 well snapwell filters with .4 um pores (Corning) gornw to confluency and experiments were conducted after 10-14 days when monolayer resistance exceeded 750 ohms/cm^2. Short-circuit current Isc and electrical resistance (RT) across the cell monolayers was measured by conventional Ussing chamber technique (24). CACO2-BBe cell monolayer on snapwell filters were mounted in Ussing chambers and incubated in Tyrode's solution (140 mM NaCl, 4 mM KCl, 1 mM MgCl2, 1.25 mM CaCl2, 12 mM glucose, and 10 mM Na-HEPES (pH 7.4) and mixed continuously by gas (100% O_2) lift). 1 uM of 2fAP was added to apical or basolateral surfaces of CACO2-BBe and changes in current across monolayers were observed accordingly.

Microscopy: Cells prepared as described previously (26). Serial sections (100X and 63X objectives) were taken on Zeiss LSM-510. Three dimensional reconstructions of the images were done using Imaris 6.3 software.

Immunofluorescence labeling of proximal jejunum: Segments of mouse proximal jejunum were fixed in ice-cold formalin for 5 h, infiltrated with cryoprotectant (30% sucrose in PBS) overnight, and frozen in OTC medium (Triangle Biomedical Sciences) at

-35^{o}C. Sections of 10 µm thickness were cut on a cryostat microtome (Microm) and mounted on polylysine-coated glass slides (Fisher Superfrost Plus). Rabbit antibody against PAR-2 was employed. AB9717, obtained from the Center for Ulcer Research and Education (UCLA), was used on undenatured sections. When co-labeling with mouse monoclonal antibody against zonula occludens-1 (ZO-1), endogenous mouse IgG was blocked by preincubation in goat anti-mouse IgG for 1 hour. Sections were incubated sequentially with blocking solution (1 hour), primary antibody (overnight at 4^{o}C), and secondary antibodies conjugated to Alexa Fluor-488 and/or -546 (Molecular Probes). Confocal images were acquired with a Zeiss LSM-510 microscope and assembled using Adobe Photoshop.

Data and statistical analysis: All graphs and statistical analysis were performed using Prism or Microsoft Excel 2003. All experiments were performed a minimum of three times. Phospho-protein levels were normalized to total protein levels before calculating fold change with respect to untreated controls. One way ANOVA and paired t tests were used to determine statistical significance of and significant differences between values under different conditions.

2.4 RESULTS

Apical and basolateral PAR-2 are expressed in polarized CACO2-BBe monolayers and are accessible to rhodamine labeled 2fAP. Previous studies showed administration of 2fAP to colonic lumen triggered inflammatory responses (16, 20, 21, 24, 27), suggesting apical PAR-2 is accessible to peptide agonist. Furthermore, labeled agonist derivatives applied to the lumen labeled cells in wild type but not PAR-$2^{-/-}$ mice (19). Serine proteases released by leukocytes and mast cells can also activate PAR-2 in the gastrointestinal system evoking inflammation (20, 21, 27) and administration of PAR-2 agonists to the basolateral side of epithelial monolayers activates PAR-2 (44), suggesting basolateral PAR-2 is also accessible to circulating agonists. We confirmed these findings in polarized colonic epithelial cell monolayers (CACO2-BBe) grown on permeabilized supports, which mimic the intestinal epithelium. Proper monolayer formation was confirmed by measuring transepithelial resistance (TER); only monolayers with TER above 1200Ohms/cm2 were used in subsequent experiments. Rhodamine labeled PAR-2 agonist peptide (2-furoyl-LIGRL (Rh-2fAP), was administered to either the apical or basolateral surface to label PAR-2. Labeling was performed for 1 hour at 0^{o}C (to prevent receptor trafficking) after which cells were fixed and stained with antibody to ZONA-1 to mark tight junctions. Confocal microscopy revealed that Rh-2fAP bound to PAR-2 at the apical surface of the epithelial monolayers (Fig. 2-1A, B), whereas reverse 2fAP (Rh-RAP) did not, indicating that the binding is not due to non-specific binding of labeled peptides (Fig. 2-1C, D). 3-dimensional reconstruction of Z-sections revealed that apical

PAR-2 was localized in the same plane as the tight junctions, consistent with their presence on the apical surface (Fig. 2-1I). Since PAR-2 agonist peptides promote removal of receptor from the surface by clathrin mediated receptor endocytosis (28), we next investigated whether the labeled peptide was internalized along with PAR-2. After treatment with Rh-2fAP on ice, monolayers were incubated at 37^{o}C and Rh-2fAP (Fig. 2-1E, F), but not Rh-RAP (Fig. 2-1G, H) was observed in vesicles below the region demarcated by the Zona-1 (Fig 2-1J). Similarly, when Rh-2fAP was added to the basolateral surface of CACO-2 monolayers, PAR-2 was labeled along the basolateral membrane, a pool of receptor not labeled by apically applied agonist (Fig 2-2A, B). When cells were warmed to 37^{o}C, we observed internalization of bound Rh-2fAP peptides as well as redistribution of ZO-1 from the tight junctions (Fig. 2-2E, F). Reconstruction of the microscopy images confirmed localization of basolateral PAR-2 with Rh-2fAP at 0^{o}C and PAR-2 induced internalization of both PAR-2 and ZO-1 at 37^{o}C (Fig. 2-2I, J). We conclude that both apical and basolateral pools of PAR-2 exist and both are expressed on the cell surface as they are both accessible to agonist. Furthermore, while basolateral PAR-2 activation promotes redistribution of tight junction proteins, apical administration does not. These cell culture studies suggest that PAR-2 must be present at both the apical and basolateral surfaces in vivo. To identify lumenal and basolateral PAR-2 *in vivo* we isolated mouse proximal jejunum and labeled with PAR-2 antibody followed by confocal microscopy. Cells were co-stained with ZO-1 to mark tight junctions and with phalloidin to demarcate the brush border. Confocal microscopy revealed expression of both apical and basolasteral pools of PAR-2 in mice ileum,

consistent with what was observed in cultured epithelial monolayers (Fig 2-3A). Interestingly, the apical pool of PAR-2 appeared to be on the microvillus membrane, consistent with a role in intestinal absorptive functions. Specificity was confirmed by comparing labeling of wild type (WT) and PAR-2 knockout (KO) mice; labeling of PAR-2 in jejunum epithelium was absent in knockout mice, although some serosal labeling was observed in both. (Fig. 2-3B, C).

Basolateral PAR-2 activation promotes decreased transepithelial resistance and increases short circuit current. PAR-2 has been reported to compromise barrier function, reflected by a decrease in TER, redistribution of tight junction proteins and macromolecule translocation across the epithelial monolayer (20-21). Furthermore, we and others have observed redistribution of tight junction proteins upon PAR-2 activation (24). These studies involve both apical and basolateral administration of agonist. To investigate whether there was a difference in the effect of PAR-2 on barrier function in response to apically or basolaterally administered agonist, we measured TER, in CACO2-BBe cells grown on permeabilized supports. TER in resting monolayers was 1428.26 ± 163.98 ohms/cm^2 and activation of basolateral 2fAP caused a transient decrease in TER, to 899.69 ± 149.18 ohms/cm^2 with basolateral addition. Moreover, apically applied PAR-2 promoted only a modest decrease in TER (1040.10 ± 160.67 ohms/cm^2) (Fig. 2-4). These data are consistent with reports that PAR-2 is capable of increasing epithelial permeability (11, 24) and suggest that this effect could be mediated by agonists present in the lumen of the intestine acting on apical PAR-2 or agonists present in the blood

accessing basolateral PAR-2, although basolaterally accessible receptors are more potent mediators of this effect.

Recent study showed PAR-2 activation induced intestinal epithelial Cl^- secretion through the activation of PLC and selective isoforms of PKC (37). Thus, we examined ΔI_{sc} upon activation of apical or basolateral PAR-2 in CACO2-BBe monolayers. The activation of basolateral PAR-2 evoked a transient Cl^- secretory response while activation of apical PAR-2 did not (Fig. 2-5). The basolateral PAR-2 induced transient Cl^- secretory response generally lasted for approximately 60 seconds with maximal ΔI_{sc} observed at 14.51±2.50 $\mu A/cm^2$. We conclude that only activation of basolateral PAR-2 pools can mediate the ion secretory response, while changes in tight junction permeability can be mediated by both apical and basolateral receptor pools.

Apical PAR-2 induces a biphasic and early maximal activation of ERK 1/2 while basolateral PAR-2 induces a gradual and late maximal activation of ERK 1/2. To further investigate the possibility that the two pools of PAR-2 mediate different responses, we examined downstream signaling events. It is well established that PAR-2 activates ERK 1/2 via multiple mechanisms; best characterized are the Gαq dependent and β-arrestin dependent pathways (22, 28-33). After introduction of 2fAP to the apical or basolateral side of CACO2-BBe monolayers grown on permeabilized supports (Fig. 2-6A and B respectively), we observed a temporal difference in the activation of ERK1/2. Apical administration promoted a robust increase in ERK1/2 phosphorylation at 5

minutes after 2fAP treatment compared with a modest increase with basolateral addition (4.86±.70 and 1.75±.23 fold change over baseline respectively). At 30 minutes after 2fAP treatment, both apical and basolateral PAR-2 induced a moderate increase in ERK 1/2 phosphorylation (2.58±.39 and 2.58±.36 fold baseline respectively). At 60 minutes after 2fAP treatment, apical PAR-2 induced a moderate activation of ERK 1/2 while basolateral PAR-2 induced a robust and maximal phosphorylation of ERK 1/2 (3.49±.36 and 6.20±.96 fold change over baseline respectively). Thus, apical PAR-2 induced a biphasic activation of ERK 1/2 with maximal phosphorylation at 5 minutes, while basolateral PAR-2 induced a gradual, delayed ERK 1/2 activation, with maximal phosphorylation at 60 minutes. We conclude that both receptor pools are capable of signaling to the ERK1/2 pathway.

Apical and basolateral PAR-2 activation of ERK ½ involves multiple distinct mechanisms. It has previously been demonstrated that many GPCRs, including PAR-2, can activate ERK1/2 via multiple pathways. Downstream of PAR-2, the classical Gαq signaling pathway results in activation of PLCβ, generation of IP3 and DAG, mobilization of intracellular Ca2+ and activation of classical PKC isoforms. This pathway can lead to ras-dependent nuclear ERK1/2 activation and proliferation. PAR-2 can also promote ERK1/2 activation via G-protein independent pathways involving β-arrestins (28-33, 35). Thus, we investigated whether the the different pools of PAR-2 utilized distinct mechanisms of ERK1/2 activation. To examine the contribution of the Gαq pathway, we used three different inhibitors: PLCβ inhibitor U73122, calcium

chelator BAPTA-AM, and the broad spectrum PKC inhibitor GF109203X. To examine the contribution of β-arrestins, cells were transfected with a dominant negative mutant of β-arrestin consisting of the clathrin binding domain. Both apical and basolateral PAR-2-induced ERK1/2 activation were inhibited BAPTA-AM and U73122 (Fig. 2-7A-D). However, while inhibition of PKC drastically inhibited ERK 1/2 phosphorylation induced by apical PAR-2 (Fig. 2-7E); basolaterally induced ERK 1/2 activation was relatively unaffected by PKC inhibition (Fig. 2-7F). Thus, while both basolateral and apical PAR-2 can activate ERK1/2 through Gαq/Ca-mediated pathways, only the apical PAR-2 pathway requires PKC. Transfection of dominant negative β-arrestin (DNarr) inhibited ERK1/2 activation by basolaterally, but not only slightly to apically administered 2fAP, suggesting that only the basolateral PAR-2 pool required input from this pathway (Fig. 7G-I). We conclude that PAR-2 uses multiple mechanisms for ERK1/2 activation in intestinal epithelial cells, and that the apical PAR-2 pool requires PKC, while the basolateral PAR-2 requires β-arrestins.

Basolateral PAR-2 activation results in activation of cofilin, while apical PAR-2 activation does not. One of the major pathways activated by β-arrestins downstream of PAR_2 is the dephosphorylation and activation of the actin filament severing protein, cofilin (1). Cofilin promotes rapid actin reorganization by severing existing actin filaments and creating free barbed ends to which actin monomers can spontaneously add. Since only basolateral PAR-2 required β-arrestins for ERK1/2 activation, we examined whether it also resulted in cofilin dephosphorylation. Consistent with the findings above,

basolaterally administered 2fAP induced cofilin dephosphorylation after 5 and 60 minutes (Fig. 2-8B). In contrast, apical 2fAP did not trigger cofilin dephosphorylation after 5 and 30 minutes (Fig. 2-8A). A major effect of cofilin actin filament severing activity is the creation of actin free barbed ends, which promotes remodeling of the actin cytoskeleton. Barbed end formation can be monitored in cells by observing incorporation of labeled actin monomers into lightly permeabilized cells after agonist treatment. Actin monomers will not incorporate into stable filaments; thus they can be used to monitor sites of filament severing. Consistent with cofilin activation, we observe that actin monomers incorporate into cells upon basolateral, but not apical PAR-2 activation in both cultured CACO-2 monolayers (Fig. 2-8C) and intestinal mucosa (Fig. 2-8D). We conclude that basolateral PAR-2 is capable of triggering β-arrestin-dependent and G-protein dependent signals, while apical PAR-2 does not trigger β-arrestin-dependent signaling.

2.5 Discussion

Previous studies have indicated that both lumenal introduction of 2fAP and exposure to mast cell tryptase in the gastrointestinal system can trigger inflammatory responses (e.g. tight junction reorganization, cytokine up-regulation), suggesting the functional expression of both apical and basolateral PAR-2 in colonic epithelial cells. However, while changes in ion secretion appear to be primarily mediated by basolaterally activated PAR-2, many of the pro-inflammatory events are activated by either apical or basolateral agonists. The studies described here demonstrate the existence of spatially and temporally distinct signaling pathways in colonic epithelial cells by apical and basolateral pools of PAR-2. Both pools of receptor are accessible to agonist activation and promote activation of ERK1/2. However, the mechanisms of ERK1/2 activation differ between apical and basolateral receptors, with only basolateral PAR-2 utilizing a β-arrestin-dependent signaling component. Furthermore, only basolateral agonist promoted activation of the actin filament severing protein, cofilin, increased Cl- secretion, and significantly lowered transepithelial resistance. The use of different signaling mechanisms by apically and basolaterally exposed PAR-2 may allow for a distinct set of cellular events in response to serine proteases present in the lumen or those released from immune cells present in the serosa at the basolateral surface.

Intestinal epithelial cells are the first line of defense against the lumenal pathogens (17, 18). Studies have suggested PAR-2 can lower intestinal epithelial integrity by promoting

tight junction reorganization and barrier dysfunction (20, 21). In the studies described here, introduction of the PAR-2 agonist, 2fAP to the apical surface of the epithelial monolayer induced a slight decrease in TER and did not significantly promote internalization of the tight junction protein ZO-1 or actin reorganization at tight junctions. In contrast, 2fAP added at the basolateral surface led to a drastic reduction in TER, redistribution of ZO-1 from the tight junctions, and activation of actin filament severing at tight junctions. These data suggest activation of basolateral PAR-2 by proteases such as mast cell tryptase might play a greater role in tight junction reorganization and barrier dysfunction. However, as both apical and basolateral pools of PAR-2 are able to promote activation of ERK1/2, both receptor pools are clearly capable of intracellular signaling. Apical PAR-2 activation may serve a protective function or play a role in endocytosis of luminal pathogens (C. Lau and K. DeFea, unpublished observations).

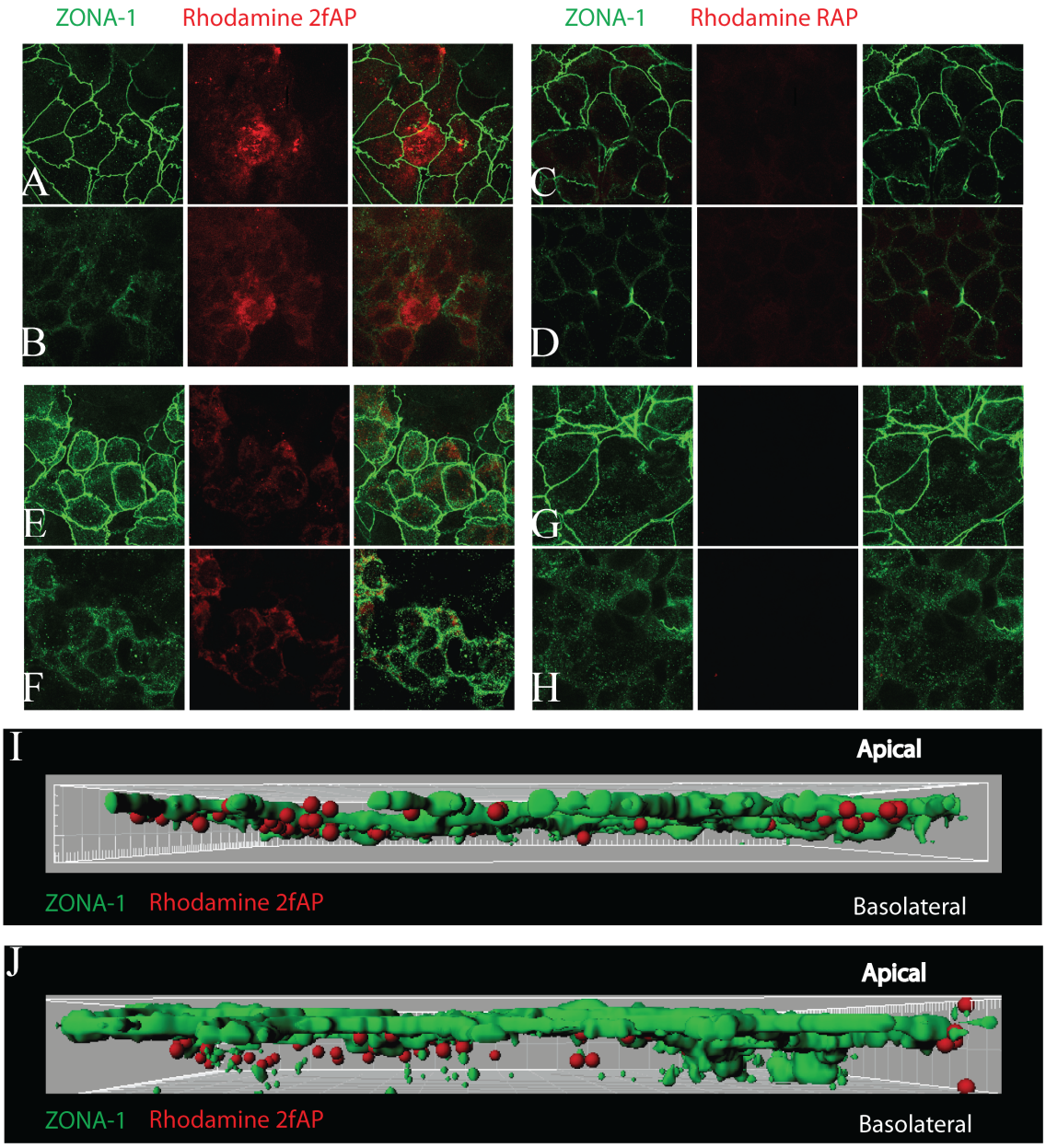
An interesting outcome of these studies is the observation that while both receptor pools can activate ERK1/2, they do so in a temporally distinct manner and by different mechanisms. Here we observed that apical PAR-2 activation resulted in biphasic ERK 1/2 phosphorylation peaking at 5 and 60 minutes, similar to what was observed in fibroblasts and breast cancer cells (31, 32, 35). In contrast, basolateral PAR-2 activation induced delayed response, with ERK 1/2 phosphorylation peaking at 60 minutes. The data indicated apical PAR-2 activation induced an early, while basolateral PAR-2 activation induced a late response in intestinal epithelial cells. This may reflect entirely separate roles of the two receptor pools or it may allow for coordination of response to

luminal pathogens. For example, apical PAR-2 might act as that "warning sensor" for the gastrointestinal system, giving an initial transient activation of a proliferative signal (23). Basolateral PAR-2 on the other hand may exhibit a prolonged response that is important for responding to insults resulting in recruitment of mast cells or the presence of tissue factors at the site of infection. We have previously reported that PAR-2 can activate ERK 1/2 through both Gαq dependent and β-arrestin dependent pathways and each pathway is associated with different physiological outcomes, e.g. proliferation versus actin reorganization (22, 28-33). In our previous studies PAR-2 activation led to cell proliferation via Gαq dependent ERK 1/2 activation and nuclear localization; whereas prolonged activation of ERK1/2 through β-arrestins was associated with cytoskeletal reorganization and cell migration. Here we observed that basolateral PAR-2 activated ERK1/2 by a β-arrestin-dependent pathway that was independent of PKC, while apical PAR-2 utilized a PKC-dependent, β-arrestin-independent pathway. Interestingly, unlike β-arrestin-dependent cofilin activation, the β-arrestin-dependent ERK1/2 pathway in polarized cells also required input from the traditional Gαq pathway, as blocking intracellular Ca^{2+} mobilization or inhibiting PLC-β prevented ERK1/2 activation by both apical and basolateral PAR-2. Since PAR-2 can activate numerous isoforms of PLC (α, β and γ), our finding suggested that basolateral PAR-2 activated isoforms that stimulated ERK 1/2 phosphorylation that is PKC independent (36, 37). Further, since a recent study suggested activated PAR-2 can interact with toll like receptors (TLRs) to activate corresponding pathways and instigate immune responses. Hence, another potential explanation for our results is that basolateral PAR-2 cross-activated interacting receptors

leading to activation of an isoform of PLC that triggered calcium influx and activate ERK 1/2 that is independent of PKC (38).

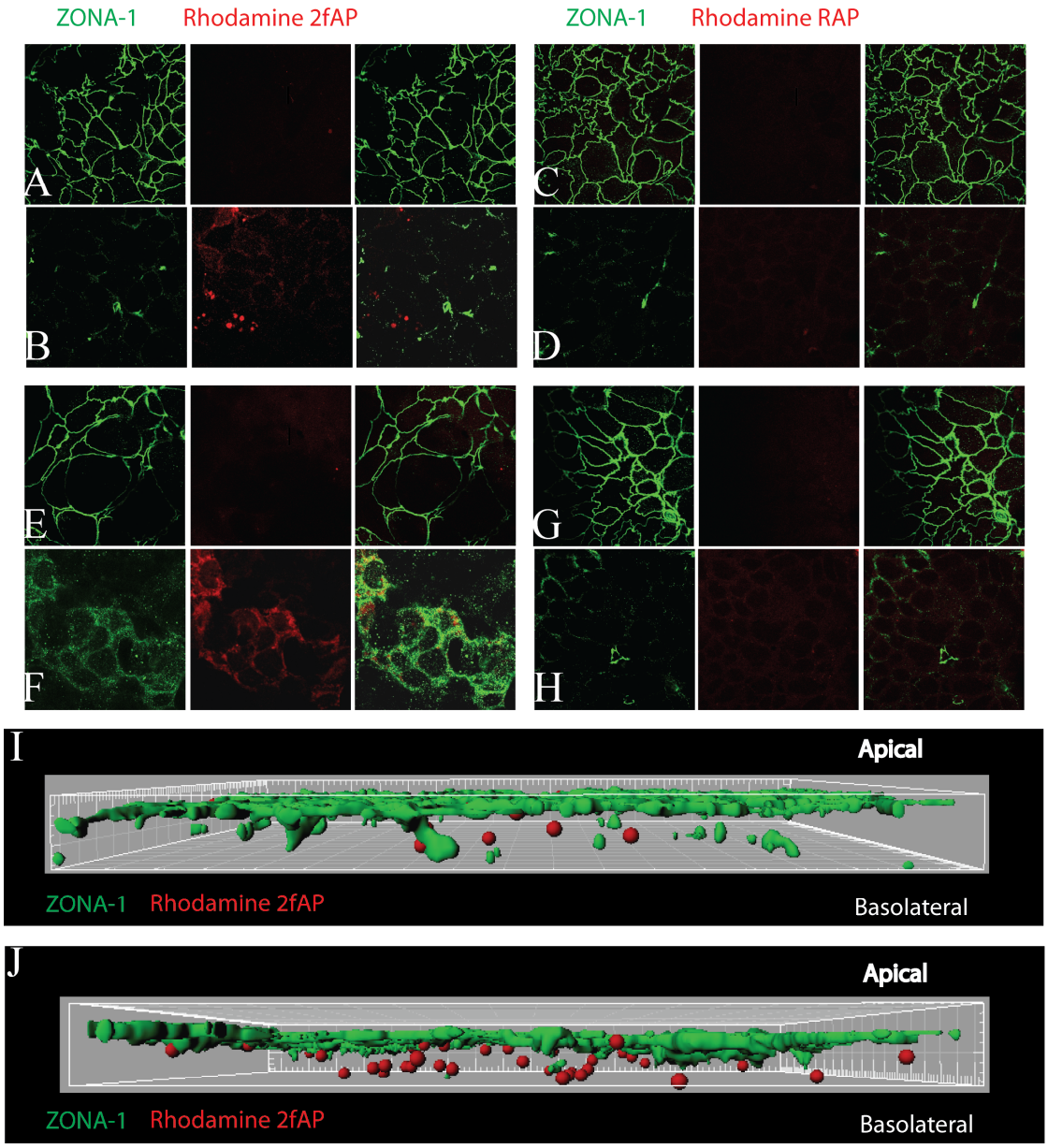
2.6 Figures and Legends

Figure 2-1: Rhodamine labeled 2fAP is able to bind to PAR-2 and induce receptor internalization when introduced to the apical surface of polarized CACO2-BBe monolayers. Rhodamine labeled 2fAP (Rh-2fAP) peptides were introduced to the apical surface of polarized CACO2-BBe monolayers. Rhodamine labeled reverse 2fAP (Rh-RAP) peptides were used to ensure the specificity of the Rh-2fAP peptides for PAR-2 and antibody for tight junction protein ZONA-1 was used to ensure proper tight junction formation and cell polarity. We observed that Rh-2fAP peptides were present and bound to PAR-2 at the apical surface of the epithelial monolayers (A, B). We did not find binding of Rh-RAP peptides indicating binding of Rh-2fAP peptides at the apical surface are specific to PAR-2 (C, D). Incubated monolayers at 37^{o}C after one hour treatment with Rh-2fAP peptides induced receptor internalization from the apical surface (E, F). Rh-RAP peptides did not get internalized, which indicates the internalization of Rh-2fAP peptides are PAR-2 induced and specific (G, H). Reconstruction of the microscopy images via Imaris® software further shows accessibility of apical PAR-2 by Rh-2fAP at 4^{o}C on the apical membrane surface and PAR-2 induced internalization at 37^{o}C (I, J).



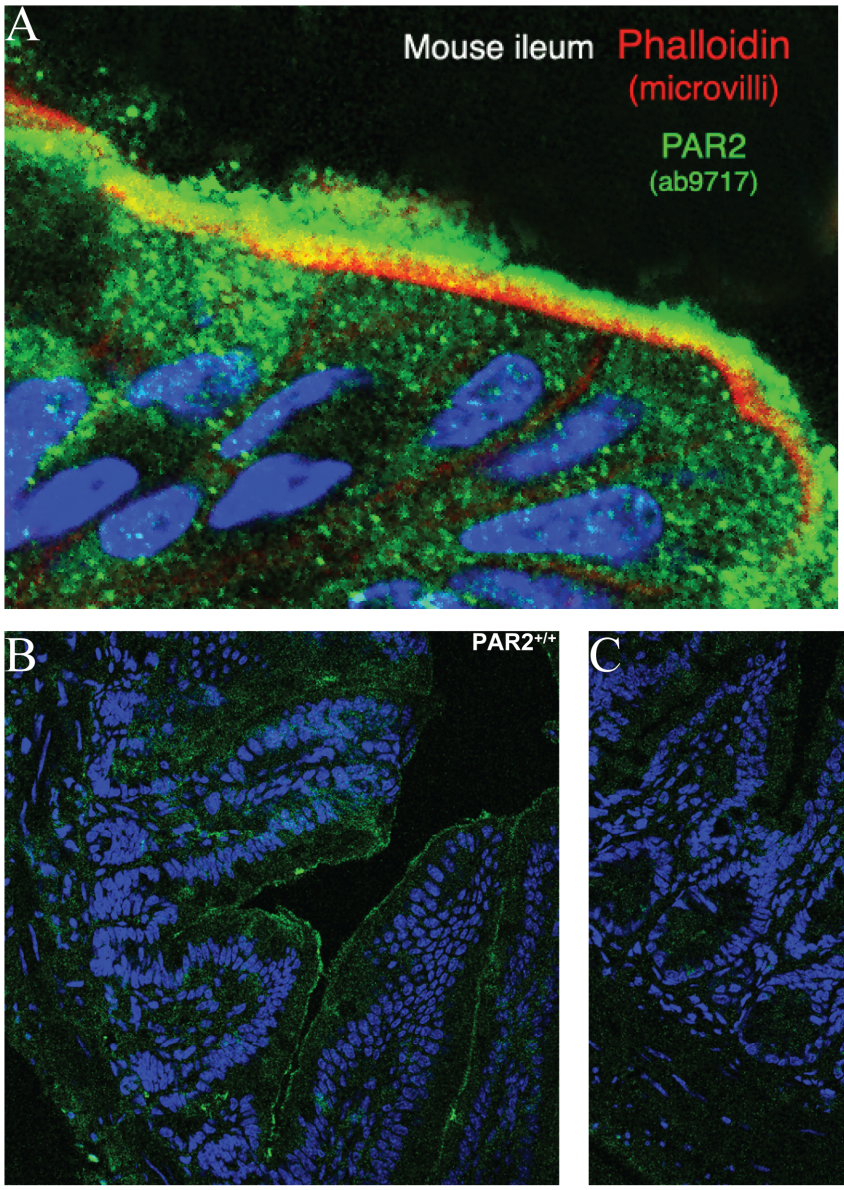
Lau et al Figure 1

Figure 2-2: Rhodamine-2fAP is able to bind to PAR-2 and induce receptor internalization when introduced to the basolateral surface of polarized CACO2-BBe cells. Basolateral surfaces of CACO2-BBe monolayers were treated with Rh-2fAP peptides. Binding of Rh-2fAP peptides 4^{o}C to the basolateral surfaces of the monolayers were seen (A, B). We did not observe binding of Rh-RAP peptides, thus indicating Rh-2fAP peptides binding are specific for PAR-2 (C, D). Further, internalization of bound Rh-2fAP peptides from the basolateral surfaces were observed at 37^{o}C (E, F). Rh-RAP peptides did not get internalized signifying that the internalization of Rh-2fAP peptides at the basolateral surfaces were PAR-2 induced and specific (G, H). Reconstruction of the microscopy images via Imaris® further confirmed and illustrated the accessibility of basolateral PAR-2 by Rh-2fAP at 4^{o}C and PAR-2 induced internalization at 37^{o}C (I, J).



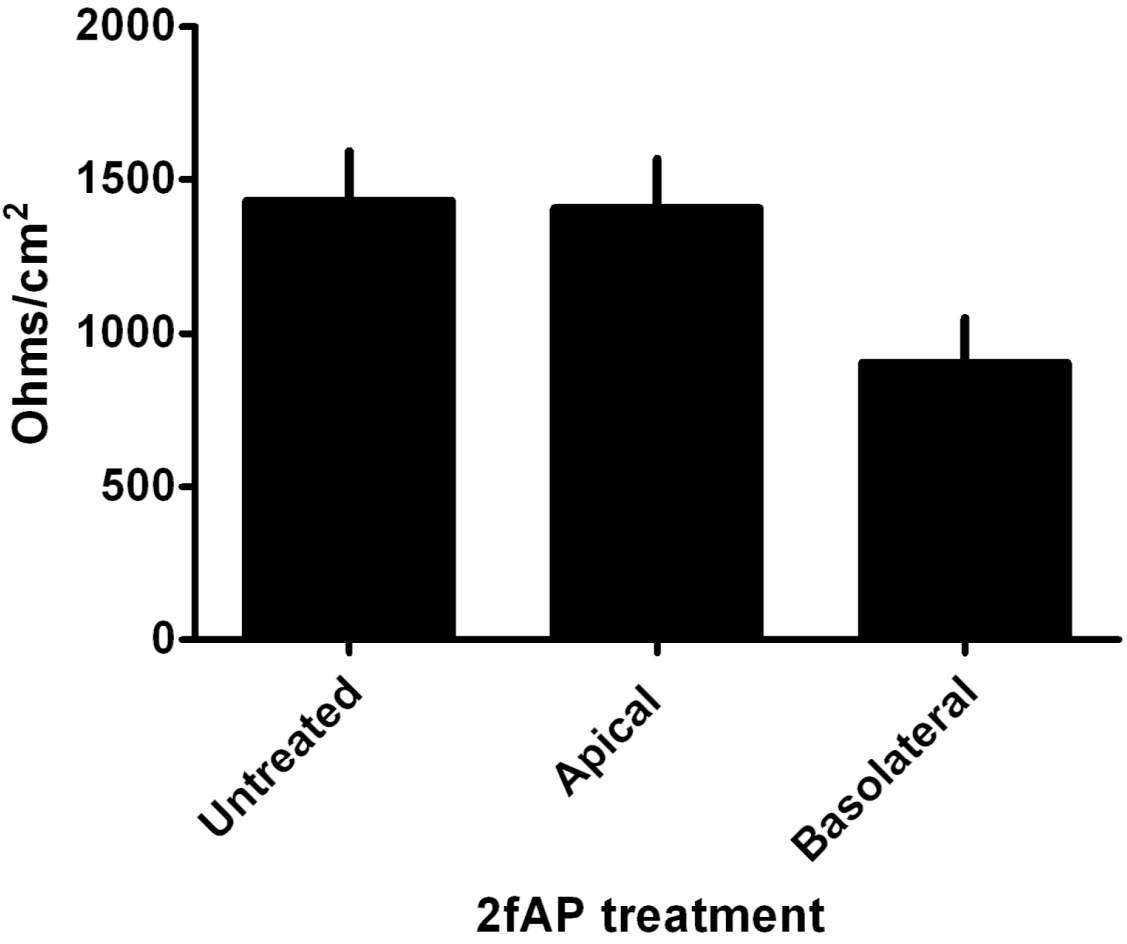
Lau et al Figure 2

Figure 2-3: Isolated mice proximal jejunum labeled with PAR-2 antibody 9717 showed both lumenal and serosal expression of PAR-2. Isolated mouse proximal jejunum and labeled with PAR-2 antibody 9717. Mice proximal jejunum sections were also labeled with TOPRO3 (blue) for detection of cell nuclei and phalloidin for the detection of microvilli to create a distinct border between the lumen and the serosa. Mice proximal jejunum were stained with 9717 for PAR-2 (green), expression of both serosal and lumenal PAR-2 were detected (A). To address the specificity of the antibodies for PAR-2, we labeled wild type and PAR-2 knockout mice proximal jejunum. 9717 (in green) labeled PAR-2 in the wild type mice proximal jejunum and not in the PAR-2 knockout, confirming the specificity of the antibody for PAR-2 (B, C).



Lau et al Figure 3

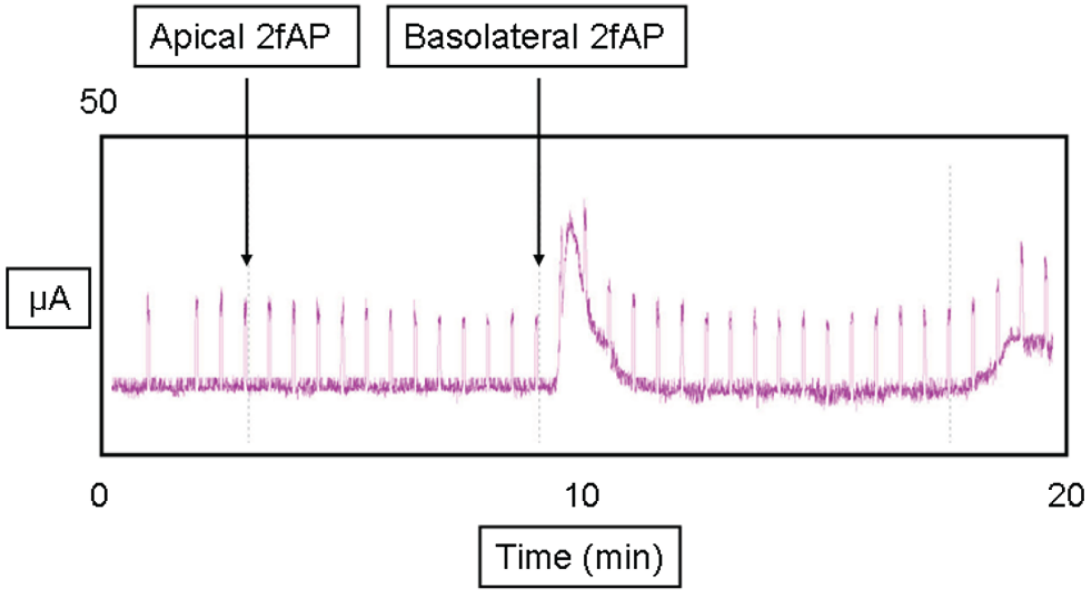
Figure 2-4: Transepithelial resistance (TER) measurements using Ussing Chamber showed CACO2-BBe formed "tight" monolayers when grown on transwell filters. CACO2-BBe monolayers were grown on transwell filters with .4 um pores to mimick intestinal epithelial barrier. To detect proper monolayer formation, we examine the TER to determine the "leakiness" of the monolayer. We measured the TER of the monolayer before treatments with 2fAP peptides, then after treatments with 2fAP peptides to the apical surface and after treatments with 2fAP peptides to the basolateral surface. TER before treatments with 2fAP peptides were detected at 1428.26 ± 163.98 ohms/cm^2, while TER after treatments with 2fAP peptides to the apical surface yielded a slight decrease at 1040.10 ± 160.67 ohms/cm^2. Treatments with 2fAP peptides to the basolateral surface triggered a drastic decrease of TER at 899.69 ± 149.18 ohms/cm^2.



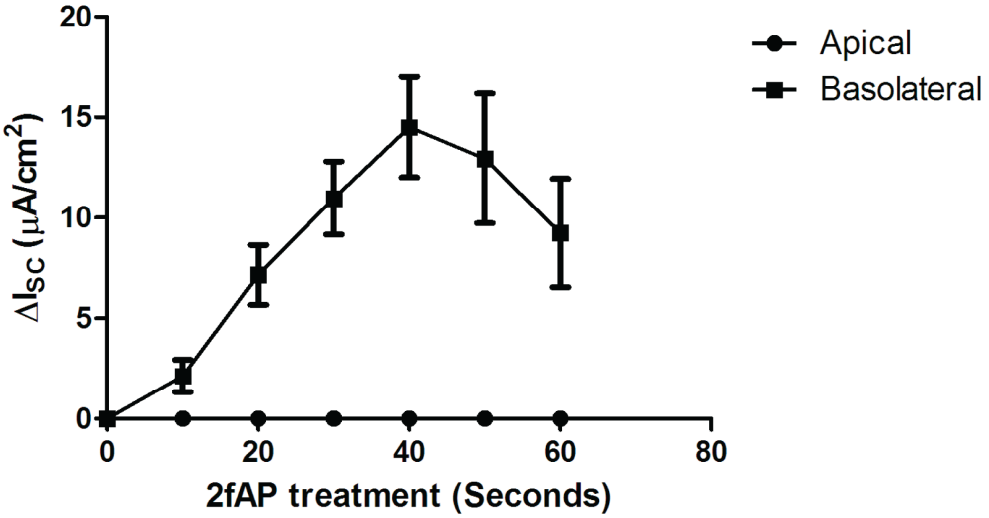
Lau et al Figure 4

Figure 2-5: Activation of basolateral PAR-2 induces an increase in short circuit current (ΔI_{sc}) while activation of apical PAR-2 does not. Activation of apical PAR-2 did not evoke ΔI_{sc} in CACO2-BBe monolayer (A). Activation of basolateral PAR-2 evoked a transient increase in short circuit current with a response of ~ 60 seconds and maximal ΔI_{sc} of 14.51±2.50 $\mu A/cm^2$ (B).

A.

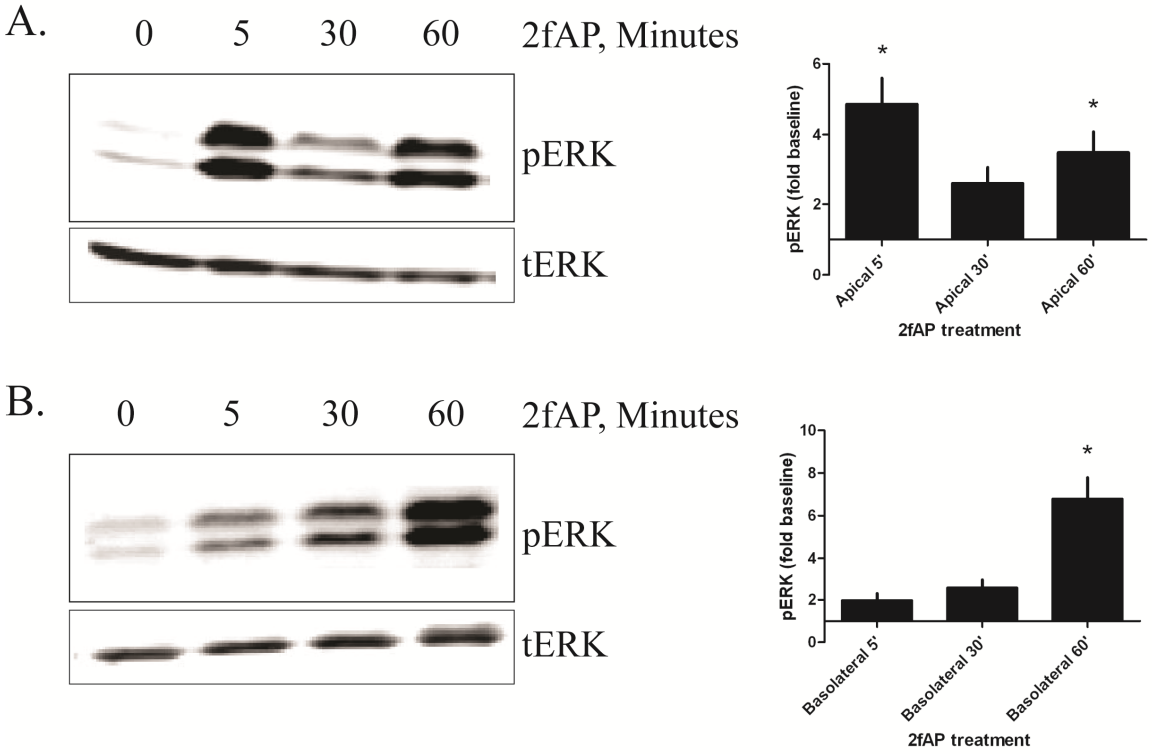


B.



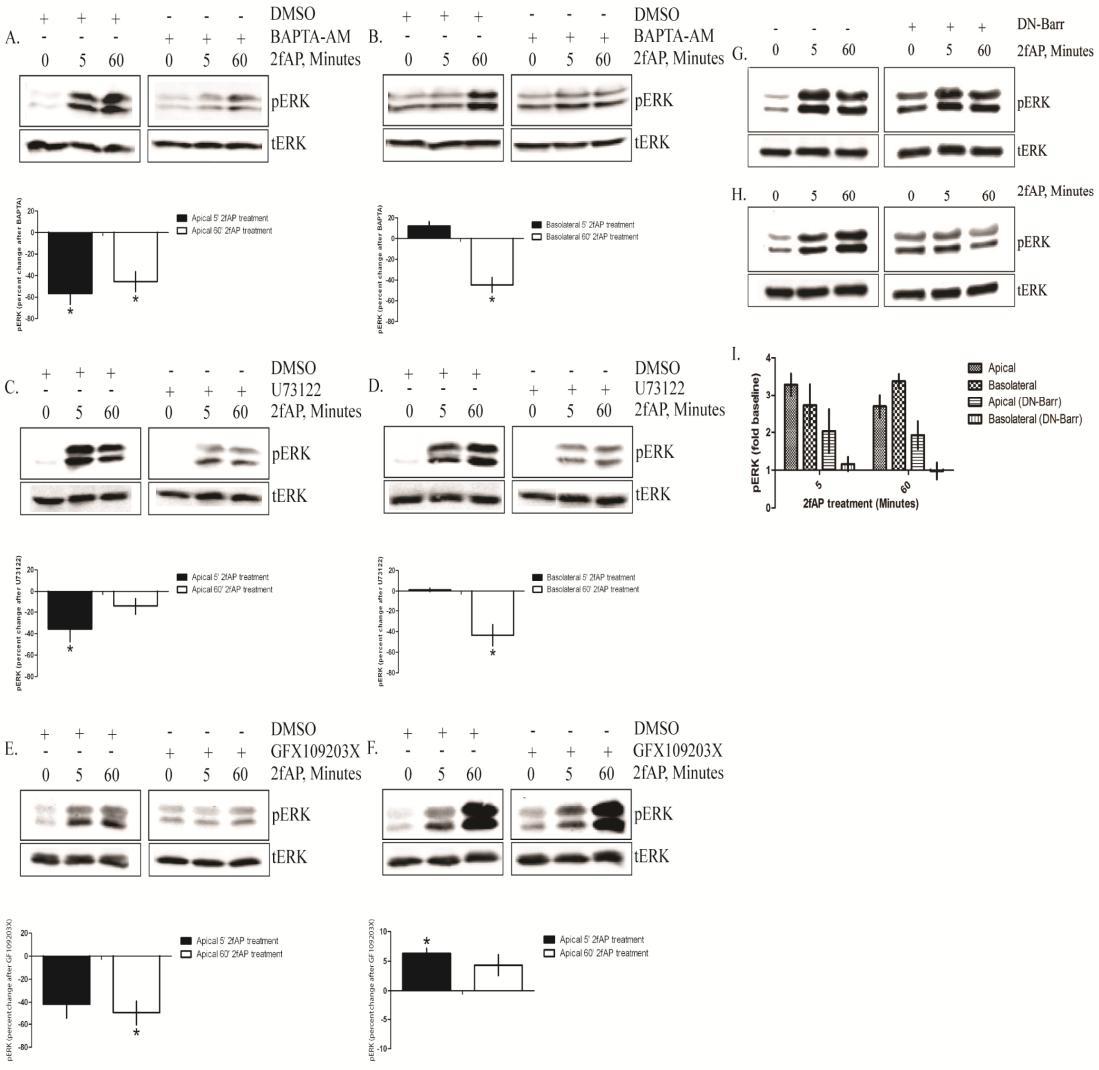
Lau et al Figure 5

Figure 2-6: Apical PAR-2 induces a biphasic and early maximal activation of Extracellular Signal-Regulated Kinases 1 and 2 (ERK 1/2) while basolateral PAR-2 induces a gradual and late maximal activation of ERK 1/2. Polarized CACO2-BBe monolayers were treated with with 2fAP at either the apical (A) or basolateral surface (B) for 5, 30 and 60 minutes. We observed a robust increase of ERK1/2 activation at 5 minutes after 2fAP treatment at the apical surface and observed a mild increase with treatment to the basolateral surface (4.86±.70 and 1.75±.23 fold change over baseline respectively). At 30 minutes after 2fAP treatment, both apical and basolateral PAR-2 induced a moderate increase in ERK 1/2 phosphorylation (2.58±.39 and 2.58±.36 fold baseline respectively). At 60 minutes after 2fAP treatment, apical PAR-2 induced a moderate activation of ERK 1/2 while basolateral PAR-2 induced a robust and maximal phosphorylation of ERK 1/2 (3.49±.36 and 6.20±.96 fold change over baseline respectively). A graph summarizes temporal distinctions of apical or basolateral PAR-2 induced ERK 1/2 activation (C). Significant values revealed by two tailed ANOVA are indicated by (*) symbol where $p<.05$.



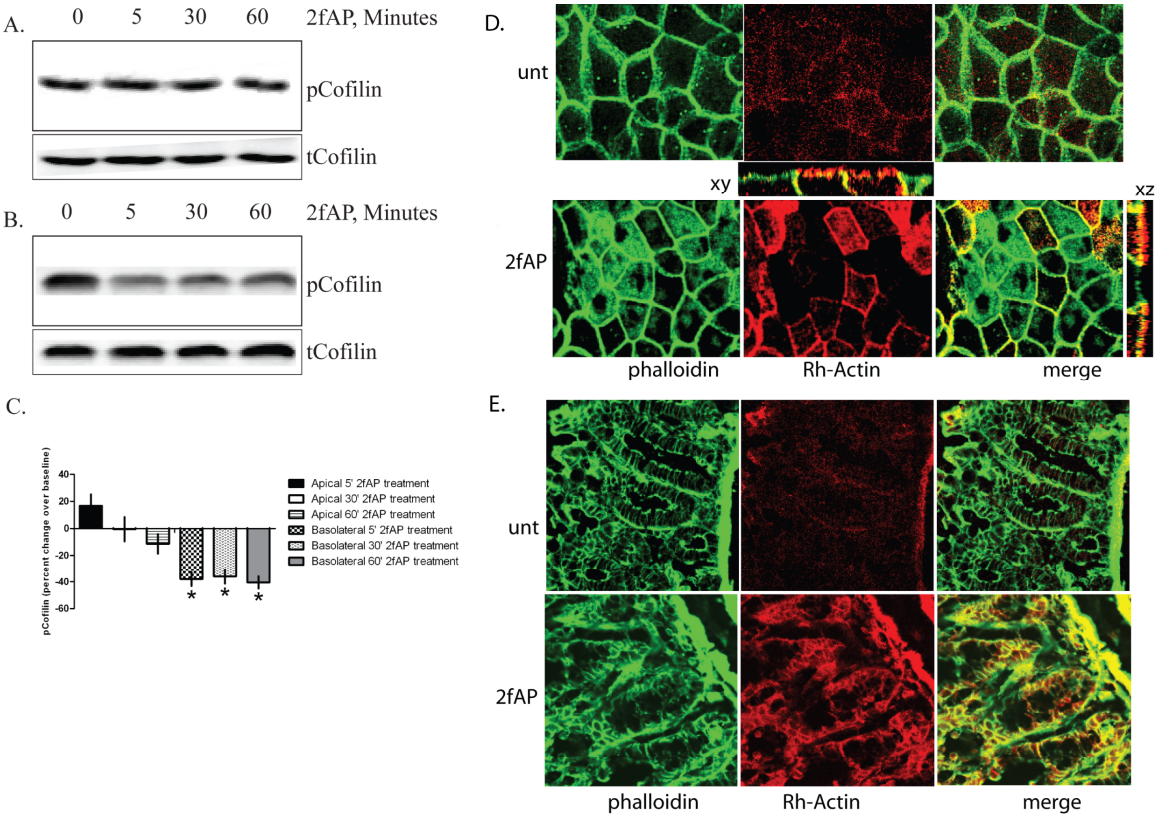
Lau et al Figure 6

Figure 2-7: Apical and basolateral PAR-2 activation of ERK 1/2 involves multiple distinct mechanisms. Polarized CACO2-BBe monolayers on filters were pretreated with appropriate inhibitors then treated with 2fAP. We observed that ERK 1/2 activation induced by apical PAR-2 decreased substantially at 5 and 60 minutes after inhibition of calcium mobilization using BAPTA-AM (57±10% decrease at 5 minutes and 46±7% decrease at 60 minutes)(A). Further, BAPTA-AM treatment led to decrease in basolateral PAR-2 induced ERK 1/2 activation at 60 minutes, however, increased slightly at 5 minutes post receptor activation (12±4.5% increase at 5 minutes and 45±7% at 60 minutes) (B). Inhibition of PLC using U73122 inhibited apical PAR-2 reduced ERK 1/2 activation induced by basolateral PAR-2 at both 5 and 60 minutes of 2fAP treatment (36%±12% decrease at 5 minutes and 14±8% decrease at 60 minutes) (C). Also, inhibition of PLC reduced ERK 1/2 activation induced by basolateral PAR-2 at 60 minutes of 2fAP treatment, however, only mildly decreased activation at 5 minutes (1±1.73% increase at 5 minutes and 44±10.5% decrease at 60 minutes) (D). Inhibition of PKC using GF109203X drastically diminished ERK 1/2 phosphoyrlation induced by apical PAR-2 at both 5 and 60 minutes (from 42±13% decrease at 5 minutes and 50±11% decrease at 60 minutes) (E); while ERK 1/2 induced by basolateral PAR-2 were relatively unaffected (6±1% increase at 5 minutes and 4±2% increase at 60 minutes) (F). Transfection DNarr drastically inhibited ERK 1/2 activation at 5 and 60 minutes stimulated by basolateral 2fAP treatment (56±9% decrease for 5 minutes and 71±6.66% decrease for 60 minutes) (G, I) and inhibited activation apically administered 2fAP (40±9% decrease for 5 minutes and 52±7.37% decrease for 60 minutes) (H, I). Statistically significant results ($p<.05$) revealed by one way ANOVA are indicated by (*).



Lau et al Figure 7

Figure 2-8: Basolateral PAR-2 activation results in activation of cofilin, while apical PAR-2 activation does not. Cofilin promotes rapid actin reorganization by severing existing actin filaments and creating free barbed ends to which actin monomers can spontaneously add. Since only basolateral PAR-2 required β-arrestins for ERK1/2 activation, we examined whether it also resulted in cofilin dephosphorylation. Consistent with the findings above, basolaterally administered 2fAP induced cofilin dephosphorylation after 5 and 60 minutes (B). In contrast, apical 2fAP did not trigger cofilin dephosphorylation after 5 and 30 minutes (A). A major effect of cofilin actin filament severing activity is the creation of actin free barbed ends, which promotes remodeling of the actin cytoskeleton. Barbed end formation can be monitored in cells by observing incorporation of labeled actin monomers into lightly permeabilized cells after agonist treatment. Actin monomers will not incorporate into stable filaments; thus they can be used to monitor sites of filament severing. Consistent with cofilin activation, we observe that actin monomers incorporate into cells upon basolateral, but not apical PAR-2 activation in both cultured CACO-2 monolayers (C) and intestinal mucosa (D). We conclude that basolateral PAR-2 is capable of triggering β-arrestin-dependent and G-protein dependent signals, while apical PAR-2 does not trigger β-arrestin-dependent signaling.



Lau et al Figure 8

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Chapter 3

PAR-2 activation stimulates bacterial uptake in the intestinal epithelial both *in vitro* and *in vivo*

3.1 ABSTRACT

Protease-activated-receptor-2 (PAR-2) is a G-protein coupled receptor (GPCR) expressed in colonic, pulmonary epithelial cells and gastrointestinal tract where its activation by serine proteases/coagulation factors can be both inflammatory and protective (13-20). Further, PAR-2 activation can disrupt tight junctions and trigger actin reorganization *in vitro*, and thus may be a contributing factor to bacteria infections through enhanced uptake (10). There is a great deal of interest in PAR-2 as a therapeutic target for gastrointestinal inflammation and barrier dysfunction; however, its role in intestinal bacterial infection remains unknown. Here we illustrate that PAR-2 activation enhanced bacteria uptake *in vitro* via a clathrin and phosphotidyl-inositol-3-kinase dependent manner. Further, we show that PAR-2 activation stimulated bacteria uptake in mice intestines. These results suggested that activation of the receptor plays a crucial role in gastrointestinal barrier dysfunction and is indeed a contributing factor to bacterial infection, further allowing for better understanding of PAR-2 and its physiological role in the gastrointestinal system.

3.2 INTRODUCTION

PAR-2 is a member of the G-protein coupled receptor family that is activated by serine proteases including trypsin, coagulation factor VIIa/ Xa, and mast cell tryptase. Exposure to such proteases triggers cleavage of the N-terminal domain of PAR-2 exposing a tethered ligand that binds and activates the receptor. There are synthetic peptides corresponding to the tethered ligand, activating peptide SLIGRL (AP) and 2-furyol-LIGRL-Ornithine (2fAP) that specifically activate PAR-2 in the absence of enzymatic cleavage. Studies suggested activation of PAR-2 is involved in both protective and inflammatory processes where PAR-2 activation can lead to broncho-protective prostaglandin release in intrapulmonary epithelium, triggering protective processes in colitis and inhibiting cytokine production in lamina propria lymphocytes (14, 17, 18). Conversely, PAR-2 expression can be up-regulated by Tumor Necrosis Factor Alpha (TNF-α) and Interleukin-1-alpha (IL-1α) release, and PAR-2 activation can enhance leukocyte recruitment and adhesion to inflammatory sites, trigger cytokine production, vasodilation, and colonic inflammation (16, 18-20). Additionally, in the gastrointestinal tract, release of mast cell tryptase in response to stress and inflammation can activate PAR-2. Consequently, activation of the receptor induces redistribution of ZO-1, F-actin and enhances paracellular permeability in colonocytes (10).

The gastrointestinal barrier is the crucial first line of defense against pathogenic invasion. The barrier selectively allows nutrients to traverse the apical surface and be absorbed into

the bloodstream while barring harmful toxins and pathogens from exiting the lumen. This fluid impermeable barrier selectively transcytose essential molecule from the apical to the basolateral surfaces via specialized processes such as receptor mediated transcytosis (12, 13, 17). Moreover, there are many environmental factors that can manipulate the integrity of the gastrointestinal barrier and thus making it porous and vulnerable to pathogenic invasions. These factors include cytokines, pathogenic toxins and enzymes. For example, Staphylococcus Aureus (S. Aureus) release enzymes that degrade tight junction proteins while Salmonella Typhimurium (S. Typhimurium) inject toxins to alter host cytoskeleton by affecting cell signaling pathways (6, 7). Further, invasions of these pathogens trigger the release of host pro-inflammatory cytokines such as Interleukins (IL) and Tumor Necrosis Factors (TNF), causing further barrier dysfunction via unknown mechanisms (2, 5, 8). Here, we proposed a novel mechanism in bacterial invasion that involves the activation of PAR-2 and examined the following: (1) whether activation of PAR-2 in colonic epithelial cells will enhance bacteria endocytosis, (2) whether the internalization of bacteria is a product of clathrin mediated receptor endocytsosis, (3) whether the bacterial endocytosis is a signaling process involving the activation of phosphoinositide 3-kinases (PI3K), lastly we investigated whether gastrointestinal dysfunction are induced as a result of PAR-2 activation in isolated mice proximal jejunum.

3.3 MATERIALS AND METHODS

All chemicals were from Sigma unless otherwise stated. Antibodies and final dilutions immunofluorescence (IF) was as follows: from BD Biosciences, mouse anti-EEA-1 (early endosome) (1:300 for IF) and mouse anti-clathrin (1:250 for IF); from Cell Signaling mouse anti-P85 (PI3K) (1:150 for IF); from Santa Cruz Biotechnology mouse anti-LAMP-1 (1:250 for IF). Phalloidin Alexa 594 (1:20 IF) from Invitrogen; Pharmacological inhibitors were used as follows: Monodansylcadaverine (Cayman Chemical) was prepared in dimethyl sulfoxide (DMSO) and used at 10 uM final concentration; PI3K inhibitor LY294002 (Alexis Biochemicals) reconstituded to 100 mM in DMSO immediately before use at 10 uM final concentration.

Transfection and cell lines: Human intestinal cell line CACO2-BBe were gifts from Dr. David Lo (UC Riverside Biomedical Sciences) and grown in Advanced Dulbecco's modified Eagle's medium, 15 mM Hepes, 10% fetal calf serum. Transient transfections were performed on 95% confluent cells using FuGENE6 (Roche) and experiments were performed between 48 and 72 hours after transfection.

Microscopy: Cells prepared as described previously (26). Serial sections (100X and 63X objectives) were taken on Zeiss LSM-510. Three dimensional reconstructions of the images were done using Imaris 6.3 software.

Immunofluorescence labeling of proximal jejunum: Segments of mouse proximal jejunum were fixed in ice-cold formalin for 5 h, infiltrated with cryoprotectant (30% sucrose in PBS) overnight, and frozen in OTC medium (Triangle Biomedical Sciences) at -35^{o}C. Sections of 10 µm thickness were cut on a cryostat microtome (Microm) and mounted on polylysine-coated glass slides (Fisher Superfrost Plus). Phallodin Alexa 594 was used to stain the sections. Confocal images were acquired with a Zeiss LSM-510 microscope and assembled using Adobe Photoshop.

Data and statistical analysis: All graphs and statistical analysis were performed using Prism or Microsoft Excel 2003. All experiments were performed a minimum of three times. Phospho-protein levels were normalized to total protein levels before calculating fold change with respect to untreated controls. One way ANOVA and paired t tests were used to determine statistical significance of and significant differences between values under different conditions.

In vitro **bacterial uptake assay:** Cultured cells are grown in Advance DMEM (4500 mg/ L D. Glucose, Non-essential amino acid, 110 mg/L sodium pyruvate, 1M hepes, 1x PSQ (Penicillin, streptomycin, L glutamine, 10 mM citrate buffer) to confluency in 6 well (6 cm) plates. Cells are serum starved for 2 hours. 1 ug of SA488 is added to the cultured cells and incubated on ice for 30 minutes to allow the bacterial particles to settle onto the cell surface. 1 uM of 2fAP is then added to appropriate Ills (no 2fAP treatment in control samples) and incubated in 37 degrees Celsius for 4 hours. The samples are

rigorously washed with phosphate buffered solution (PBS, 140 mM NaCl, 3mM KCl, 5 mM Na2HPO4, 1.5 mM KH2PO4) 3 times for the removal of membrane bound bacterial particles, and detached using Cellgro Cellstripper. Trypsin is not used to detach the cells because it activates PAR-2. The samples are then analyzed via flow cytometry (FACS).

FACS analysis: To find the region on the histogram that corresponded to the cells containing SA488. The laser intensity is at the level which every cell counted is detected on the histogram. The histogram were separated into 3 regions: (1) Cell auto-fluorescence (background), (2) Cells containing bacteria and (3) External bacteria, or bacteria only (bacteria outside, not endocytosed into cell). Once the region which corresponds to bacterial uptake was determined, cells that have taken up bacteria were detected and quantified using the program Cellquest (Fig. 3-1).

***In vivo* mice small intestinal loop assay:** Mice are anesthetized using Avertin (2-2-2 Tribromoethanol). Next, a small abdominal incision is made and small intestine is pulled out. One inch of the intestine is isolated by tying off the ends and injected with 1 uM of 2fAP or RAP + 1 ug of SA488 + PBS. One hour treatment takes place on a warm plate to prevent hypothermia and the isolated intestines are removed and fixed. Samples are they sliced at 10 uM, stained with phalloidin, and visualized via immuno-fluorescence.

3.4 RESULTS

PAR-2 activation stimulates S. Aureus uptake in CACO-2. Our results showed that PAR-2 activation stimulated S. Aureus uptake in cultured epithelial cells. Confocal microscopy images showed 2fAP treated CACO-2 cells (Fig. 3-1A) were able to internalize S. Aureus 594 particles more readily compared to the untreated cells (Fig. 3-1B). FACS analysis quantified and confirmed the confocal microscopy results. Treatment with 2fAP exhibited a 72% increase in S. Aureus 594 positive cells when compared to untreated samples (1286±369.1 events (untreated); 2214.0±364.7 events) (Fig. 3-1C).

PAR-2 vesicles upon treatment with 2fAP colocalized with endocytosed S. Aureus particles in CACO-2 cells. Since activation of PAR-2 induces clathrin mediated receptor endocytosis, we investigated whether S. Aureus particles are endocytosed upon PAR-2 internalization. Thus we began by examining if endocytosed S. Aureus particles colocalized with PAR-2 vesicles upon treatment with 2fAP. Indeed, our confocal microscopy results showed colocalization between PAR-2 vesicles (red) and S. Aureus particles (green) upon 2fAP treatment (Fig. 3-2A). In untreated samples, PAR-2 did not vesicularize, nor colocalized with the S. Aureus particles (Fig. 3-2B).

PAR-2 induced S. Aureus uptake in CACO-2 cells is clathrin dependent. As mentioned previously, since PAR-2 activation induces clathrin mediated receptor

endocytosis, we investigated whether PAR-2 stimulated S. Aureus uptake is a clathrin mediated process. Immuno-fluorescence revealed upon 2fAP treatment, endocytosed S. Aureus particles are surrounded by clathrin (Fig 3-3A) and not in the untreated samples (Fig. 3-3B). Moreover, FACS analysis revealed inhibition of clathrin mediated endocytosis by using MDC inhibited PAR-2 stimulated bacterial up take (increase from 2387±306.3 to 3026±389.6 after 2fAP treatment, a 27% increase; post MDC treatment, 2fAP activation yielded a slight increase from 1637±333.8 to 1746±359.7, a 7% increase) (Fig. 3C).

PAR-2 induced S. Aureus uptake is a signaling process involving PI3K.

Phosphatidlyinositol 3-kinase (PI3K) family controls numerous biological processes including cell growth, survival, cytoskeletal remodeling, differentiation, migration and metabolism (22-25). Further, studies by Kierbel et al. verified that PI3K, and its second messenger, phosphatidlylinositol 3,4,5-triphosphate (PIP3) to be involved in host cell entry by *pseudomonas aeruginosa* (6,7). Since we previously showed that PAR-2 promotes Gαq/Ca^{2+}/Src Family Kinase (SFK)-dependent-stimulation class 1A PI3K activation (but also PAR-2 mediated B-arrestin-dependent-inhibition) PI3K may play an important role in PAR-2 stimulated S. Aureus endocytosis. A decrease was observed in PAR-2 stimulated bacteria uptake upon LY treatment (increase from 2155±340.0 to 2737.5±429.2 after 2fAP treatment, a 23% increase; post LY treatment, 2fAP activation did not induce bacterial uptake, from 1821.8±424.6 to 1821.3±426.5) (Fig. 3-4C).

Confocal microscopy shows recruitment and colocalization of PI3K with endocytosed S. Aureus upon 2fAP treatment (Fig. 3-4A, B).

PAR-2 stimulated endocytosed S. Aureus enters the early endosome and lysosome. PAR-2 activation leads to recruitment of adapter proteins β-arrestins triggering clathrin mediated receptor endocytosis. Moreover, endocytosed receptors have been shown to enter the early endosome for sorting, and cleaved PAR-2 enters the lysosome for degredation (21, 26, 27). Hence we examined whether PAR-2 stimulated endocytosed S. Aureus entered the early endosome and also the lysosome. Confocal microscopy revealed endocytosed S. Aureus enters the early endosome as early as after 5 minutes after treatment with 2fAP (Fig. 3-5A). Also, lysosomal antibody LAMP-1 showed internalized S. Aureus induced by PAR-2 enters the lysosome as well (Fig. 3-5B).

PAR-2 activation stimulated S. Aureus uptake *in vivo*. PAR-2 expression is abundant in the gastrointestinal tract, found in endothelial cells, colonic myocytes, enterocytoes, enteric neurons, terminals of mesenteric afferent nerves and immune cells (14-19). Studies show luminal proteases activate PAR-2 and trigger inflammation and gastrointestinal dysfunction. A "mouse intestinal loop" experiment was conducted to confirm PAR-2 enhanced bacteria uptake *in vivo*. Results showed PAR-2 activation allowed S.A. 488 to traverse the apical barrier (Fig. 3-6A-C). Although results were not quantified, noticeable increases of S.A. 488 were present beyond the gastric mucosa upon 2fAP treatment (compared to untreated).

3.5 DISCUSSION

PAR-2 activation stimulates S. Aureus uptake in CACO-2 and in mice ileum. PAR-2 is heavily expressed in gastrointestinal tract and is found in colonic and intestinal epithelial cells (13, 15-20). Receptor activation enhances paracellular permeability triggering reorganization of F-actin and ZO-1 (13). Further, PAR-2 is internalized for desensitizing purposes via a clathrin-dependent process upon activation (27-31). Our results showed that PAR-2 activation stimulated S. Aureus uptake in cultured epithelial cells. The FACS analysis and confocoal quantitatively and visually confirmed the biological phenomenon. Since the gastrointestinal barrier is the crucial first line of defense against pathogenic invasion, our data suggests PAR-2 may be a target utilized by invading pathogens for traversing this tight junction fortified barrier. Another possibility may be that PAR-2 activation induces epithelial bacterial uptake for antigen presentation and sample in preparation of the oncoming attack inducing proper immune responses. Epithelial cells with the aid of dendridic cells have been shown to possess the ability to sample antigens from the lumen (33, 34, 35). Hence, upon attachment of pathogenic microbes, PAR-2 may be activated either via the proteases released by the pathogen themselves, or by tryptaste released by mast cells or luminal agonists. PAR-2 activation induces epithelial microbial uptake and with aid of dendritic cells allows for antigen sampling and preparation for the oncoming attacks.

PAR-2 vesicles upon treatment with 2fAP colocalized with endocytosed S. Aureus particles in CACO-2 cells and stimulated bacterial uptake is a clathrin mediated process. PAR-2 activation induces clathrin mediated receptor endocytosis upon recruitment and binding of adapter proteins β-arrestins (27-31). Our results suggest the receptor internalization process aid the entry of pathogenic microbes. Confocal microscopy revealed S. Aureus particles colocalized with both clathrin and PAR-2, indicating that both internalized PAR-2 and S. Aureus are within the same clathrin vesicle, endocytosed simultaneously. Further, FACS analysis confirmed clathrin mediated endocytosis are involved in PAR-2 simulated bacterial uptake, where inhibition of clathrin internalization drastically reduced the 2fAP effect.

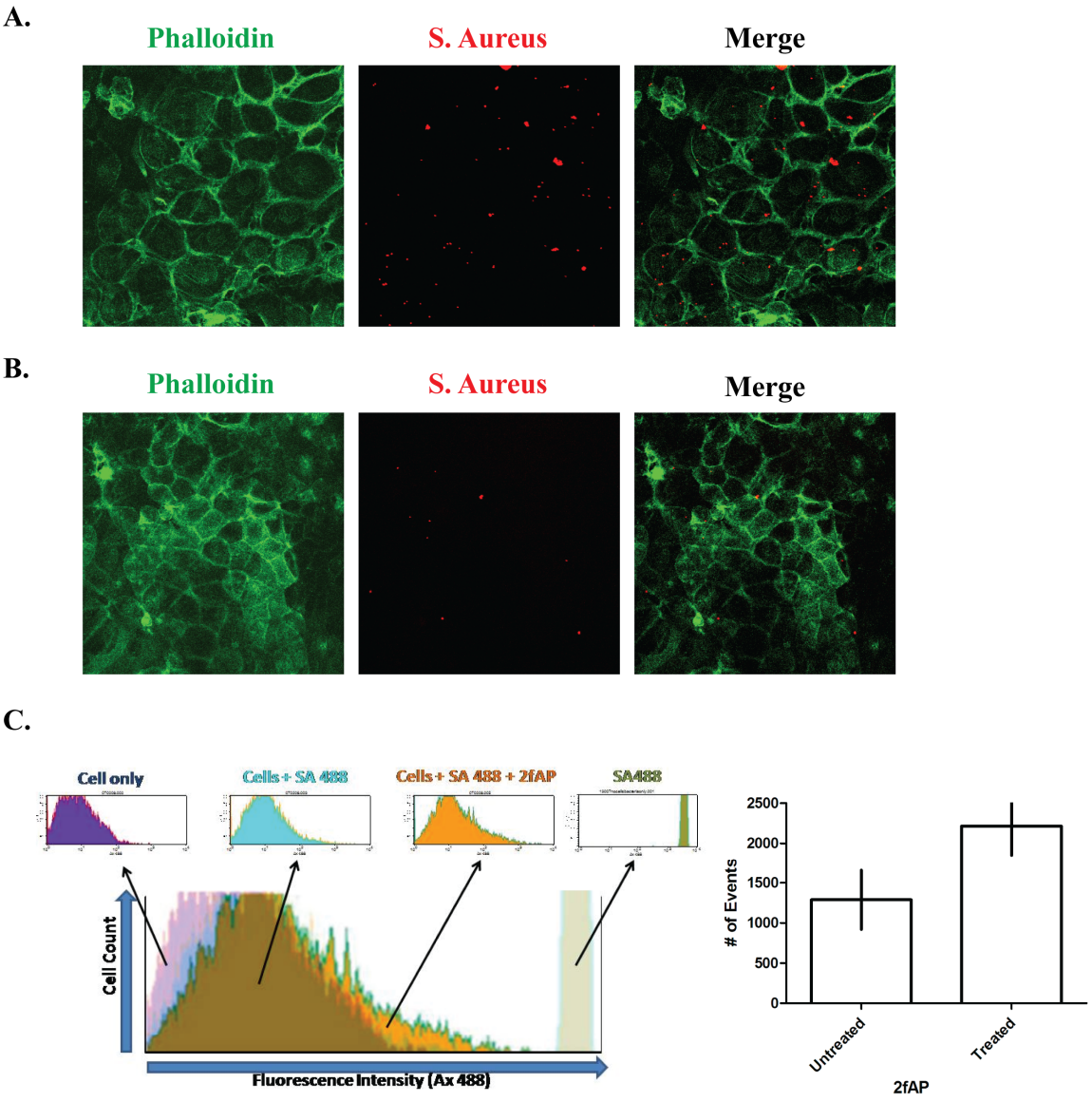
PAR-2 induced S. Aureus uptake is a signaling process involving PI3K. As mentioned previously, studies suggest PI3K pathway is important and required for the endocytosis of bacterial *Pseudomonas Aeruginosa* (P. Aeruginosa) into epithelial cells (93). Moreover, the same group showed in response to *P. Aeruginosa* attachment to the apical surface of the epithelial cells, many of the basolateral proteins including PI3K and actin translocated and recruited to the site of bacterial adhesion (6, 7). The proteins at the apical surface were removed as well from the site of bacterial attachment. Furthermore, these studies demonstrated the basolateral proteins that are found at the protrusion site of bacterial adhesion were shuttled from the basolateral membrane and directed via a dynamin and PI3K dependent transport mechanism. Hence, since we have shown PAR-2 activation can induce activation of both p110α and p110β catalytic subunits of PI3K via G-protein signaling, hence only in cell lines which with low endogenous expression of β-arrestins, we examined whether PI3K activation was significant in PAR-2 stimulated

bacterial uptake. Confocal microscopy revealed internalized S. Aureus particles upon 2fAP treatment colocalized with PI3K, indicating PI3K are recruited to the site to permeabilize the cell membrane allowing microbial entry. Inhibition using LY and quantification using FACS analysis confirmed PI3K is indeed involved in the process, where 2fAP effects were abolished. This suggests PAR-2 stimulated bacterial endocytosis is not only a process mediated by mechanical methods (clathrin mediated) however, via a signaling mechanism as well.

PAR-2 stimulated endocytosed S. Aureus enters the early endosome and lysosome. PAR-2 activation induces internalization of the receptor mediated by β-arrestins triggering clathrin mediated receptor endocytosis and where internalized receptors enter early endosome for sorting, then lysosome for degredation (21, 26, 27). Hence we examined whether PAR-2 stimulated endocytosed S. Aureus entered the early endosome and also the lysosome. Our results demonstrated indeed the internalized bacterial particles enter early endosome and lysosomes for sorting and degredation. This may be an important process for the antigen presentation where the internalized bacterial particles upon degradation are utilized as the antigens which allows the induction of proper immune responses.

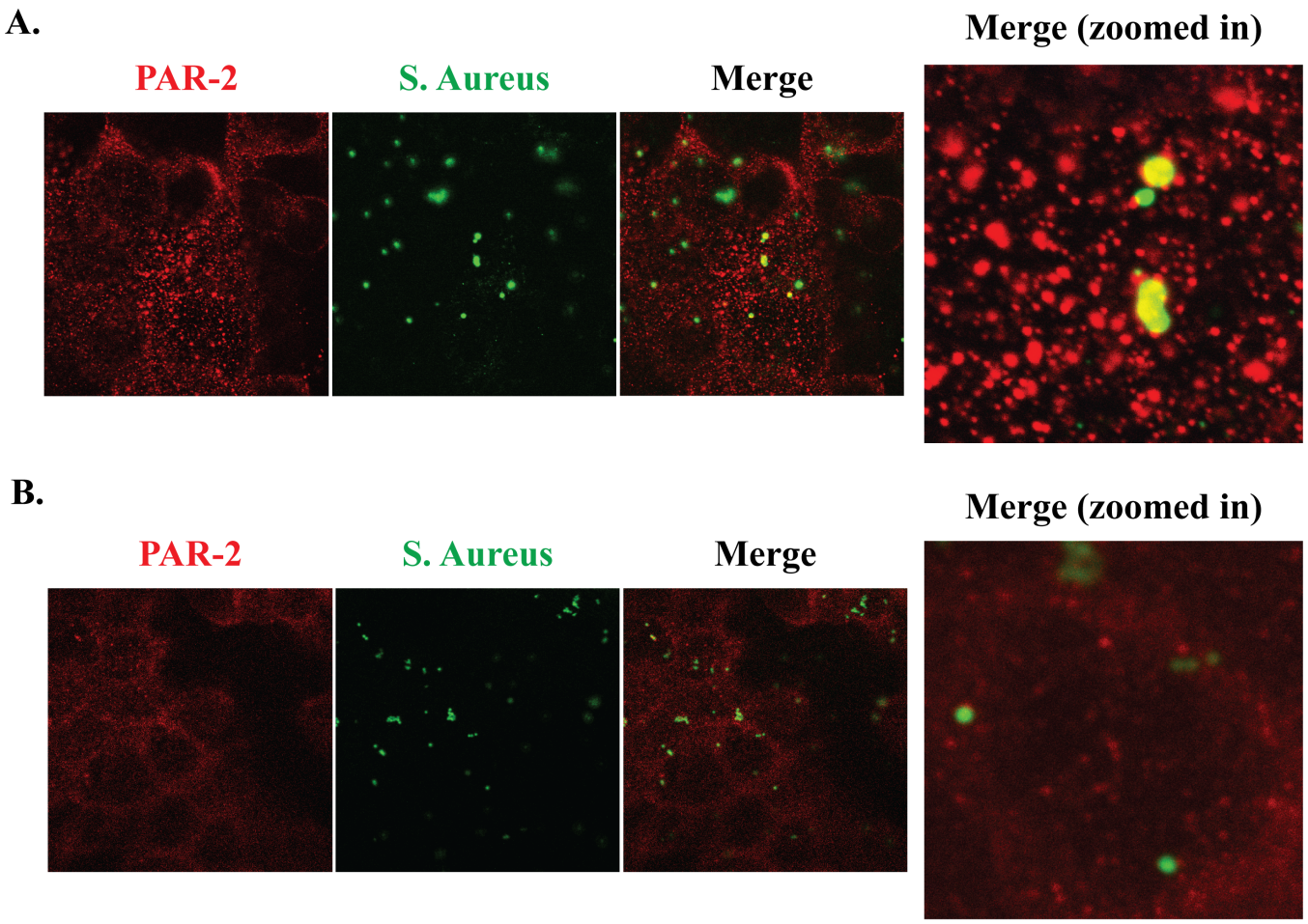
3.6 FIGURES AND LEGENDS

Figure 3-1: PAR-2 activation stimulates S. Aureus uptake in CACO-2. PAR-2 activation stimulated S. Aureus uptake in cultured epithelial cells. Confocal microscopy images showed 2fAP treated CACO-2 cells (A) were able to internalize S. Aureus 594 particles more readily compared to the untreated cells (B). FACS analysis quantified and confirmed the confocal microscopy results where the histogram shows the shift of the peaks upon treatment with 2fAP and graph illustrates the corresponding numbers (C).



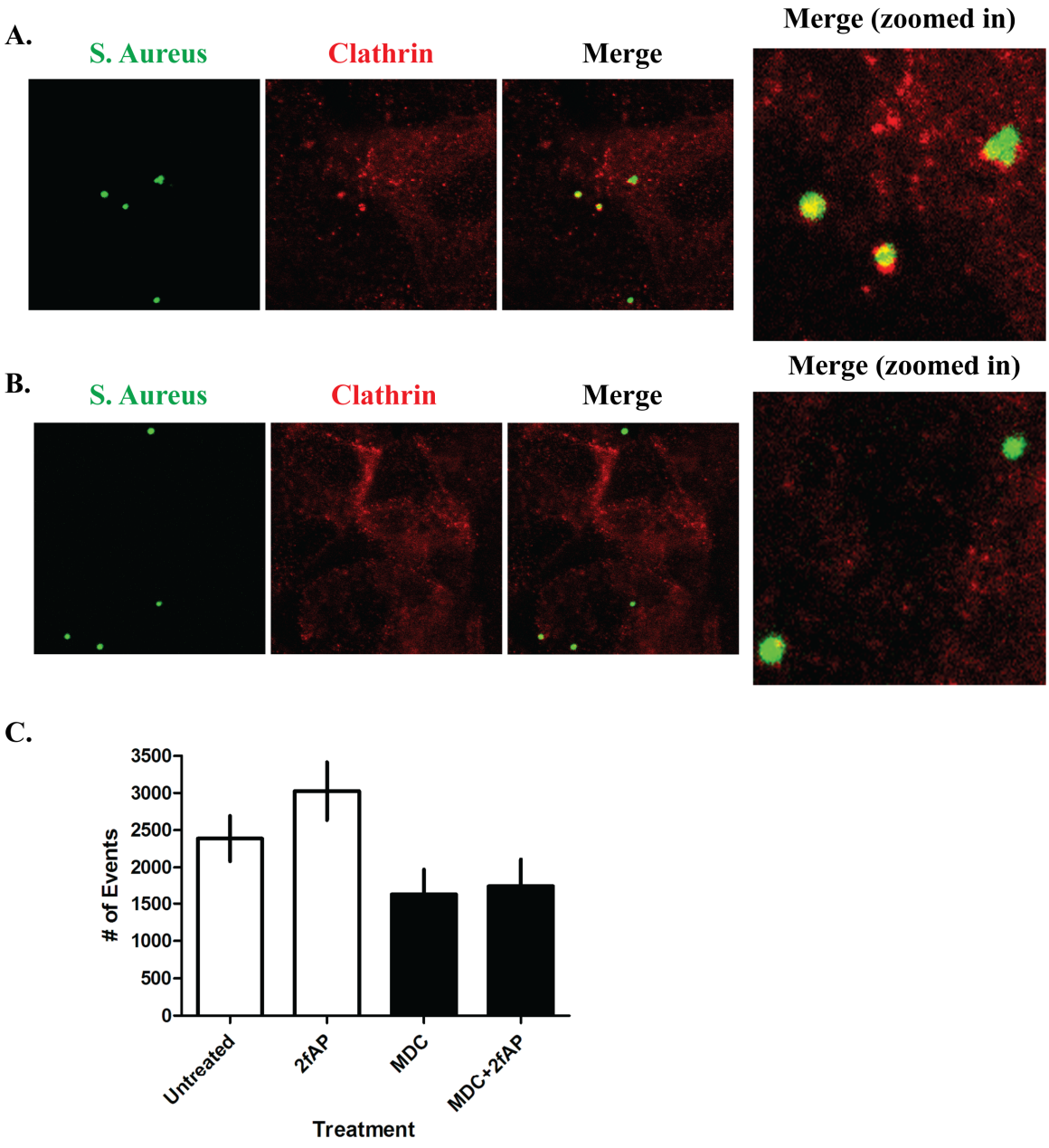
Lau et al Figure 1

Figure 3-2: PAR-2 vesicles upon treatment with 2fAP colocalized with endocytosed S. Aureus particles in CACO-2 cells. Confocal microscopy results showed colocalization between PAR-2 vesicles (red) and S. Aureus particles (green) upon 2fAP treatment (A). In untreated samples, PAR-2 did not vesicularize, nor colocalized with the S. Aureus particles (B).



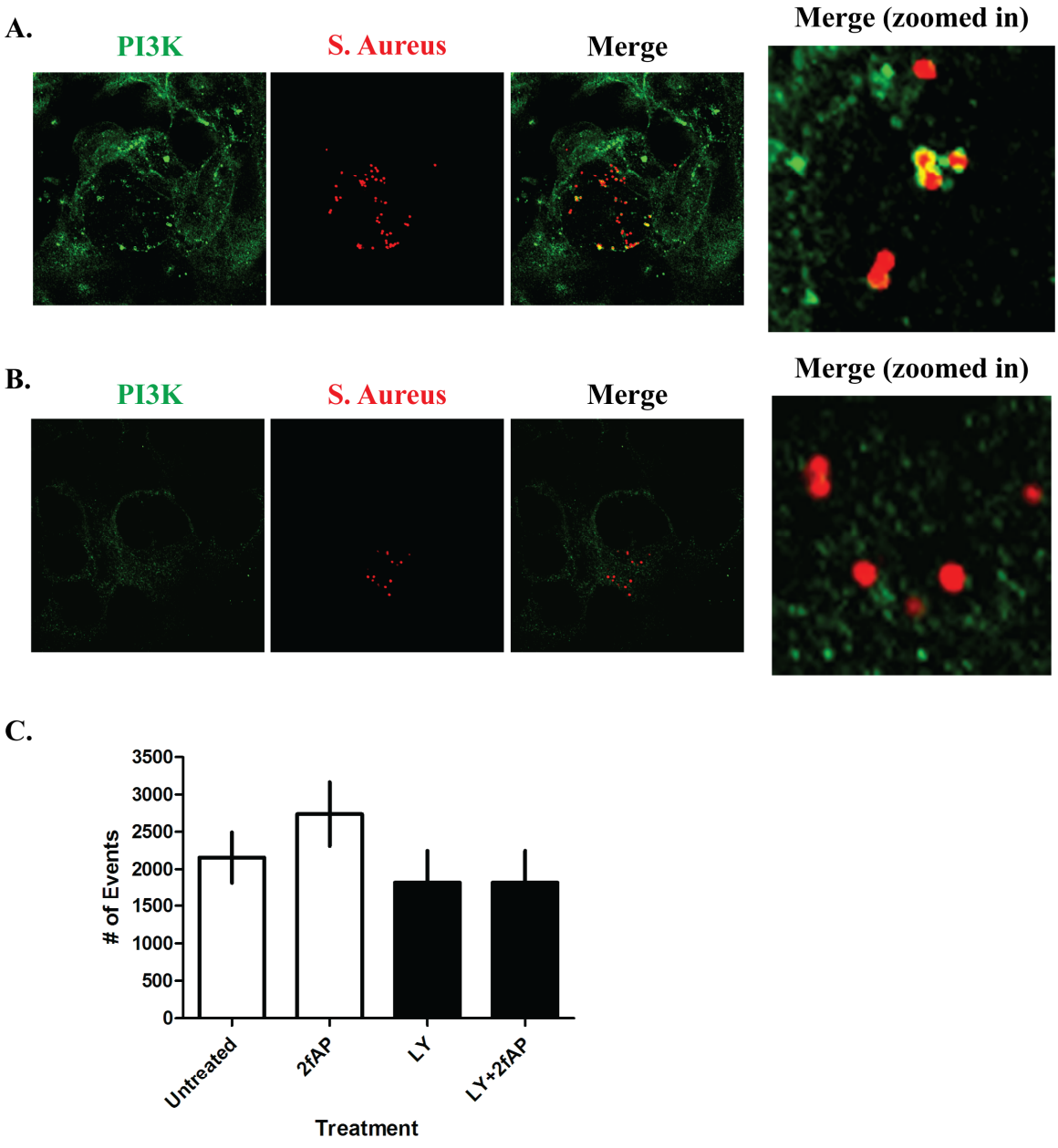
Lau et al Figure 2

Figure 3-3: PAR-2 induced S. Aureus uptake in CACO-2 cells is clathrin dependent. Immuno-fluorescence revealed upon 2fAP treatment, endocytosed S. Aureus particles are surrounded by clathrin (A) and not in the untreated samples (B). FACS analysis revealed inhibition of clathrin mediated endocytosis by using MDC inhibited PAR-2 stimulated bacterial up take (C).



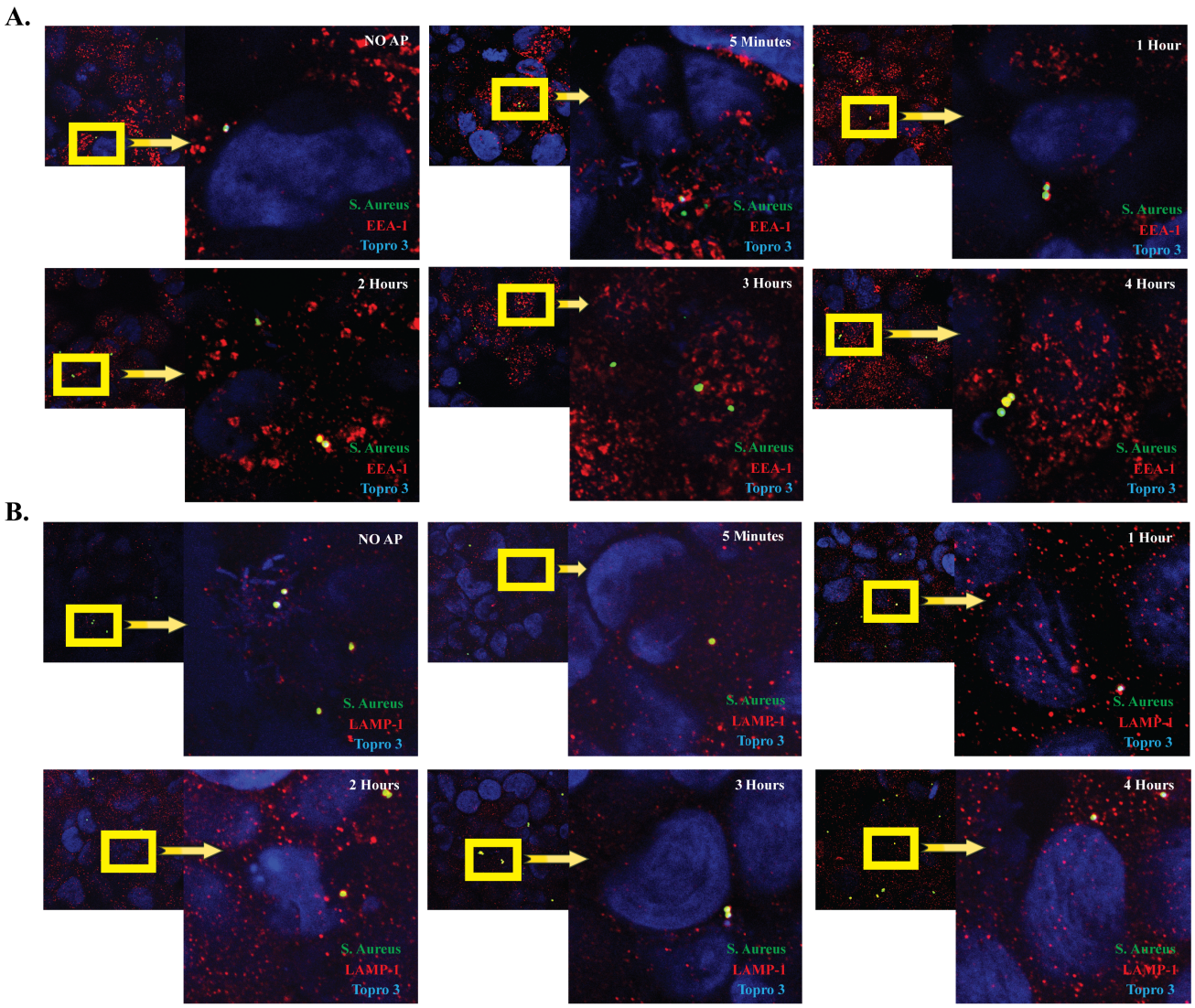
Lau et al Figure 3

Figure 3-4: PAR-2 induced S. Aureus uptake is a signaling process involving PI3K. We examined whether PI3K may play an important role in PAR-2 stimulated S. Aureus endocytosis. A decrease was observed in PAR-2 stimulated bacteria uptake upon LY treatment (C). Confocal microscopy shows recruitment and colocalization of PI3K with endocytosed S. Aureus upon 2fAP treatment (A, B).



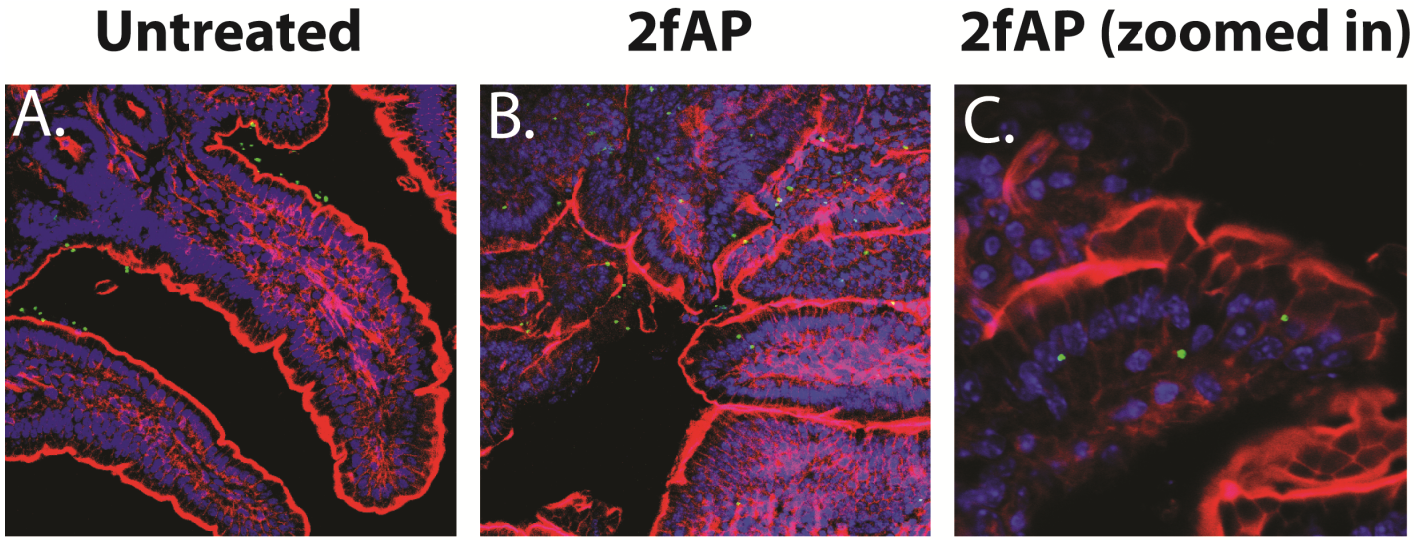
Lau et al Figure 4

Figure 3-5: PAR-2 stimulated endocytosed S. Aureus enters the early endosome and lysosome. Confocal microscopy revealed endocytosed S. Aureus enters the early endosome as early as after 5 minutes after treatment with 2fAP (A). Also, lysosomal antibody LAMP-1 showed internalized S. Aureus induced by PAR-2 enters the lysosome as well (B).



Lau et al Figure 5

Figure 3-6: PAR-2 activation stimulatd S. Aureus uptake *in vivo*. A "mouse intestinal loop" experiment was conducted to confirm PAR-2 enhanced bacteria uptake *in vivo*. Results showed PAR-2 activation allowed S.A. 488 to traverse the apical barrier (A-C). Although results were not quantified, noticeable increases of S.A. 488 were present beyond the gastric mucosa upon 2fAP treatment (compared to untreated).



Lau et al Figure 6

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Chapter 4

Differential effects of beta arrestins on the internalization, desensitization & ERK 1/2 activation downstream of Protease Activated Receptor-2

4.1 ABSTRACT

Beta-arrestins 1 & 2 are known to play important roles in desensitization of membrane receptors and facilitation of signal transduction pathways. It has been previously shown that beta-arrestins are required for signal termination, internalization and ERK 1/2 activation downstream of Protease-Activated-Receptor-2 (PAR-2), but it is unclear whether they are functionally redundant or mediate specific events. Here we demonstrate that in mouse embryonic fibroblasts (MEF's) from beta-arrestin knockout mice, Gαq signaling by PAR-2, as measured by mobilization of intracellular Ca^{+2}, is prolonged. Only expression of beta-arrestin-1 shortened the signal duration, whereas either beta-arrestin-1 or 2 was able to restore Protein kinase-C induced receptor desensitization. Beta-arrestin-1 also mediated early, while beta-arrestin-2 mediated delayed, receptor internalization and membrane associated ERK 1/2 activation. While beta-arrestin-1 colocalized with a lysosomal marker (LAMP-1), beta-arrestin-2 did not, suggesting a specific role for beta-arrestin-1 in lysosomal receptor degradation. Together these data suggest distinct temporal and functional roles for beta-arrestins in PAR-2 signaling, desensitization and internalization.

4.2 INTRODUCTION

Beta-arrestins 1 and 2 are multi-faceted proteins that can promote signal termination of many heterotrimeric G-protein initiated signals, as well as regulate the localization and activity of downstream signaling molecules and facilitate chemotaxis (4, 11, 12, 16, 17, 18, 21, 23, 25, 26, 39, 43). Signal termination can be broken down into 2 parts: desensitization of the receptor and removal of the receptor from the cell surface by endocytosis. G-protein coupled receptor (GPCR) desensitization is usually defined as a molecular event that renders the receptor insensitive to further activation and/or attenuates the initial signal generated. Beta-arrestins participate in both processes, by binding to receptors (usually via phospho-serine/threonine residues in the C-terminus and third cytosolic loop) and uncoupling them from their cognate G-proteins (28) and by binding to both clathrin and the clathrin adaptor proteins, AP-1 and AP-2, to facilitate endocytosis (24, 32).

Protease-Activated-Receptor-2 is a GPCR that mediates a diverse array of cellular processes in response to serine proteases. Previous studies on PAR-2 have demonstrated that it undergoes agonist-induced (homologous) desensitization and internalizes via a beta-arrestin-dependant mechanism. Both processes are impaired in mouse embryonic fibroblasts (MEF's) from beta-arrestin double knockout mice; internalization is also blocked by a dominant negative mutant of beta-arrestin 1 (15, 38). Furthermore, over-expression of either beta-arrestin 1 or 2 is reported to increase accumulation of IP3 (a $G_{\alpha}q$ signaling intermediate), suggesting that beta-arrestins terminate IP3 generation through uncoupling of the receptor from $G_{\alpha}q$. For desensitization and internalization of

some GPCR's, the two beta-arrestins are interchangeable, whereas for others they have distinct roles (1, 2, 4, 37, 44). Previous studies have also demonstrated that PAR-2 requires both beta-arrestins for efficient ERK 1/2 activation and chemotaxis (13, 17, 18, 38), suggesting that they may regulate different events in PAR-2 signaling and signal termination. Furthermore, several lines of evidence suggest PKC activation can promote desensitization and internalization of PAR-2, leading to the hypothesis that either or both beta-arrestins might bind to Protein Kinase C (PKC) phosphorylated sites in the PAR-2 C-terminus (7, 13). Upon agonist-induced endocytosis, many receptors are recycled back to the plasma membrane; however, since the physiological agonists of PAR-2 are serine proteases that remove the extreme N-terminus rendering it unusable, most is degraded rather than recycled. Restoration of surface PAR-2 levels requires exocytosis from Golgi stores, by a process involving the small GTPase-Rab11 (7, 34). Whether beta-arrestins play a role in receptor exocytosis has not been examined.

Because previous studies demonstrating a requirement for beta-arrestins in PAR-2 internalization were done in mouse embryonic fibroblasts (MEF's) from mice lacking both beta-arrestins, or in cells stably transfected with a dominant negative mutant of beta-arrestin 1 that can block both beta-arrestin 1 and 2 (15, 38), it is unclear whether the two beta-arrestins regulate distinct processes during PAR-2 desensitization, internalization and signaling. In one study, over-expression of either beta-arrestin-1 or 2 attenuated accumulation of the second messenger IP3, suggesting that either one can promote receptor uncoupling from $G_{\alpha}q$ (38), while in another study, knockdown of either beta-arrestin 1 or 2 nearly abolished ERK 1/2 activation suggesting that both are required for

signaling (18). Furthermore, no studies have investigated their role in heterologous desensitization such as that promoted by PKC activation.

Because beta-arrestins were first identified as mediators of signal termination and receptor endocytosis, it has often been assumed that their role in other downstream signaling events such as ERK 1/2 activation is secondary to their ability to mediate endocytosis. However, recent studies have revealed that beta-arrestins can elicit signals, independent of G-protein coupling, suggesting that their roles as mediators of signal termination can be separated from their roles as facilitators of signal transduction. In this study, we probe the role of individual beta-arrestins in PAR-2 desensitization, internalization and ERK 1/2 activation. We show not only can the individual arrestins promote their "arresting" (desensitizing) effects differently but can also mediate internalization and downstream signaling of a receptor in different ways.

4.3 MATERIAL AND METHODS

Rabbit anti-PAR2 B5 antibody was a generous gift from Dr. Morley Hollenberg (University of Calgary, Canada), Rabbit anti-LAMP1, rabbit anti-ERK1 and mouse PAR2 antibodies were from Santa Cruz Biotechnology, Mouse anti-EEA1 was from BD Transduction Laboratories, anti-phospho-ERK1/2 was from Cell Signaling Technologies, anti-FLAG M2 antibody was from Sigma. Fluorescent secondary antibodies anti-rabbit Alexa 488/546/633 and anti-mouse Alexa 488/546 were from Molecular Probes, 2-furoyl-LIGRL (2-fAP) was synthesized by Genemed Inc., Trypsin was from Worthington Chemicals, Fura2-AM and Pluronic F127 were from Molecular Probes, Phorbol Myristoyl Acetate (PMA) was from Sigma Aldrich, FLAG-tagged beta-arrestin-1 & 2 constructs and anti-β-arrestin-1/2 antibodies were a kind gift from Dr. Robert Lefkowitz (Duke University Medical Center), Cell Stripper and 1X PBS were from GIBCO BRL.

Cell Culture: Mouse Embryonic Fibroblasts Wild Type (MEF WT), β-arrestin Double Knockout (MEFβarr DKO), β-arrestin1 (MEF DKO + βarr1), β-arrestin2 (MEF DKO + βarr2) were a gift from Dr. Robert Lefkowitz and MDA MB 468 and CHO cells were from ATCC. MEF's and MDA MB-468 cells were grown in DMEM medium supplemented with 10% Cosmic Calf Serum and 10U/ml Penicillin/Streptomycin and CHO's were grown in DMEM/F-12 supplemented with 10% FCS and Pen/Strep. All cells were maintained at 37^{o}C with 5% CO_2. For passaging, cells were detached using Cell Stripper solution. All transfections were done using Lipofectamine and Plus Reagent

(Invitrogen) using standard procedures. For CHO cells stably transfected with PAR-2-GFP, cells were selected in G418 for 2 weeks until GFP-positive colonies formed. To avoid clonal cell artifacts, all positive colonies were pooled and selected for PAR-2-GFP expression using a FACS-Aria. Stable cells lines were maintained in the absence of G418 for at least 2 passages before all experiments.

Flow cytometry analysis of surface expression of PAR-2: Flow cytometry was used to quantify removal of PAR-2 from the plasma membrane and to thereby determine the rate of endocytosis. Cells were treated with 100 nM 2f-AP for 0-60 minutes, washed in ice-cold PBS, detached with ice-cold Cell Stripper and incubated with a 1:500 dilution of anti-PAR-2 (B5 antibody) in DMEM + 1% BSA for 2 hours on ice followed by FITC conjugated secondary antibody for 1 hour on ice in dark. Surface cell fluorescence was determined by flow cytometry using a BD FACScan flow cytometer. Cellquest Pro software was used to determine the mean fluorescence and number of FITC-positive cells.

Immunofluorescence and confocal microscopy: Cells were seeded onto collagen-coated coverslips ($3x10^4$ cells/coverslip) and allowed to grow overnight, after which they were transfected using Lipofectamine and Plus Reagent with the appropriate plasmids, according to manufacturer's instructions. Twenty four hours post-transfection media was changed to serum free DMEM and cells were treated with 100 nM 2-fAP or 50 nM Trypsin for 0-60 minutes, after which they were washed in ice cold 1X PBS, fixed in 4%

Neutral Buffered Formalin and immunostained as described previously (17). Antibody concentrations were as follows: anti-FLAG (1:1000), anti-LAMP1 (1:500), anti-EEA1 (1:500), Texas Red-Transferrin (10μg/ml), anti-PAR2 (1:250). The coverslips were then mounted using DAKO Cytomaton fluorescent mounting medium onto slides and imaged using a Zeiss LSM 510 confocal microscope. All images were collected and analyzed using LSM browser software.

Single cell Calcium Concentration measurements: PAR2-GFP transfected MEF's or untransfected MDA MB-468 cells were incubated in physiological salt solution (PSS: 137 mM NaCl, 4.7 mM KCl, 0.56 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM Na_2HPO_4, 10 mM HEPES, 2 mM L-Glutamine and 5.5 mM D-Glucose pH 7.4), containing 0.1% BSA and a 1:1 mixture of 5 μM Fura-2/AM & 50% Pluronic F-127 detergent for 40 minutes at 37^{o}C. They were then washed and mounted in a perfusion chamber containing 1.5 ml of PSS-BSA at 37^{o}C on the stage of a Nikon TE300 microscope. Agonists were directly added to the bath. Cells were observed with a 40X objective and fluorescence was detected in individual cells using a Nikon video camera and a video microscopy acquisition program (Metafluor). Fluorescence was measured at 340 and 380 nm excitation and 510 nm emission. The ratio of the fluorescence at the two excitation wavelengths, which is proportional to the [Ca^{+2}], was determined. For dose response experiments, cells were exposed to increasing concentrations of agonist; for all other experiments a single concentration of agonist was used (50 nM Trypsin and 100 nM 2-fAP). To examine desensitization, cells were pretreated with 1 μM PMA for 20 minutes

before agonist challenge. All readings were followed by an application of the calcium ionophore, ionomycin, to determine maximum Ca^{+2} levels. The concentration of intracellular Ca^{+2} in response to agonist was determined using the Grynkiewicz equation (20).

Protein analysis and Western Blotting: For all protein analysis, Laemmli sample buffer was added to each sample (Final concentration: 50 mM Tris-Cl pH 6.8, 2.5% SDS, 10% Glycerol, 5% β-mercaptomethanol, 0.1% Bromophenol blue). Equal amounts of protein were loaded onto polyacrylamide mini gels (10% for β-arrestin analysis, 12% for ERK analysis) and run for ~ 2 hours at 100 V. Proteins were transferred to PVDF membrane for 30 minutes, blocked in TBS + 1% Fish gelatin and incubated with primary antibody solutions (p-ERK, 1:1000 + tERK, 1:1000 and β-arrestin-1+2 (A1CT, 1:500)) overnight at 4^{o}C. Blots were washed 3 times in TBS supplemented with 1% Tween-20, followed by incubation with either Alexa 680- or IR800- conjugated secondary antibodies (1:45000) for 1 hour at room temperature. Blots were washed 3X in TBS-Tween and analyzed using the LICOR-Odyssey infrared imaging system. Odyssey software was used to calculate integrated intensities for each band for quantification. Western blots shown are representative of at least 3 independent experiments.

pERK1/2 assays: For total phosphor-ERK analysis, cells (~ 80% confluent) were serum starved in DMEM + 0.1% BSA overnight and treated with 50 nM Trypsin for 0-60 minutes, then lysed in RIPA buffer (1X PBS containing 1% Triton-X 100, 2 mM EDTA,

10 mM NaF, 5 mM activated Na_3VO_4, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 10 μg/ml benzamidine and 2 mM PMSF). Ten micrograms of protein from each time point was analyzed by SDS-PAGE followed by western blotting with phosphor-ERK1/2 antibody and a total ERK1/2 antibody as described above. For determination of membrane vs. nuclear ERK1/2, cells were treated for 0-90 minutes and ruptured by homogenization with a glass dounce in hypotonic lysis buffer (10 mM Triethanolamine-acetic acid, pH 7.6, 250 mM sucrose, 1 mM EGTA, 10 mM NaF, 2 mM Na_3VO_4, 1 mM PMSF, 1 mM DTT, plus 10 μg/ml of leupeptin, aprotinin and benzamidine). Nuclear fractions were collected by centrifugation at 500*g* and pellets resuspended in Laemmli sample buffer. Membrane fractions were collected by centrifugation of low speed supernatants at 100,000*g* for 1 hour. Pellets were resuspended in Laemmli sample buffer. SDS-PAGE and western analysis was conducted as described above.

4.4 RESULTS

Role of beta-arrestins in receptor mediated desensitization: Upon proteolytic activation by trypsin, PAR-2 can couple to $G_{\alpha}q$, which activates Phospholipase Cβ (PLCβ), leading to PIP2 hydrolysis and the generation of IP3 and DAG. IP3 mobilizes Ca^{+2} from intracellular stores; for this reason, Ca^{+2} mobilization is frequently used as a direct readout of PAR-2/Gαq-protein coupling and PIP2 hydrolysis (7, 8, 14, 30, 31). Peptides corresponding to the tethered ligand (SLIGRL or SLIGKV) can be used to activate PAR-2 in the absence of proteolytic cleavage but exhibit very low affinity and are typically used in high micro molar range (3, 8). More recently, higher affinity, synthetically modified peptides such as 2-furoyl-LIGRL (2fAP) have been developed (27) which is used in this study for non-proteolytic PAR-2 activation. Early studies on PAR-2 signaling demonstrated that treatment with agonist peptides renders the receptor insensitive to a second agonist-induced Gαq coupling, as assayed by mobilization of intracellular Ca^{+2} (8). Heterologous activation of protein Kinase C (PKC) by phorbol esters was also shown to attenuate PAR-2 induced Ca^{+2} mobilization, and mutation of putative PKC phosphorylation sites abolishes PMA-induced desensitization (7, 13). To examine whether there was a differential requirement for either beta-arrestin 1 or 2 in PAR-2 desensitization, we measured trypsin-induced Ca^{+2} mobilization in mouse embryonic fibroblasts from Wild Type (MEF WT) or beta-arrestin1/2 $^{-/-}$ mice (MEF DKO), and MEF DKO expressing endogenous levels of either beta-arrestin-1 (DKO + βarr1) or beta-arrestin-2 (DKO + βarr2). These cells have been characterized previously

and used by numerous laboratories to assess beta-arrestin dependence in different cellular events (22, 38, 42). Expression o PAR-2 is relatively low in these cells (38); thus, we expressed PAR2-GFP, which has previously been demonstrated to traffic and signal like the endogenous receptor (15), in order to monitor Ca^{+2} mobilization.

We first determine Ca^{+2} mobilization in response to proteolytic (trypsin) and non-proteolytic (2fAP) PAR-2 agonists in MEF WT and MEF βarr DKO, transfected with PAR-2 GFP to establish whether initial PAR-2 responses were altered by the absence of beta-arrestins. Additionally, dose responses of transfected MEF's were compared to that observed in a breast cancer cell line, MDA MB-468, that expresses high levels of endogenous PAR-2 and has been previously used to characterize PAR-2 signaling (17, 18, 43). Consistent with reports by others, transfected PAR-2 GFP and endogenous PAR-2 promoted similar dose dependant increases in intracellular Ca^{+2} levels in the presence and absence of beta-arrestins (compare MEF WT to MEF βarr DKO, Fig 4-1), suggesting that beta-arrestins are not necessary for PAR-2/Gαq coupling. As reported by others, non-proteolytic agonists have a lower efficiency, but are equally efficacious at promoting Ca^{+2} mobilization (8). It is noteworthy that the response to 2fAP was slightly left-shifted in MEF βarr DKO compared to MEF WT, which may reflect the fact that in the absence of beta-arrestins the soluble peptide can continue to activate the receptor. This same left shift would not be expected for trypsin because the receptor cannot be reused once it has been proteolytically cleaved. For all subsequent studies, 50 nM trypsin and 100 nM 2fAP were used; at these concentrations, the responses in both transfected MEF cell lines and MDA-MB 468 cells were comparable (Fig 4-1).

We then compared the duration of Ca^{+2} mobilization in response to PAR-2, and the effect of PMA on PAR-2 stimulated intracellular Ca^{+2} levels in MEF WT (Fig 4-2A, B), MEF βarr DKO (Fig. 2-2C, D), DKO + βarr1 (Fig. 4-2E,F) and DKO + βarr2 (Fig. 4-2G, H). While the absence of either or both beta-arrestins did not significantly affect the maximum intracellular Ca^{+2} concentration, the duration of the Ca^{+2} response was increased in MEF DKO, suggesting defective G-protein uncoupling (Fig. 4-2J). Expression of beta-arrestin 1 (MEF DKO + βarr 1) decreased the Ca^{+2} signal duration to that of MEF WT, while expression of beta-arrestin 2 (MEF DKO + βarr 2) did not, implying that initial PAR2/G-protein uncoupling is primarily mediated by beta-arrestin 1 (Fig. 4-2J). Next, we examined heterologous desensitization by pre-treating cells with PMA for 20 minutes before addition of PAR-2 agonist. As was previously reported, Ca^{+2} mobilization in MEF WT was inhibited by greater than 70% by PMA treatment, while Ca^{+2} mobilization by ionomycin was unaffected (Fig. 2-2B, K). MEF βarr DKO cells were resistant to PMA-induced receptor desensitization, while MEF DKO + βarr1 (Fig. 4-2F, K) and MEF DKO + βarr 2(Fig. 4-2H, K) were not, suggesting that both beta-arrestins are involved in mediating PMA-induced receptor desensitization.

Although it has been presumed that PMA-induced PAR-2 desensitization is due to activation of PKC, there may be other pathways activated by phorbol esters. To test this hypothesis, we examined the sensitivity of the PMA effect to inhibition by a broad spectrum PKC inhibitor, GF109203X (GFX). As shown in Fig. 4-3, the PMA-induced suppression of Ca^{+2} mobilization observed in PAR-2 GFP transfected MEF WT or in untransfected MDA MB 468 cells was rescued by inhibition of PKC with GFX.

Interestingly, homologous desensitization was not completely blocked by GFX, suggesting the involvement of additional kinases in the regulation of PAR-2 desensitization.

Differential kinetics of PAR-2 internalization in the absence of either β-arrestin-1 or 2: To examine the possibility that beta-arrestins differentially regulate the endocytosis of PAR-2, we first measured internalization of endogenous PAR-2 in MEF WT, MEF βarr DKO, DKO + βarr 1 and DKO + βarr 2 by quantifying cell surface receptor levels using an antibody to the extracellular N-terminus followed by fluorescence-activated-cell scanning. The antibody to the N-terminus recognizes an epitope that is partially removed by trypsin activation; thus, for this study and all internalization assays, a non-proteolytic agonist, 2fAP, was used. The kinetics of PAR-2 internalization appeared to be the same, in response to trypsin, as assessed by confocal microscopy (data not shown). Cells were treated with 2fAP, for 0-60 minutes and internalization was determined by loss of surface fluorescence (Fig. 4-4). In MEF WT, a 55 ± 5% loss of surface fluorescence was observed as early as 5 minutes after agonist addition and was maintained for 60 minutes. In MEF DKO, no significant internalization was observed. Interestingly, in DKO + βarr1 cells, a biphasic pattern of trafficking was observed. PAR-2 was rapidly internalized (by 58 ± 10% of control) within 5 minutes of receptor activation but by 15-30 minutes, the surface fluorescence values returned to baseline. By 60 minutes, surface fluorescence again decreased by 20 ± 5%. In DKO + βarr 2 cells internalization occurred more slowly with a significant decrease in surface fluorescence (60 ± 2%) observed by 30-60 minutes.

The differential kinetics of PAR-2 internalization in MEF's was confirmed by confocal microscopy (Fig. 4-5, 4-6). PAR-2 GFP was transfected in each of the four MEF cell lines and its trafficking and colocalization with recycling endosomal marker (Transferrin, Fig. 4-5A), early endosomal marker (EEA1, Fig. 4-5B), and lysosomal marker (LAMP1, Fig. 4-6) was monitored. As has been reported by others, PAR-2 GFP colocalized with EEA1 and transferrin within 5 minutes of activation in MEF WT and with LAMP1 after 60 minutes. Consistent with flow cytometry data, PAR-2 GFP did not colocalize with EEA1 until 30 minutes of activation in DKO + βarr 2 cells; whereas in DKO + βarr 1 cells, this colocalization was observed within 2 minutes. Colocalization of PAR-2 GFP with LAMP1 was observed in MEF WT and DKO + βarr 1, but not βarr DKO or DKO + βarr2 cells (Fig. 4-6A). Colocalization of endogenous PAR-2 with beta-arrestin 1 and LAMP 1 was also observed in MDA MB 468 cells after prolonged agonist exposure, while beta-arrestin 2 colocalized with PAR-2 but not with LAMP1 (Fig. 4-6B). Furthermore, while beta-arrestins translocated to the membrane and colocalized with PAR-2 GFP in MEF's within 2-5 minutes of PAR-2 activation, very little beta-arrestin 1 remained colocalized after 15 minutes (Fig. 4-7). In contrast, the majority of PAR-2/β-arrestin-2 colocalization was observed after 30-60 minutes of receptor activation (Fig. 4-7). These data suggest that beta-arrestin 1 may mediate a lysosomal degradation of PAR-2; whereas beta-arrestin 2 might mediate other post-internalization fates.

Role of β-arrestins in exocytosis of PAR-2: A considerable portion of PAR-2 is stored in the Golgi following its translation, and agonist induced trafficking of PAR-2 has been

reported. This trafficking event was sensitive to Brefeldin A (BFA) but not Cycloheximide, suggesting Golgi stores of PAR-2 are mobilized to the surface upon PAR-2 activation (15). Internalization assays revealed that some surface expression of PAR-2 returned after 30 minutes of agonist treatment in MEF WT and DKO + βarr 1; whereas, in DKO + βarr 2, the receptor appeared to remain internalized. To determine whether beta-arrestins were involved in the exocytosis of PAR-2, we examined agonist-induced trafficking of PAR-2 GFP after exposure to the Golgi disrupting agent Brefeldin A (BFA). The appearance of PAR-2 GFP at the surface, after 30 minutes of 2fAP treatment, was abolished by BFA treatment in MEF DKO + βarr 1 cells (Fig. 4-8).

Differential regulation of signaling molecules downstream by β-arrestins 1 & 2: PAR-2 has been shown to signal through beta-arrestins, leading to activation of some proteins (ERK 1/2) and inhibition of others (PI3K) (13, 18, 43). Studies from other labs suggest that GPCR's can activate ERK 1/2 by both G-protein-dependant and independent pathways, the latter being beta-arrestin-dependant (37, 38, 44). Since the beta-arrestins differ temporally in their regulation of PAR-2 internalization and previous studies suggest both beta-arrestins are necessary for full ERK 1/2 activation, we investigated the specific contribution of each using the four MEF cell lines. In MEF WT cells, PAR-2 activation resulted in a biphasic ERK 1/2 phosphorylation, with an early peak at 5 minutes and a later peak at 30-60 minutes (Fig. 4-9A). In DKO + βarr 1 cells only the early increase at 5 minutes was observed, while in DKO + βarr 2 cells, we observed a slow rise in ERK 1/2 phosphorylation at 5-60 minutes (Fig. 4-9C, D). In MEF DKO cells

early ERK 1/2 phosphorylation was reduced by 50% and prolonged ERK 1/2 phosphorylation at 30-60 minutes was abolished (Fig. 4-9B). PAR-2 has been promoted to promote both membrane retention and nuclear translocation of activated ERK 1/2, the former being associated with chemotaxis and the latter with proliferation (5, 9, 13, 17, 18, 29, 35). In NIH3T3 cells, beta-arrestin 1 appeared to prolong ERK 1/2 activation for up to 90 minutes in isolated pseudopodia (17). Therefore, we examined whether beta-arrestins 1 and 2 differentially regulate membrane associated ERK 1/2. Subcellular fractionation of MEF's revealed that PAR-2 promotes a transient nuclear translocation of ERK 1/2, which is prolonged in the absence of both beta-arrestins (Fig. 4-10A, B). In contrast, membrane associated phospho-ERK 1/2 is abolished in the absence of beta-arrestins (Fig. 4-10B). Expression of either beta-arrestin 1 or beta-arrestin 2 alone abolished and prolonged nuclear ERK 1/2 and resulted in appearance of membrane associated phospho-ERK 1/2. Furthermore, expression of beta-arrestin 2 appeared to specifically prolong membrane associated ERK 1/2 activation (Fig. 4-10C, D).

4.5 DISCUSSION

Beta-arrestins are known to play important roles in a variety of cellular processes, from desensitization of membrane receptors and facilitation of signal transduction pathways. In the case of PAR-2, studies have suggested that both beta-arrestins are required for efficient desensitization, internalization and ERK 1/2 activation (17, 18, 38), which suggested that they might have overlapping as well as distinct roles in these processes. Here we demonstrate that beta-arrestin 1 and 2 differ temporally in their regulation of PAR-2 desensitization, internalization and ERK 1/2 activation, suggesting it is their coordinated functions that are essential for PAR-2 signaling.

Role of β-arrestins in G-protein uncoupling and PKC-mediated desensitization: The first event in receptor desensitization is the termination of the initial signal, usually accomplished through uncoupling of the receptor from its cognate G-protein (10, 33). Receptor G-protein uncoupling results in two phenomena: 1) The initial signaling event is terminated, and 2) the receptor becomes refractory to second agonist stimulation. In these studies $G_{\alpha}q$ coupling is measured by release if intracellular Ca^{+2} in response to the PAR-2 agonists, trypsin and 2fAP. In MEF's from β-arrestin $^{-/-}$ mice, the duration of Ca^{+2} release is nearly tripled, consistent with ineffective uncoupling of PAR-2 from $G_{\alpha}q$. Expression of beta-arrestin 1, but not beta-arrestin 2 effectively restores signal termination, suggesting that beta-arrestin 1 mediates this initial uncoupling event. In many cases, desensitization is initiated upon phosphorylation of receptors by G-Protein-Receptor Kinases (GRK's) and subsequent binding of beta-arrestins. GRK's are activated

in response to the same receptors that utilize them, reflecting a negative feedback loop designed to prevent constitutive receptor signaling. Although PAR-2 utilizes beta-arrestins for internalization (15), no role for GRK's in PAR-2 signaling has been demonstrated yet. In contrast, studies have revealed that activation of PKC promotes receptor desensitization and internalization, and mutation of putative PKC phosphorylation sites impairs these same processes (7, 13). Whether PKC itself or another downstream kinase directly phosphorylates PAR-2 remains unknown, but here we demonstrate that beta-arrestins are required for PKC-mediated desensitization. Pretreatment with PMA did not block PAR-2-promoted mobilization of internal Ca^{+2} in MEF's from β-arrestin $^{-/-}$ mice or in wild type cells after treatment with a PKC inhibitor. After expression of either beta-arrestin-1 or 2 in the MEF βarr DKO cells, PMA once again inhibited PAR-2 mediated Ca^{+2} mobilization, suggesting their functions are redundant at this step in PAR-2 desensitization. Surprisingly, inhibition of PKC did not block homologous desensitization (unpublished observations), suggesting that other kinases (perhaps GRK) mediate agonist-induced G-protein uncoupling. Thus heterologous desensitization by PKC may reflect a contribution of other cellular pathways in the regulation of PAR-2 activation.

Role of β-arrestins in PAR-2 trafficking: In MEF βarr DKO cells, agonist-induced PAR-2 internalization was virtually abolished. Interestingly, expression of beta-arrestin 1 alone restored early, while expression of beta-arrestin 2 alone restored prolonged receptor internalization. Taken together with the apparent ability of beta-arrestin 1 to mediate

rapid termination of PAR-2 induced Ca^{+2} mobilization, one might presume a temporal difference in the recruitment of each beta-arrestin to the membrane. Such a distinction would have to occur within seconds after receptor activation, as both beta-arrestin 1 and 2 colocalized with PAR-2 within 5 minutes of its activation. Whether both beta-arrestins can simultaneously interact with PAR-2 or whether each regulates a distinct receptor pool remains to be determined. Colocalization with early endosomal markers was observed with expression of either beta-arrestin-1 or 2, but targeting to lysosomes, as evidenced by colocalization of PAR-2 with LAMP-1, was only rescued by expression of beta-arrestin-1. Consistent with this finding, in cells expressing high levels of endogenous PAR-2, LAMP-1 colocalized with beta-arrestin-1 after prolonged agonist exposure, but not with beta-arrestin-2, despite the fact that PAR-2 colocalized with both. Whether this result reflects a distinct fate of beta-arrestin-2 bound receptor remains to be determined. Reports by others of beta-arrestin-2 ubiquitination and ubiquitin-mediated degradation of PAR-2 raise the possibility that beta-arrestin-2 might mediate ubiquitin-directed PAR-2 degradation, while beta-arrestin-1 mediates lysosomal degradation (19, 36, 41). Another possibility is that beta-arrestin-2 associated receptors are involved in prolonged ERK 1/2 activation (discussed below).

Another surprising observation was the fact that restoration of surface PAR-2 was enhanced by expression of beta-arrestin-1 alone, and this was sensitive to the Golgi disrupting drug, Brefeldin A. One observation for this observation is that beta-arrestin-1 not only mediates early endocytosis and degradation of PAR-2, but also facilitates trafficking of receptors from Golgi stores to the membrane in response to agonist

stimulation. There is precedent for such a hypothesis, as beta-arrestin-1 facilitated anterograde trafficking was reported for Ral-GDS (6) and PAR-2 has previously been shown to exhibit BFA-sensitive agonist-induced exocytosis (15, 34). Alternatively, beta-arrestin-1 might mediate rapid endocytosis and degradation of PAR-2 which is replaced by constitutive exocytosis.

Role of β-arrestins in ERK 1/2 activation: It has previously been shown that PAR-2-evoked ERK 1/2 activation requires both beta-arrestins, although there may be a temporal difference in their involvement (13, 18, 38). Here we provide evidence that PAR-2 evoked ERK 1/2 activation involves at least three separate pathways: an early beta-arrestin-1 dependant phase (2-5 minutes), a prolonged beta-arrestin-2 dependant phase and a third beta-arrestin-independent component. The temporal involvement of each beta-arrestins is consistent with their apparent involvement in receptor internalization; moreover, the two beta-arrestins do not appear to require each other for regulation of ERK 1/2 activity. In previous studies, a mutant receptor lacking two putative C-terminal PKC phosphorylation sites was able to exert prolonged Ca^{+2} mobilization leading to activation of Src-family kinases, nuclear ERK 1/2 activation and proliferation; whereas the wild type receptor primarily utilized the beta-arrestin-dependant pathway resulting in cytosolic sequestration of activated ERK 1/2. In these studies, PAR-2 evoked nuclear translocation of active ERK 1/2 appeared to be unaffected in MEF βarr DKO; expression of beta-arrestin-1 or 2 increased membrane-associated ERK 1/2. These data further

suggest that the two beta-arrestins may regulate distinct pools of activated ERK 1/2, just as they appear to regulate distinct pools of receptor.

There are a growing number of receptors that appear to exert beta-arrestin-dependant, G-protein-independent ERK 1/2 activation. In some cases, beta-arrestins stably associate with ERK 1/2 (e.g. downstream of PAR-2) leading to its sequestration away from the nucleus, whereas in other cases, they facilitate its nuclear transport (12, 13, 39, 40). In the case of PAR-2, there is evidence to suggest that residues in the receptor C-terminus may define the duration of ERK 1/2 association with beta-arrestin, the specificity of beta-arrestin association and the mechanism of activation (13, 35, 38). Tissue and cell-type specific differences in beta-arrestin levels, may then result in differences in Ca^{+2} signal duration and receptor internalization, as well as tip the scales towards nuclear or cytosolic/membrane ERK 1/2, ultimately resulting in distinct cellular outcomes of PAR-2 activation.

4.6 FIGURES AND LEGENDS

Fig. 4-1: Dose response of Ca^{+2} mobilization to trypsin and 2f-AP. MEF WT**,** MEF βarr-DKO (both transfected with PAR-2-GFP) and MDA-MB 468 cells were loaded with Fura2-AM, treated with increasing concentrations of trypsin (**A**) or 2fAP (**B**), and the ratio of fluorescence at 340 / 380 nm was determined. Maximum concentrations of intracellular Ca^{+2} for each concentration were calculated according to the Grynkiewicz equation and plotted as a function of Log [agonist].

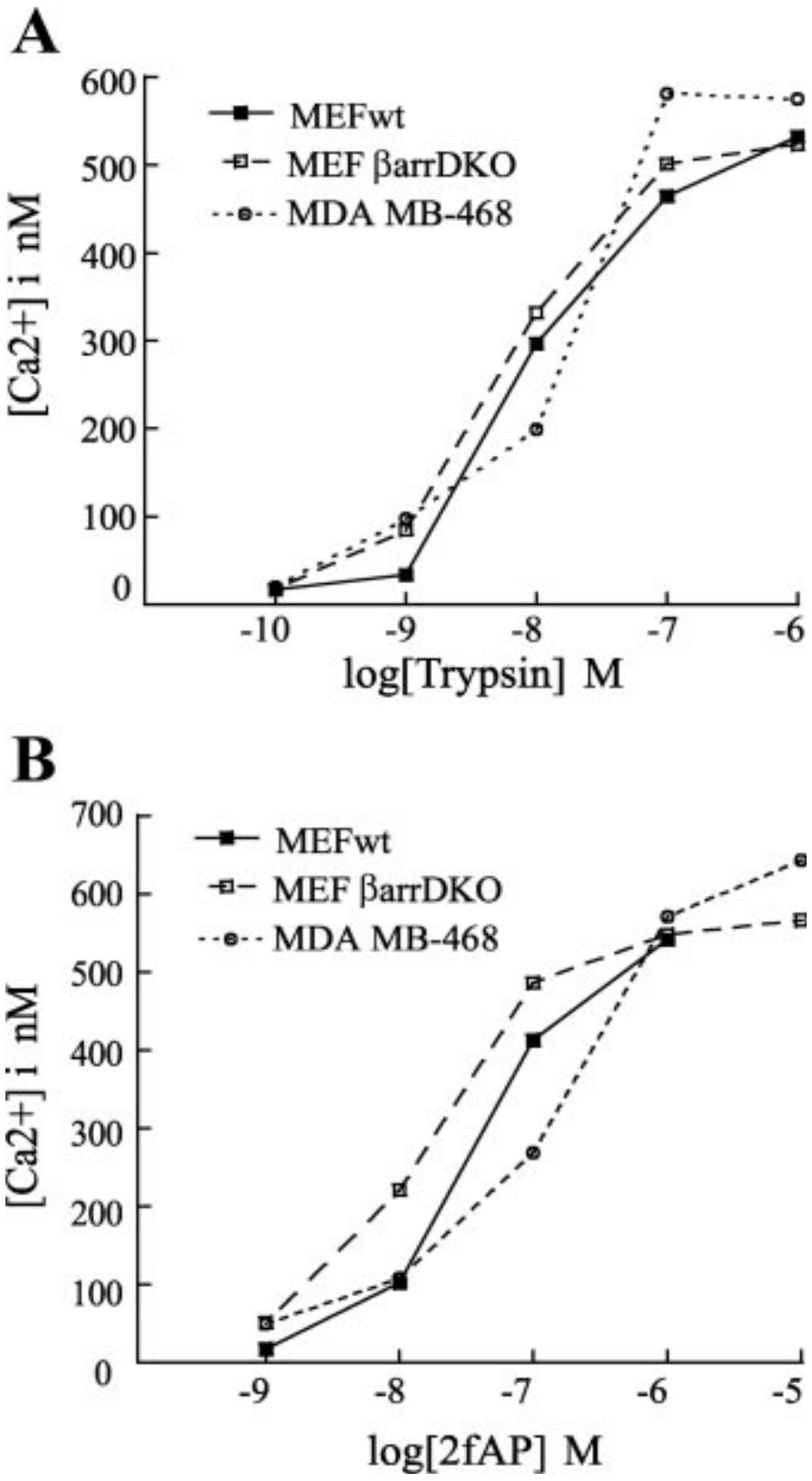

A
600
500
400
300
200
100
0
[Ca2+] i nM
MEFwt
MEF βarrDKO
MDA MB-468
-10
-9
-8
-7
-6
log[Trypsin] M
B
700
600
500
400
300
200
100
0
[Ca2+] i nM
MEFwt
MEF βarrDKO
MDA MB-468
-9
-8
-7
-6
-5
log[2fAP] M

Fig. 4-2: β-arrestins mediate PKC-induced PAR-2 desensitization and determine the duration of Ca^{+2} signaling. A-H: Representative Ca^{+2} mobilization traces from Fura2/AM loaded MEF WT (A, B), MEF β-arr DKO (C, D), DKO + β-arr1 (E, F) and DKO + β-arr2 (G, H). Cells were pretreated with either vehicle (A, C, E, G) or 1 μM PMA (B, D, F, H) before addition of 50 nM trypsin, followed by addition of a Ca^{+2} ionophore (ionomycin). **I:** Western Blot showing β-arrestin levels in MEF WT, MEF βarr DKO, DKO + βarr1 and DKO + β-arr2. **J:** Average duration of the PAR-2 evoked Ca^{+2} signal I each MEF cell line was determined from 4 independent experiments and presented as mean ± SE, $p<0.01$. **K:** Average maximal PAR-2 evoked Ca^{+2} responses in each MEF cell line, with and without PMA treatment, were calculated from 3 independent experiments and presented as mean ± SE, $p<0.05$. Statistical significance was determined by ANOVA analysis of PAR-2 responses and Tukey t-tests to compare responses between groups.

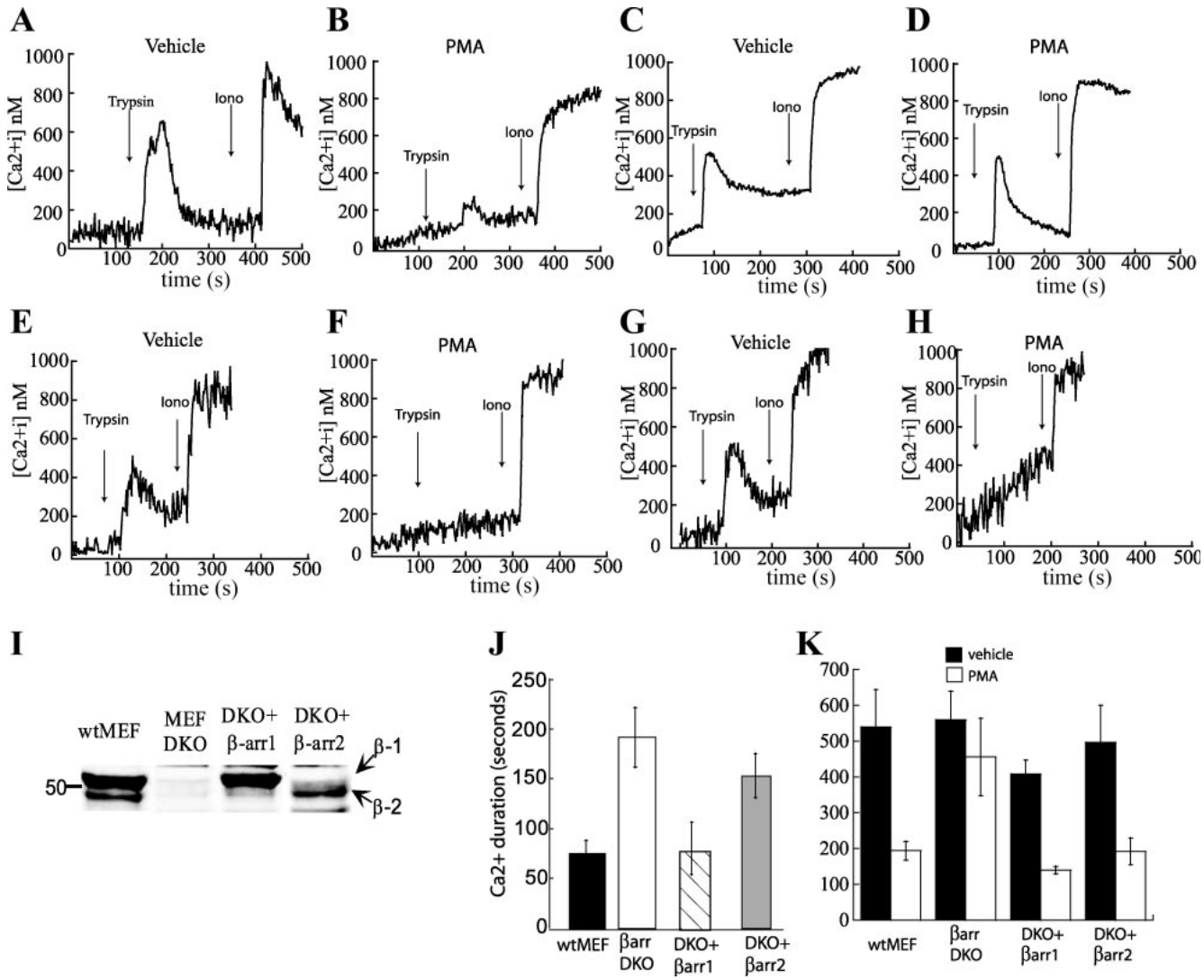

A
Vehicle
B
PMA
C
Vehicle
D
PMA
E
Vehicle
F
PMA
G
Vehicle
H
PMA
[Ca2+i] nM
time (s)
Trypsin
Iono
I
wtMEF
MEF DKO
DKO+ β-arr1
DKO+ β-arr2
β-1
β-2
50
J
Ca2+ duration (seconds)
wtMEF
βarr DKO
DKO+ βarr1
DKO+ βarr2
K
vehicle
PMA

Fig. 4-3: PMA induced desensitization is rescued by treatment with a broad spectrum PKC inhibitor (GFX). MEF WT (**A-D**) or MDA MB 468 cells (**E-H**) were treated with vehicle (A, E), 300 nM GFX alone (B, F), 1 μM PMA alone (C, G) or PMA + GFX (D, H) and PAR-2 induced Ca^{+2} mobilization was determined in response to 50 nM trypsin as described in Figures 1 and 2.

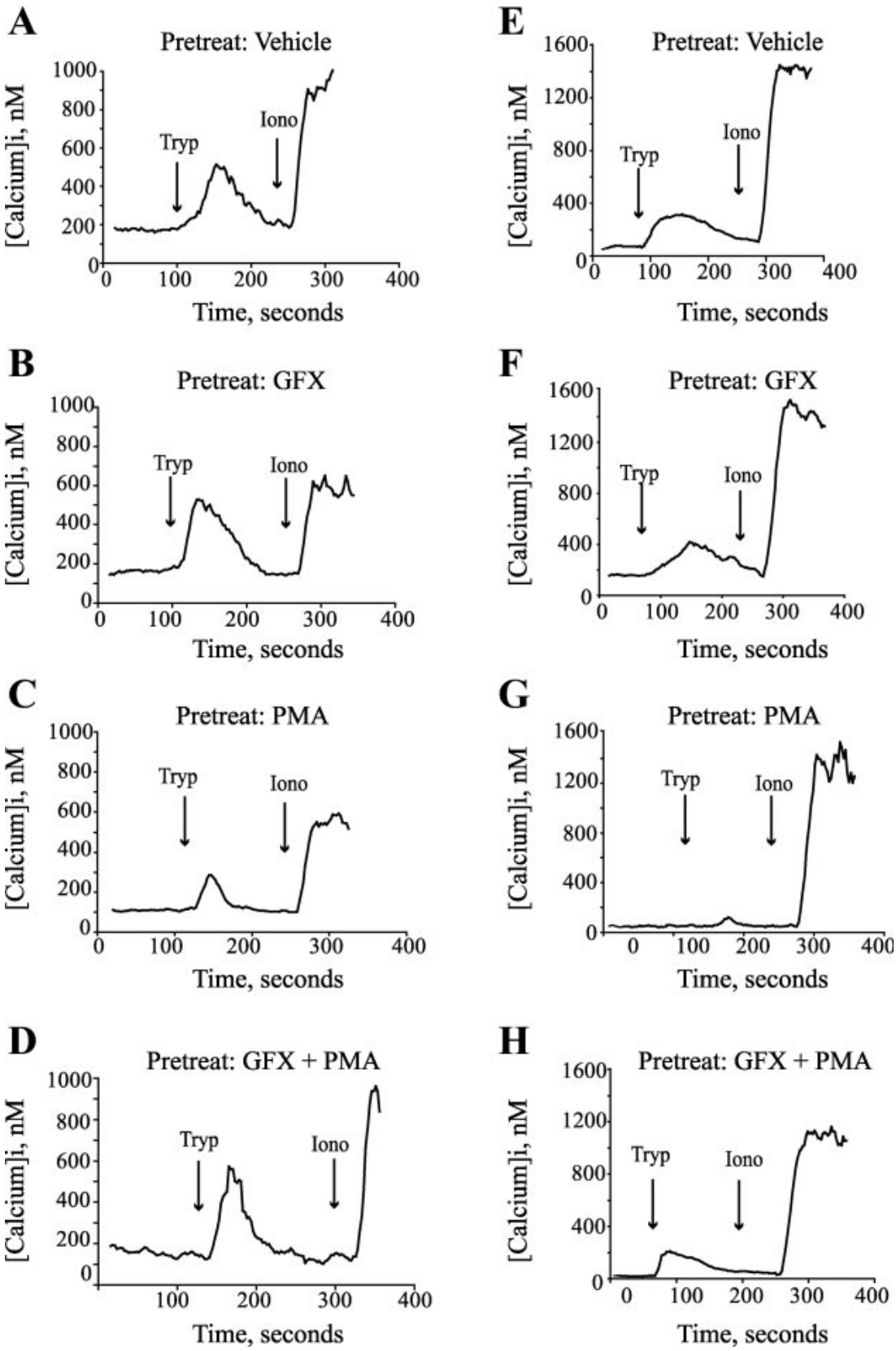

A
Pretreat: Vehicle
[Calcium]i, nM
Tryp
Iono
Time, seconds
B
Pretreat: GFX
C
Pretreat: PMA
D
Pretreat: GFX + PMA
E
Pretreat: Vehicle
F
Pretreat: GFX
G
Pretreat: PMA
H
Pretreat: GFX + PMA

Fig. 4-4: β-arrestin-1 mediates early and β-arrestin-2 mediates late PAR-2 internalization. MEF WT, MEF βarr DKO, MEF DKO + βarr1 and MEF DKO + βarr2 cells were treated with PAR-2 agonist (100nM 2fAP) for 0-60 minutes and endogenous cell surface receptor levels were determined by immunoreactivity to an antibody directed against the extracellular N-terminus using flow cytometry. Decreases in surface fluorescence relative to baseline reflect receptor internalization.

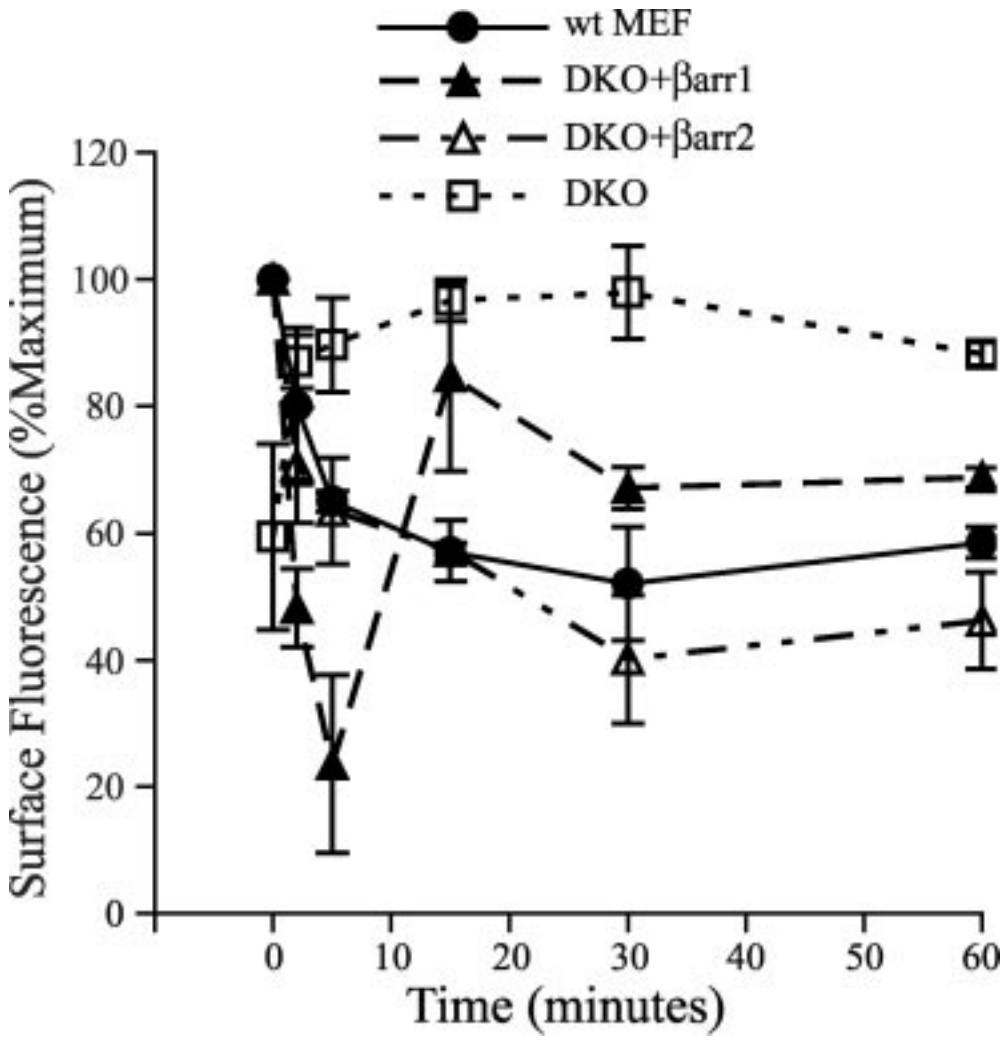

wt MEF
DKO+βarr1
DKO+βarr2
DKO
Surface Fluorescence (%Maximum)
120
100
80
60
40
20
0
0
10
20
30
40
50
60
Time (minutes)

Fig. 4-5: Localization of PAR-2 with endosomal markers. A: MEF WT, MEF βarr DKO, MEF DKO + βarr1 and MEF DKO + βarr2, transiently transfected with PAR-2, were bound with Cy5-Transferrin (TF, a marker for constitutive endocytosis) on ice, then warmed to 37^0C in the presence or absence of PAR-2 agonist (100 nM 2fAP) for 0-30 minutes. **B:** MEF's, transiently transfected with PAR-2 GFP were treated with 100 nM 2fAP for 2, 5 and 30 minutes, fixed and co-stained with a marker of early endosomes (EEA1). Arrows indicate internalized receptor colocalized with either TF or EEA1. Insets to left of each composite are 6X zooms of boxed regions.

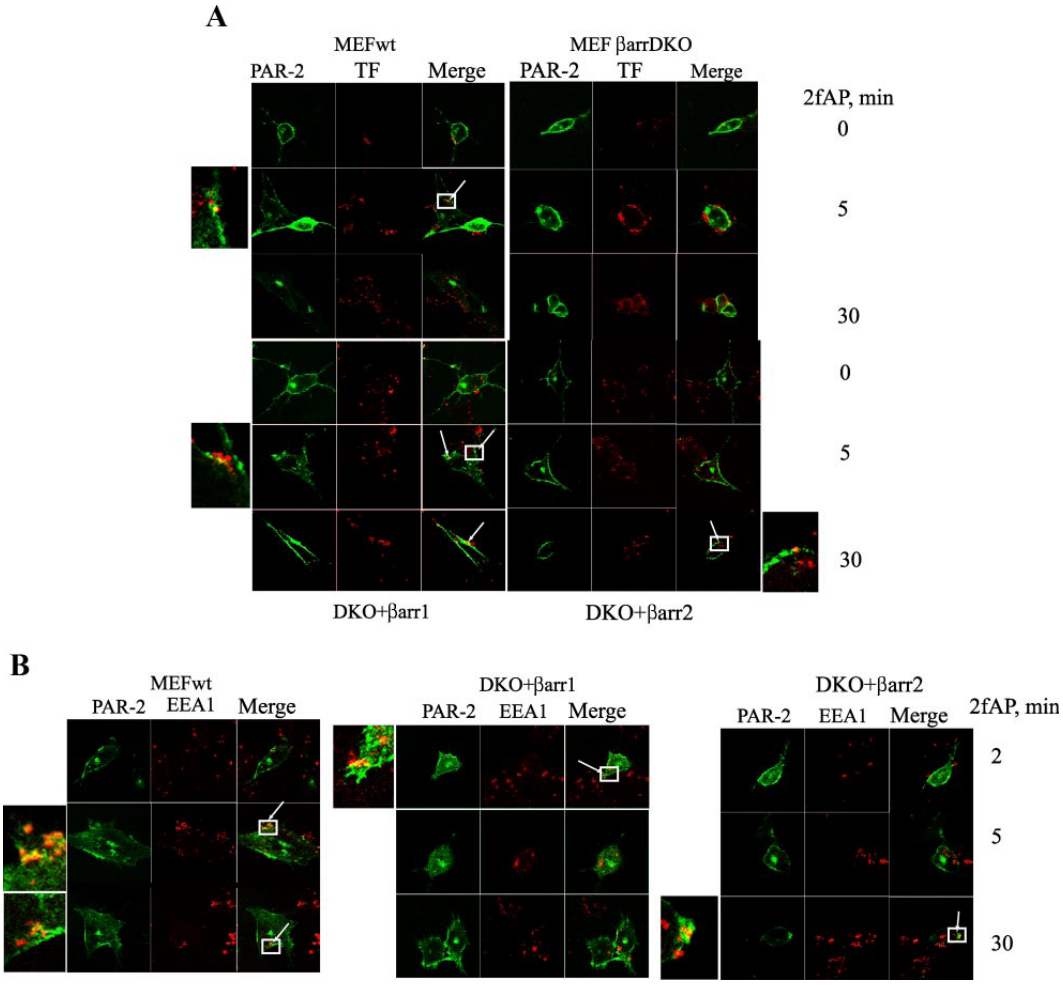
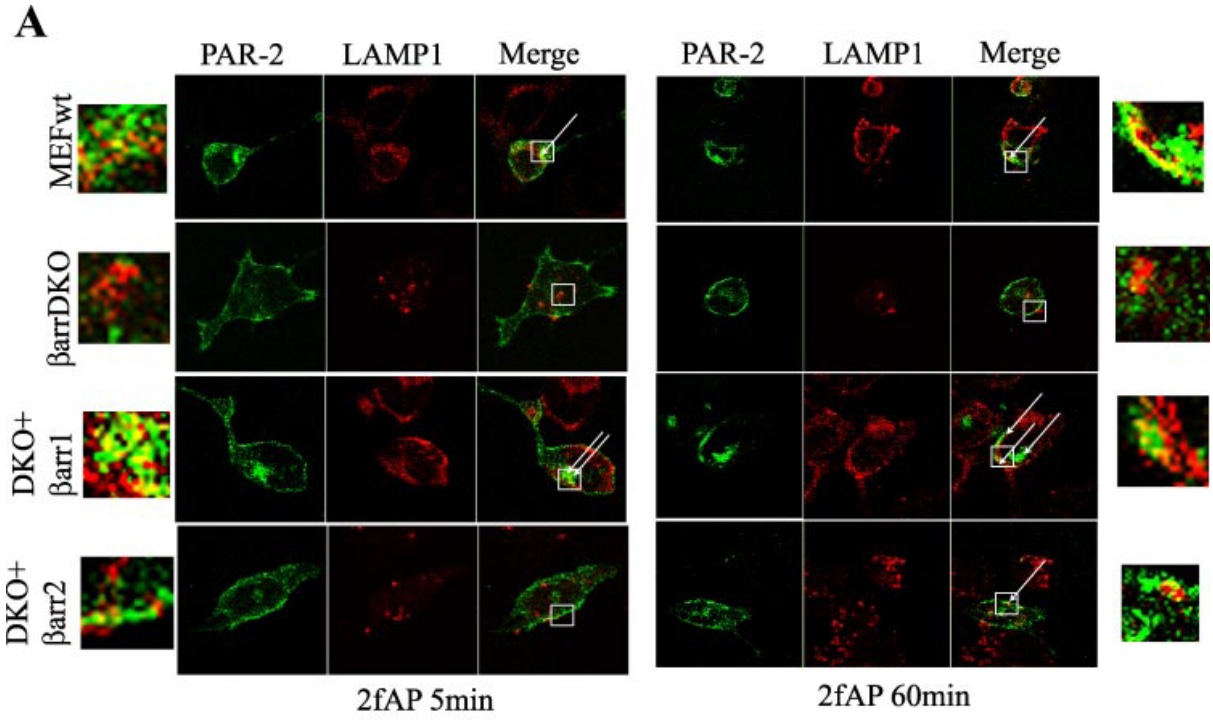

A
MEFwt
PAR-2
TF
Merge
MEF βarrDKO
PAR-2
TF
Merge
2fAP, min
0
5
30
0
5
30
DKO+βarr1
DKO+βarr2
B
MEFwt
PAR-2
EEA1
Merge
DKO+βarr1
PAR-2
EEA1
Merge
DKO+βarr2
PAR-2
EEA1
Merge
2fAP, min
2
5
30

Fig. 4-6: Localization of PAR-2 with lysosomes. A: MEF WT, MEF βarr DKO, MEF DKO + βarr1 and MEF DKO + βarr2, transiently transfected with PAR-2 GFP, were treated with 100 nM 2fAP for 0-60 minutes, fixed and stained with a marker for degradative lysosomes (LAMP-1). Arrows indicate colocalized vesicles. Insets shown to the left and right of composites represent 5X zooms. **B:** MDA MB 468 cells (which express high levels of endogenous PAR-2), transiently transfected with β-arrestin-1 and 2-GFP were treated with 100 nM 2fAP for 2 hours and colocalization of PAR-2 and LAMP-1 with β-arrestin-1 (upper panel) or β-arrestin-2 (lower panel) was visualized by confocal microscopy.


A
PAR-2
LAMP1
Merge
PAR-2
LAMP1
Merge
MEFwt
βarrDKO
DKO+ βarr1
DKO+ βarr2
2fAP 5min
2fAP 60min

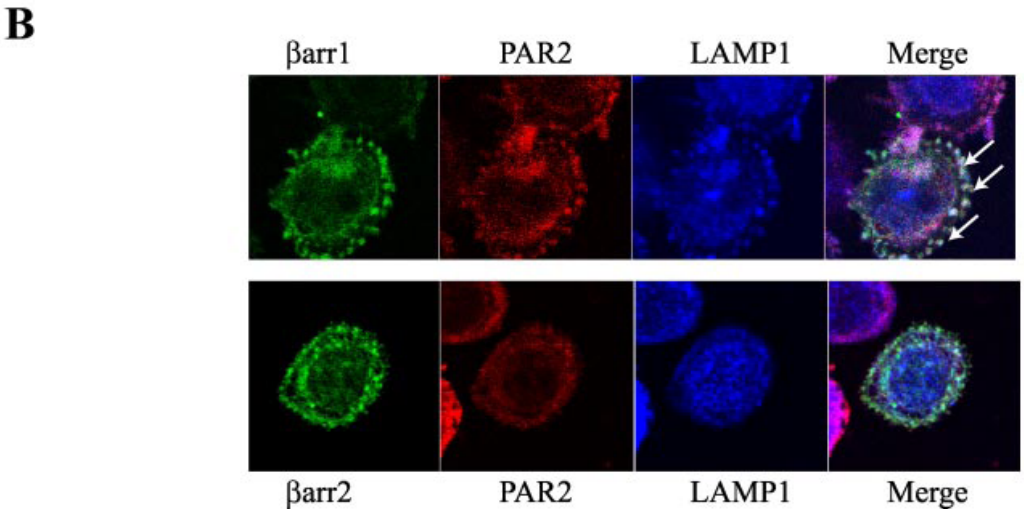

B
βarr1
PAR2
LAMP1
Merge
βarr2
PAR2
LAMP1
Merge

Fig. 4-7: Time course of β-arrestin translocation and colocalization with PAR-2. CHO-PAR2 cells (stably expressing PAR-2-GFP) were transiently transfected with either FLAG-β-arrestin1 (**A**) or FLAG-β-arrestin2 (**B**) and treated with 100 nM 2fAP for 0-60 minutes. Note that both β-arrestins colocalize with PAR-2 within 5 minutes, but β-arrestin2 remains colocalized longer. Arrows indicate colocalization.

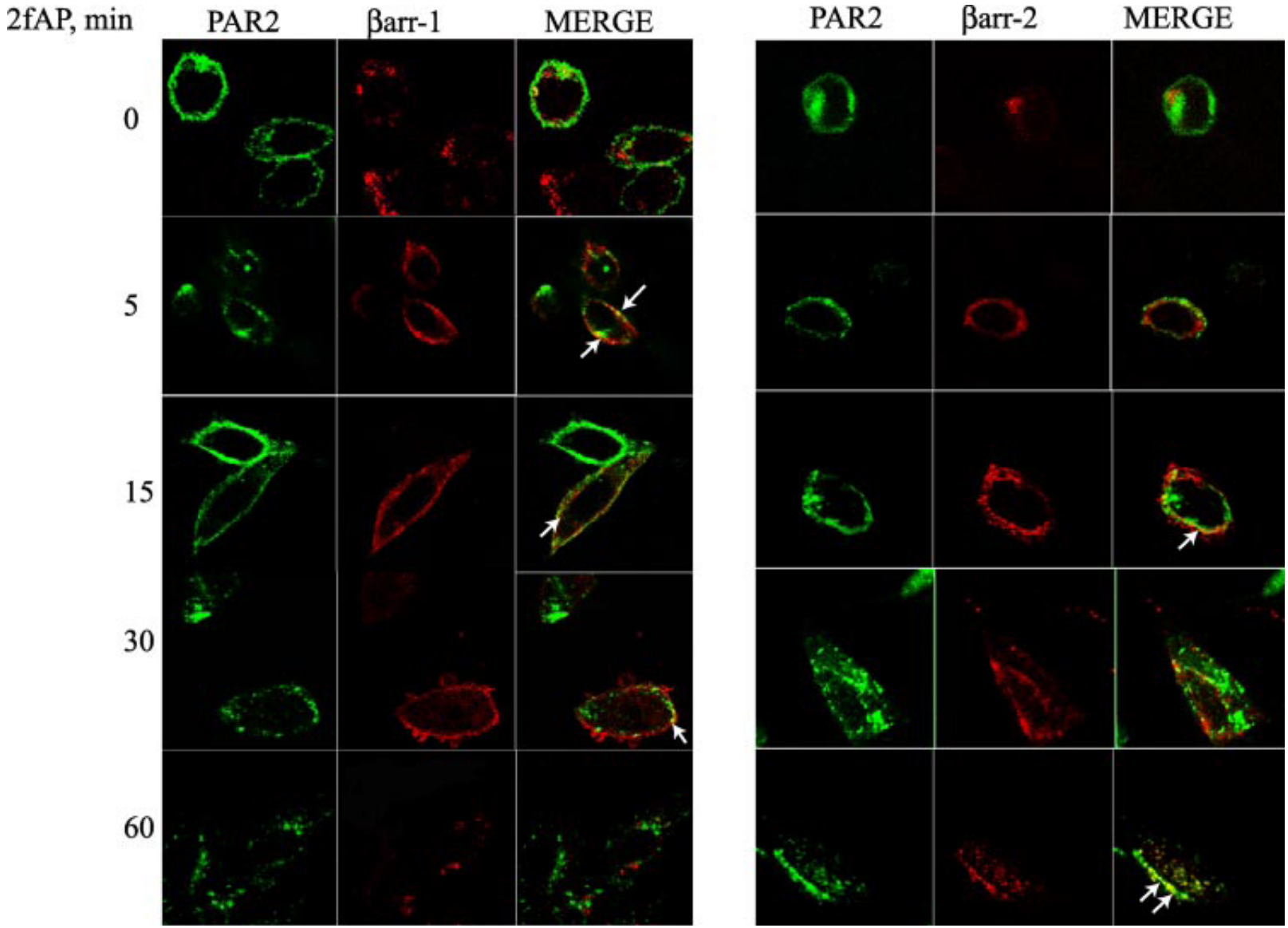

2fAP, min
PAR2
βarr-1
MERGE
PAR2
βarr-2
MERGE
0
5
15
30
60

Fig. 4-8: Disruption of Golgi apparatus with Brefeldin A blocks PAR-2 reappearance at plasma membrane. MEF WT (**A**) and MEF DKO + βarr1 (**B**) were treated with 100 nM 2fAP for 0-30 minutes in the presence and absence of 3 μg/ml Brefeldin A (BFA). Note that the reappearance of PAR-2 at the plasma membrane in DKO + βarr1 cells is blocked by BFA treatment.

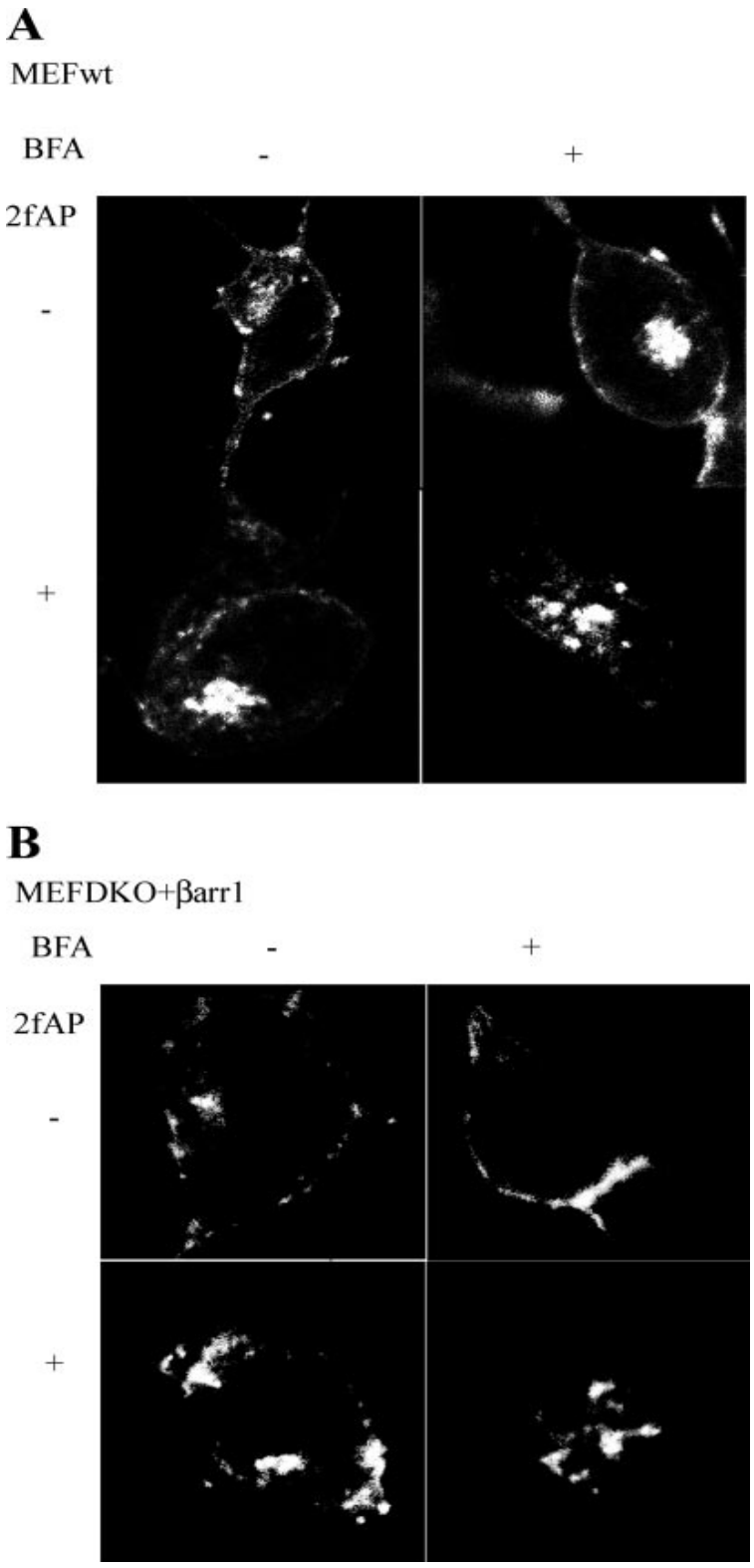

A
MEFwt
BFA
-
+
2fAP
-
+
B
MEFDKO+βarr1
BFA
-
+
2fAP
-
+

Fig. 4-9: β-arrestin-1 mediates early and β-arrestin-2 mediates late ERK 1/2 activation. MEF WT (**A**), MEF βarr DKO (**B**), DKO + βarr1 (**C**) and DKO + βarr2 (**D**) cells were treated with 50 nM trypsin for 0-60 minutes, or 20% serum (ser) for 5 minutes as a positive control, and lysates were analyzed by western blot for phosphor (pERK) and total ERK 1/2 (tERK). **E:** Graph depicting normalized phosphor-ERK levels as a fraction of baseline; values shown represent mean ± SE from 4 independent experiments.

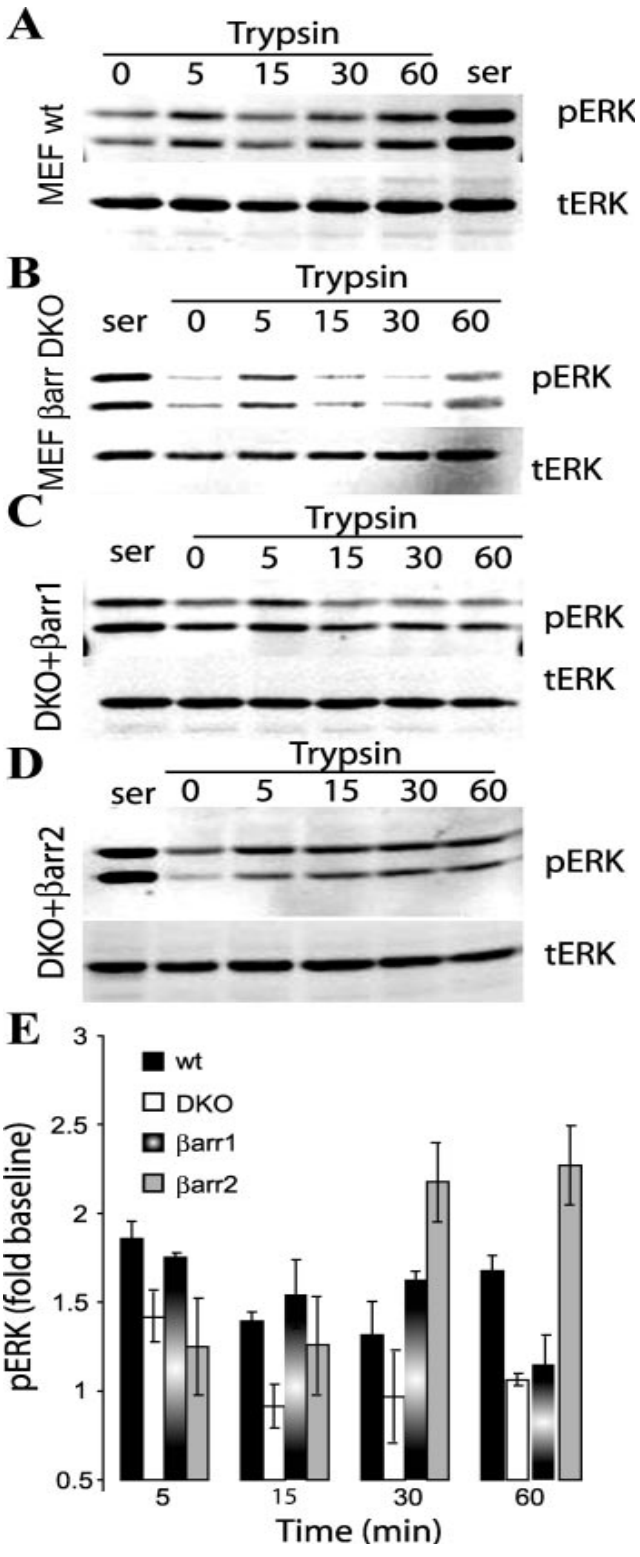
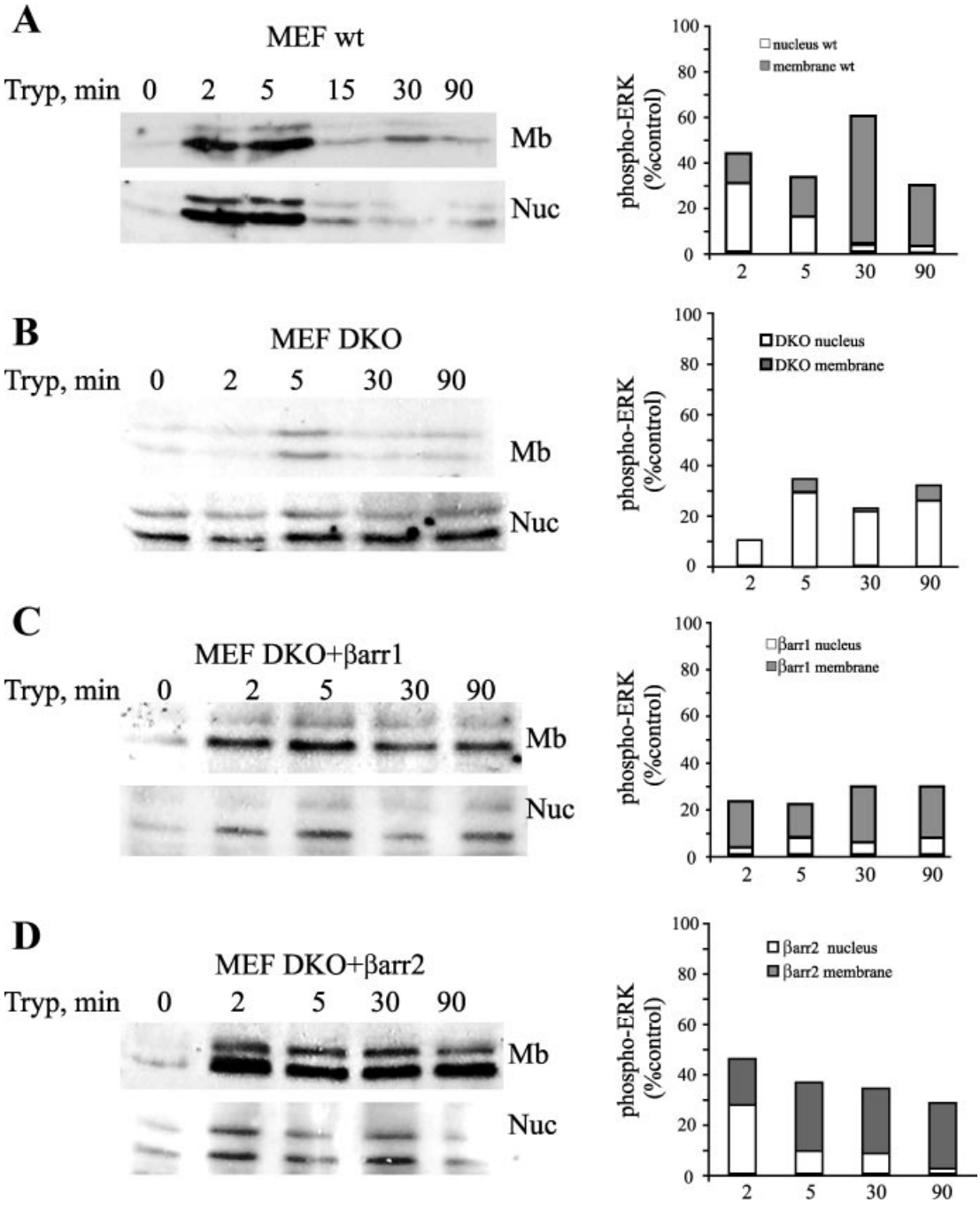

A
Trypsin
0 5 15 30 60 ser
MEF wt
pERK
tERK
B
Trypsin
ser 0 5 15 30 60
MEF βarr DKO
pERK
tERK
C
Trypsin
ser 0 5 15 30 60
DKO+βarr1
pERK
tERK
D
Trypsin
ser 0 5 15 30 60
DKO+βarr2
pERK
tERK
E
wt
DKO
βarr1
βarr2
pERK (fold baseline)
3
2.5
2
1.5
1
0.5
5
15
30
60
Time (min)

Fig. 4-10: Subcellular fractionation of pERK in MEF's. MEF WT (**A**), MEF βarr DKO (**B**), MEF DKO + βarr1 (**C**) and MEF DKO + βarr2 (**D**) cells were treated with 50 nM trypsin for 0-90 minutes after which cytosolic, nuclear and plasma membrane fractions were analyzed for phosphor-ERK by western blot. Shown are nuclear and plasma membrane fractions (left panels). Percentages of pERK in each fraction was calculated by densitometry and expressed as a bar graph to the right of each panel.


A
MEF wt
Tryp, min 0 2 5 15 30 90
Mb
Nuc
nucleus wt
membrane wt
phospho-ERK (%control)
100
80
60
40
20
0
2 5 30 90
B
MEF DKO
Tryp, min 0 2 5 30 90
Mb
Nuc
DKO nucleus
DKO membrane
phospho-ERK (%control)
100
80
60
40
20
0
2 5 30 90
C
MEF DKO+βarr1
Tryp, min 0 2 5 30 90
Mb
Nuc
βarr1 nucleus
βarr1 membrane
phospho-ERK (%control)
100
80
60
40
20
0
2 5 30 90
D
MEF DKO+βarr2
Tryp, min 0 2 5 30 90
Mb
Nuc
βarr2 nucleus
βarr2 membrane
phospho-ERK (%control)
100
80
60
40
20
0
2 5 30 90

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Chapter 5

Conclusions and Perspective

GPCR signaling has been extensively studied and has been a major target for therapeutic systemic diseases. Characterization of specific GPCR's by identifying signaling components, processes, and their cellular localization can be the key for utilization of these transmembrane proteins as the main or co-therapeutic agents. In our studies we focused on the protease activated receptor PAR-2. We characterized PAR-2 via cellular localization in the colonic epithelium and observed distinctions based on physiological response and signaling, both temporally and mechanistically. Moreover, we identified and confirmed PAR-2 acitvation stimulated bacterial uptake and identified the underlying mechanism behind the phenomenon. Lastly, we examined the differential effects between β-arrestin-1 and 2.

5.1 Apical and basolateral pools of protease-activated receptor-2 direct distinct signaling events in the intestinal epithelium

Our studies indicate that there are two pools of PAR-2 that are present in the gastrointestinal epithelial barrier, a pool exposed to the lumen and another to the bloodstream. These studies revealed that upon PAR-2 activation, both pools induces inflammatory responses such as cytokine upregulation, gastrointestinal barrier dysfunction and increase inflammatory damage. Our studies show that both pools, apical and basolateral PAR-2 are different in signaling, temporally and mechanistically and induces physiologically responses differently. Further, we identified that both pools of PAR-2 are accessible to 2fAP by creating a cell culture model which mimics the

gastrointestinal epithelium. We identified apical PAR-2 induced ERK 1/2 activation in a G-protein dependent, β-arrestins independent manner that induced early maximal activation. Basolateral PAR-2 on the other hand induced a late maximal ERK 1/2 activation that does not follow the classical Gq signaling and is β-arrestins dependent. We believe this may be in a distinction that may provide functional importance between the two pools of PAR-2. Apical PAR-2 is exposed to the lumen, to pathogenic microbes. Hence, since studies show pathogens release protease that may induce PAR-2 activation, rapid signaling is necessary for apical PAR-2 in order for the receptor to act as an "alarm" response. However, this response must not be prolonged since extensive inflammation may inflict self damage and weaken own protective mechanisms. Basolateral PAR-2's are known to induce inflammation upon release of tryptase via degranulating mast cells. Hence, these inflammatory effects triggered by basolateral PAR-2 must be gradual and precise; hence, false inflammatory responses are prevented.

We also illustrated that the activation of basolateral PAR-2 activation increased short circuit current via induction of chloride secretion (ΔI_{sc}) decreased transepithelial resistance (TER) and induced cofilin activation (important for tight junction reorganization) while activation of apical PAR-2 did not. This suggests basolateral PAR-2 actvation induces chloride secretion which induces movement of water and "flushing" which washes out pathogens which may have traverse the gastrointestinal epithelial barrier. Tight junction reorganization induced by basolateral PAR-2 activation of cofilin may contribute to gastrointestinal barrier dysfunction. These studies illustrating the distinction between the two receptor pools provides us with a better understanding of

PAR-2 in the gastrointestinal system and may allow for clarification of PAR-2 as therapeutic agent.

5.2 PAR-2 activation stimulates S. Aureus uptake in vitro and in vivo

We showed PAR-2 is localized apically and basolaterally and both are accessible to 2fAP in the colonic epithelial cells. Thus, we progressed to examine its role in bacterial infections in colonic epithelial cells. We observed an enhancement in bacterial uptake in CACO-2 cells upon 2fAP treatment. Further, we determined the process to be clathrin and PI3K dependent. Further, internalized bacteria appear to utilize internalization of proteolytically cleaved PAR-2 as a shuttle to gain entry into the cell. These findings indicate PAR-2 plays a role for either absorption of bacterial particles for antigen presentation purposes or a method for which bacterial particles gain entrance into the cell. Studies indicate gastrointestinal epithelial cells play a crucial role in antigen presentation with the aid of dendritic cells. Since the data suggest internalized bacterial particles enter the lysosome for degredation, PAR-2 activation may present a mechanism for which epithelial cells induce uptake of microbes to determine whether they are pathogenic or commensal. This prevents unwarranted inflammatory responses towards commensal microbes that could cause potential self harm. Further, if pathogenic antigens are found, since PAR-2's are shown to induce inflammatory responses, proper immune responses allow for preparation of defense against invading pathogens.

Another possibility is PAR-2 may be a target utilized by pathogens to gain entrance and traverse the gastrointestinal epithelial barrier. Numerous studies imply pathogens release proteases that are capable of activating PAR-2. Hence, inducing receptor internalization provides pathogens a viable means of entering a cell for replication, lyse, and consequent full blown attack while evading the immune system during preparation. Hence, PAR-2 antagonists may be a viable therapeutic agent against microbial attacks.

These findings mentioned above provide a step towards understanding and fundamentals for PAR-2 in signaling and physiological responses in the gastrointestinal system. Understanding the differences between apical and basolateral PAR-2 provides a fundamental understanding that may instigate other physiological responses, and consequent therapeutic options that may entail on the activation of one pool of the receptor. Further, findings that indicate PAR-2 activation stimulate bacterial uptake may provide an understanding towards the side effects that PAR-2 activation induces if PAR-2 becomes a therapeutic option in the gastrointestinal system. Overall, these studies have provided a better understanding of PAR-2 and may contribute to PAR-2 as an important therapeutic target in the future.