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Molecular mechanism of action of opioids in human neuroblastoma cells

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MOLECULAR MECHANISM OF ACTION OF OPIOIDS IN HUMAN NEUROBLASTOMA CELLS

by

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DISSERTATION

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Dedicated to S.H.,

and all those who have died

as a result of the cancer, neuroblastoma.

We should be careful to get out of an experience only the wisdom that is in it--and stop there; lest we be like the cat that sits down on a hot stove-lid. She will never sit down on a hot stove-lid again--and that is well; but also she will never sit down on a cold one anymore.

Mark Twain

ABSTRACT

A series of human neuroblastoma cell lines was screened for the presence of opioid receptor sites. Of these cell lines, SK-N-SH was found to express approximately 50,000 μ and 10,000 δ opioid receptor sites/cell. In vitro characterization revealed that the binding properties of these receptor sites closely resembled those of human and rodent brain.

Phosphatidylinositol turnover as a potential second messenger system for the μ receptor was examined in SK-N-SH cells. Carbachol was capable of stimulating a rapid and prolonged accumulation of inositol phosphate metabolites. However, neither opiates nor opioid peptides elicited a phosphatidylinositol turnover response in these cells.

Neurotransmitter receptor systems were determined in the three subclones of SK-N-SH cells. The neurotransmitter receptor systems in SH-SY5Y (neuroblast-like) and SH-IN (intermediate) clones were found to be similar to the parent cells. In contrast, SH-EP (epithelial-like) clones did not express any of the receptor systems measured.

Cells of the SH-SY5Y line, a phenotypically stable subclone of SK-N-SH cells, were induced to differentiate by treatment with various inducing agents, and changes of several neurotransmitter receptor systems were determined. Nerve growth factor (NGF) and retinoic acid (RA) up-regulated, while dBcAMP down-regulated opioid receptor sites. [^{3}H]Dopamine uptake was slightly enhanced only in RA-treated cells. Strikingly, the efficacy of PGE_1-stimulated accumulation of cAMP was enhanced by 15- to 30-fold upon RA treatment.

Efficacy, potency and tolerance of narcotic drugs were studied in RA-differentiated SH-SY5Y cells. Efficacy of opiates and opioid peptides in inhibiting PGE_1- or forskolin-stimulated cAMP accumulation was greatly enhanced after RA treatment. Potency and efficacy profiles of a series of agonists strongly suggest that the inhibition was predominantly mediated by μ receptors. Narcotics that are known to be partial agonist analgesics also gave partial response in this assay system. Furthermore, chronic exposure to morphine resulted in a right shift of the morphine dose-response curve in inhibiting forskolin-stimulated cAMP accumulation in these cells.

In summary, this dissertation establishes conditions for the study of molecular function of the μ opioid receptor in SH-SY5Y cells.

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INTRODUCTION

A. OVERALL OBJECTIVE

The overall objectives of this dissertation are to: identify suitable human neuroblastoma cell lines that express opioid receptor sites relevant to the understanding of the molecular mechanisms of opioid action; determine the neurotransmitter receptor systems in three morphologically distinct subclones of SK-N-SH cells; compare the effects of differentiation on the neurotransmitter receptor systems in SH-SY5Y cells by different inducing agents; and determine the second messenger system coupled to μ opioid receptors in SH-SY5Y cells.

B. BACKGROUND

1. Opioid Receptor System

Demonstration and Properties of Opioid Receptors

The first direct demonstration of opioid receptor binding sites in nervous tissues was simultaneously reported in 1973 by three independent laboratories (Pert and Snyder, 1973; Simon *et al.*, 1973; Terenius, 1973). The binding was stereospecific, saturable and specific for narcotic analgesics and antagonists. That the binding site was indeed the receptor responsible for mediating narcotic analgesic effect was further supported by the observation that the rank order affinities of a

series of opiates correlated well with their relative analgesic potency (Stahl et al., 1977).

Opioid receptor binding was reduced by proteolytic enzymes and protein-modifying reagents interacting with the sulfhydryl groups (Simon et al., 1973; Pasternak and Snyder, 1974; Simon and Groth, 1975). Sodium ions and guanine nucleotide (GTP) were found to have different effects on agonist and antagonist binding. Agonist binding was reduced, while antagonist binding was relatively unaffected by sodium or guanine nucleotides (Blum, 1978b). The degree of depression of binding among groups of agonists varied greatly, the ketazocine-like agonists being affected much less than the morphine-like compounds (Kosterlitz and Leslie, 1978).

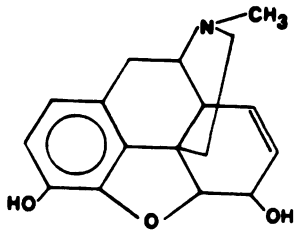
Multiple Opioid Receptors

The concept of multiple opioid receptors was first proposed by Portoghese (1965). On the basis of pharmacological profiles of morphine and certain synthetic analogues in chronic spinal dogs, Martin and colleagues proposed the existence of three types of opioid receptors which they named μ for morphine, κ for ketozocine, and σ for N-allynormetazocine (SKF 10047) (Martin et al., 1976; Gilbert and Martin, 1976). After the discovery of enkephalins, Lord et al. (1977) found enkephalins to be more potent than morphine in inhibiting electrically-induced contraction of mouse vas deferens, while the reverse was true in guinea pig ileum. They therefore concluded that the μ receptor is predominant in the guinea pig ileum, while in mouse vas deferens a different opioid receptor type predominates, it was named the δ receptor. The finding in rat vas deferens that β-endorphin was highly

Chemical Structure of Selected Opioid Ligands

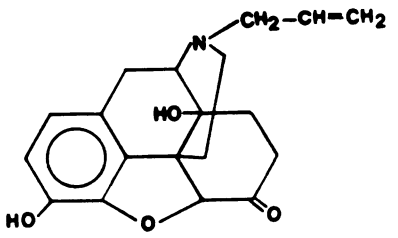
μ Selective agonist

Morphine



μ Selective antagonist

Naloxone



μ Specific agonist

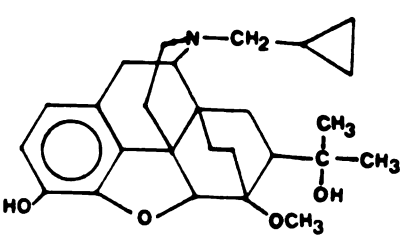
Morphiceptin Tyr-Pro-Phe-Pro-$CONH_2$

δ Selective agonist

[D-Ala,D-Leu]enkephalin Tyr-D-Ala-Gly-Phe-D-Leu-OH

μ, δ, κ Antagonist

Diprenorphine



potent, while other opioids were not in inhibiting the electrically-induced contraction has led to the postulation of yet another receptor type, ε (Schülz et al., 1979). Besides μ, δ, κ and ε receptors, an additional class of high-affinity binding sites (λ) (Grevel and Sadée, 1982) and subtypes (μ_1, μ_2, κ_1, κ_2, and κ_3) (Nishimura et al., 1984; Pfeiffer et al., 1981; Castanas et al., 1984) have also been proposed.

Pharmacology of Opioid Drugs

The major pharmacological effects of the opioid agonists result from their interactions with receptors in the central nervous system (CNS) and the bowel. These include analgesia, respiratory depression, decrease in gastrointestinal motility, drowsiness, euphoria, and mood alterations, and the induction of tolerance and physical dependence (Jaffe and Martin, 1980). Opioid antagonists like naloxone reverse the above effects, and will precipitate withdrawal in opiate-dependent subjects.

The relationship between pharmacological effects of opioids and their receptor subtypes was first proposed by Martin and colleagues (Gilbert and Martin, 1976; Martin et al., 1976). The μ receptor mediates supraspinal analgesia, respiratory depression, euphoria and physical dependence. The κ receptor mediates spinal analgesia, miosis, and sedation. The dysphoric effects of some opiates are thought to be mediated via the σ receptor. For the past decade, considerable new evidence has been submitted suggesting that the μ opioid receptor is primarily responsible for the analgesic actions of narcotics. The evidence includes:

(1) There is a good correlation between analgesic potency and activity

in the guinea pig ileum--which is known to contain predominantly μ receptors (Lord *et al.*, 1977)--bioassay for a large number of opioid ligands (Kosterlitz and Waterfield, 1975).

(2) Opioid peptides that have μ-receptor selectivity are also more potent in inducing analgesia (Gacel *et al.*, 1981; Handa *et al.*, 1981; Chang *et al.*, 1983).

(3) μ Receptors are enriched in the brain regions that are known to be important in analgesia (Goodman *et al.*, 1980).

(4) Correlation of analgesic effects of narcotics to their *in vivo* receptor binding profiles also indicates that the μ receptor has a predominant role in mediating the analgesic actions (Rosenbaum *et al.*, 1984).

In contrast to μ and κ receptors, the *in vivo* functions of δ receptors remain largely undefined.

Second Messenger System

Opioid agonists have been shown to lower the basal and hormone-stimulated cAMP level in rat brain homogenates (Law *et al.*, 1981; Collier and Roy, 1974) and cultured cells (Law *et al.*, 1982; Sharma *et al.*, 1975). Since these experiments were done in the presence of a phosphodiesterase inhibitor, it is reasonable to assume that the observed effects of opioids are on adenylate cyclase.

Since only δ receptors were expressed in cultured cells (West and Miller, 1983), opioid effect on adenylate cyclase in cultured cells was presumably mediated via δ receptors. Furthermore, opioid ligands that are more selective at δ receptors were also more potent in inhibiting the adenylate cyclase activity in these cells (Law *et al.*, 1983). The

opioid receptor-adenylate cyclase link in rat brain, on the other hand, is much less clear because of the coexistence of multiple receptor types in the CNS.

Results from electrophysiological studies in neurons of the myenteric plexus and locus coeruleus have suggested that μ (Miller, 1984; Williams and North, 1983) and possibly δ (Miller, 1984) receptors were involved in calcium mobilizing action triggered by opioids (Henderson, 1983; Miller, 1984; Egan and North, 1983).

Recently, it has been shown that an opioid peptide had an inhibitory effect on calcium currents in NG108-15 hybrid cells (Hescheler et al., 1987). Similar to the opioid effect on adenylate cyclase in these cells (Sharma et al., 1975), this effect can also be abolished by pretreating the cells with pertussis toxin (Hescheler et al., 1987). However, unlike the opioid adenylate cyclase coupling system which is known to involve the inhibitory guanine nucleotide binding protein, Gi (Rodbell, 1982), the effect on calcium channels was thought to be transmitted via a receptor-Go coupling unit (Hescheler et al., 1987).

Of the three major opioid receptor subtypes (μ, δ and κ), only the δ receptor has been clearly shown to be coupled to adenylate cyclase; therefore, it is important to determine the second messenger system for the μ and κ receptors in order to facilitate our understanding of narcotic actions at the molecular level.

2. Neuroblastoma Cells

Human Neuroblastoma

Neuroblatoma is the most common extracranial solid tumor in children (Evans, 1980). Most children present with the disease before 3 years of age, although some are first discovered in their second decade of life. The most common site of origin is the abdomen, with the tumor arising from the adrenal medulla (Pochedly, 1976). Neuroblastoma tends to disseminate early. The most common sites of metastasis are cortical bones, regional lymph nodes, liver, bone marrow, and subcutaneous tissues (Fernbach et al., 1977). Two-thirds of children with neuroblastoma present with a disseminated form of the disease, and most are incurable by currently known methods.

Characteristics of Human Neuroblastoma Cells

Neuroblastoma arises from the neural crest. Whereas neural crest cells normally mature into several types of differentiated cells (including melanoblasts; ganglial cells of the sympathetic systems; chromaffin cells of the adrenal medulla; and structural cells of the nervous system such as microglial cells and Schwann cells) (Shinke, 1980), malignant neuroblasts have been shown essentially to express morphologic and biochemical characteristics of neural cells such as the presence of neurosecretory granules (Seeger et al., 1977) and neurotransmitter synthesis (Biedler, 1978). Furthermore, the neuroblasts isolated from neuroblastoma can be readily differentiated into mature neuronal cells marked by morphological, biochemical and

Cultured cells of neural origin which express opioid receptors.

Cell line	Derivation	Receptor type	Coupled to adenylate cyclase	References
N18TG2	Subclone C1300 mouse neuroblastoma	δ, ε?	Yes	Amano et al., 1972; Law et al., 1982; Hammonds & Li, 1981.
NG108-15	N18TG2 X rat C6 glioma hybrid	δ, ε?	Yes	Klee & Nirenberg, 1974; Sharma et al., 1975; Hammonds et al., 1981
N4TG1	Subclone C1300 mouse neuroblastoma	δ	Yes	Amano et al., 1972; Chang & Cuatrecasas, 1979; Hazum et al., 1980
TCX17	N18TG2 x mouse sympathetic ganglion cell	δ	No	Blosser et al., 1976
NCB20	N18TG2 X Chinese hamster brain cell	δ, σ?	Yes	McLawhon et al., 1981
N1E-115	Subclone C1300 mouse neuroblastoma	δ	Yes	Amano et al., 1972; Gilbert et al., 1982
NMB	Human	δ	?	Ard et al., 1985; Hochhaus et al., 1986
Kelly	Human	δ	?	Hochhaus et al., 1986
IMR-32	Human	δ	?	Hochhaus et al., 1986

electrophysiological changes (Påhlman et al., 1984; Kuramoto et al., 1981; Sonnenfeld and Ishii, 1982). Therefore, neuroblastoma cells in vitro are useful tools for advancing our understanding of the molecular functions of neurotransmitter receptor systems.

Neuroblastoma Cell Lines as Models for Opioid Receptor Studies

The opioid receptor system consists of multiple receptor subtypes. Establishing a clonal cell line with homogenous population of a single cell type which expresses opioid receptors would provide us with a well-defined condition for the elucidation of biochemical events between agonist-receptor binding and pharmacological response. Opioid receptor research in this direction has been fruitful over the past decade and numerous cell lines of murine origin have been established (West and Miller, 1983). Although only δ opioid receptors were found in these cells (West and Miller, 1983), these cell lines have proven excellent model systems for the study of the molecular functions of the opioid receptor (Klee and Nirenberg, 1974; Sharma et al., 1975; Gilbert and Richelson, 1983).

The human neuroblastoma cell lines IMR-32, NMB and Kelly have also been found to express δ opioid receptors (Hochhaus, 1986, Ard et al., 1985) and non-opioid β-endorphin binding sites (Westphal and Li, 1984). The molecular functions of the δ receptors in these human neuroblastoma cell lines, however, have not yet been determined.

A great deal has been learned from cultured cells since the establishment of the NG108-15 hybrid cell line for opioid receptor studies (Gilbert and Richelson, 1983; Sharma et al., 1975). However, what we have learned from murine cell lines may not apply to the opioid

receptor system in general. Therefore, identification of cell lines that express opioid receptor types other than δ would undoubtedly facilitate our efforts to achieve a more comprehensive understanding of the molecular functions of the opioid receptor systems.

CHAPTER I

A HUMAN NEUROBLASTOMA CELL LINE EXPRESSES μ AND δ OPIOID RECEPTOR SITES

1. SUMMARY

A series of neuroblastoma cell lines were screened for the presence of opioid receptor sites with the tracers [^{3}H]diprenorphine (μ, δ, κ ligand) and [^{3}H]naloxone (μ-selective ligand). One human neuroblastoma cell line, SK-N-SH, displayed avid binding for both tracers. Binding experiments with multiple tracers revealed the presence of both μ and δ sites. These sites were stereospecific, saturable and proteinaceous in character. Saturation binding experiments provided an estimate of 50,000 μ and 10,000 δ sites/cell. NaCl (100 mM) and guanine nucleotide, GppNHp (50 μM), reduced opioid agonist but not antagonist binding to these sites. The opioid binding sites on SK-N-SH cells closely resemble the previously reported μ and δ sites in human and rodent brain. Therefore, the SK-N-SH neuroblastoma cell line represents a useful tool to study the molecular functions of opioid receptors.

2. INTRODUCTION

The opioid receptor system consists of several types of binding sites (e.g., μ, δ, κ) with different biological functions (Wood, 1982; Maurer, 1982; Chang et al., 1981; Gillan and Kosterlitz, 1982; Lord et

al., 1977; Rosenbaum et al., 1984). Additional classes of putative receptor sites (σ, ε) (Zukin and Zukin, 1981; Wüster et al., 1980) or high affinity binding sites (λ) (Grevel and Sadée, 1983) and sub-types (μ_1, μ_2, κ_1, κ_2, κ_3) (Nishimura et al., 1984; Pfeiffer et al., 1981; Castanas et al., 1984) have also been proposed. In view of the multitude of binding sites in heterogenous tissue preparations, it is important to identify transformed neuronal cell lines in which the distinct opioid binding sites can be individually studied. Among cell lines that have been examined for the presence of opioid receptor, only the δ type of binding sites was found in murine neuroblastoma cells (West and Miller, 1983). Nevertheless, these cell lines have proven excellent model systems for studying the molecular functions of the opioid receptor (Klee and Nierenberg, 1984; Sharma et al., 1975; Gilbert and Richelson, 1983). However, the in vivo functions of the δ site remain largely unknown. On the other hand, the role of μ and κ sites in regulating pain perception, among other functions, is well documented (Wood, 1982; Martin et al., 1976; Rosenbaum et al., 1984; Goodman et al., 1980).

In order to identify transformed cells with opioid receptor types other than the δ sites, we have screened a series of human neuroblastoma cell lines that are usually derived from metastases of peripheral primary neuronal tumors, but whose characteristics are thought to resemble those of CNS neurons. Of the cell lines tested, only one line, SK-N-SH, displayed readily measurable specific binding for both the μ, δ, κ-ligand [^{3}H]diprenorphine and the μ-selective ligand [^{3}H]naloxone, which suggested the presence of μ sites. This human neuroblastoma cell line was derived from a metastatic tumor in the bone marrow of a four-year-old girl (Biedler et al., 1973).

3. MATERIALS AND METHODS

3.1 Materials

SK-N-SH, NJB90, SK-N-MC, AG2272, IMR-32, AG2203, Y79 NHT C10, Neuro 2A, and NB 41 A3 cell lines were provided by the Tissue Culture Center of the University of California, San Francisco. The NG108-15 cell line was provided by Dr. Horace Loh, UCSF. The NMB and Kelly cell lines were obtained from Dr. Fred Gilbert, Mount Zion School of Medicine, New York, NY. [^{3}H]DAGO* (60 Ci/mmol), [^{3}H]DADL (50 Ci/mmol), [^{3}H]naloxone (60 Ci/mmol) and [^{3}H]diprenorphine (41 Ci/mmol) were purchased from Amersham Corp. DAGO, DADL, morphiceptin, leu-enkephalin, met-enkephalin, β-endorphin, (1-13) dynorphin, bestatin, and thiorphan were purchased from Peninsula Lab. Morphine sulfate was obtained from Mallinckrodt, Inc., naloxone HCl from Endo Lab., and guanylyl-imidodiphosphate, GppNHp, tetralithium salt from Boehringer Mannheim. L-Leucyl-l-leucine, trypsin, EC 3.4. 21.4, from bovine pancreas, 9100 units/mg, and STREPTOMYCES GRISEUS neutral proteinase (pronase E), EC 3.4. 21.4, were purchased from Sigma Chemical Co. Captopril was obtained from E.R. Squibb & Sons, Inc. Diprenorphine HCl, etorphine HCl, bremazocine HCl and ethylketocyclazocine methane sulfonate were obtained from the National Institute on Drug Abuse. (+)-Naloxone was provided by Dr. A.E. Jacobson of the National Institute of Health, NIADDKD Bethesda, MD.

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*Abbreviations used: DAGO, [D-Ala^2, MePhe^4, Gly-ol^5] enkephalin; DADL, [D-Ala^2, D-Leu^5] enkephalin.

3.2 Methods

3.2a Cell Cultures

SK-N-SH cells were grown at 37°C in monolayer in RPMI 1640 medium supplemented with 10% fetal calf serum. NB 41 A3, NJB 90, SK-N-MC, NMB, Kelly, IMR-32 and AG 2272 were all grown under the same conditions as SK-N-SH. Y79 NHT C10 cells were grown in suspension in RPMI 1640 medium containing 10% fetal calf serum. Mouse neuroblastoma x rat glioma hybrid cells NG 108-15 were grown in monolayer in DME H21 medium containing HAT buffer (0.1 mM hypoxanthine, 10 µM aminopterin and 17 µM thymidine) supplemented with 10% fetal calf serum in a humidified atmosphere of 10% CO_2. Neuro-2A cells were grown under the same conditions as NG 108-15 cells except no HAT buffer was added.

3.2b Preparation of Membrane Homogenates

At confluency, monolayer (except NG 108-15) cells were harvested by replacing the culture medium with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline containing 0.04% EDTA. After 3 min at 20°C, the cells were detached by mechanical agitation. The NG 108-15 cells were detached by mechanical agitation after replacing the medium with phosphate buffered saline. After cells were harvested, the suspension was centrifuged at 1000 rpm for 7 min. For crude membrane homogenates, the pellet was immediately homogenized in ice-cold 50 mM Tris-HCl (pH 7.4) buffer with a Brinkman blender to yield a protein concentration ~ 1 mg/ml. In the case of SK-N-SH cells, washed membrane homogenates were also prepared. To obtain washed membrane homogenates, the pellet was homogenized and then incubated at 20°C for one hour in 50 mM Tris-HCl pH 7.4 buffer containing 100 mM NaCl. After the incubation, the suspension was

centrifuged at 40,000 g at 4°C for 20 minutes, and the pellet was washed twice in ice-cold 50 mM Tris-HCl buffer. The final pellet was resuspended in ice-cold 50 mM Tris-HCl buffer containing a mixture of peptidase inhibitors (bestatin (30 μM), L-leucyl-L-leucine (2 mM), thiorphan (0.6 μM), and captopril (10 μM)) to yield a protein concentration of ~ 0.8 mg/ml. The resulting crude or washed homogenates were used immediately for binding assay. Protein content was measured spectrophotometrically by the method of Lowry (Lowry et al., 1951).

3.2c Binding Experiments

Ligand binding to the opioid receptor was measured at equilibrium at 20°C with a centrifugation assay. Time required to reach equilibrium was determined for each tracer in SK-N-SH cell membrane homogenates. The crude or washed membrane homogenate (1 ml) was incubated for 40 to 60 min with ligand and tracer in polypropylene micro-centrifuge tubes (1.5 ml). Tracer concentration in all binding experiments was no more than 50% of the K_D value towards its respective binding site. Each ligand concentration was assayed in duplicate. After incubation the tubes were centrifuged (Eppendorf-Brinkman 5412) for 4 min at 12,000 g at 4°C, the supernatants removed by aspiration and the inner surface of the tube rinsed 3x with 1 ml ice-cold 50 mM Tris-HCl buffer, pH 7.4. [^{3}H] Activity was measured directly in the tubes by liquid scintillation counting after dissolving the pellet with 1 ml Scinti Verse II (Fischer Scientific Co., NJ). Nonspecific tracer binding, measured in the presence 10^{-6} M diprenorphine accounted for less than 20% of total [^{3}H] binding.

3.2d Data Analysis

Data obtained from saturation binding experiments performed with constant concentration of tracer (under labeling conditions that are specific for only one type of binding site) were fitted to a single site law of mass action model by the extended least squares nonlinear regression program (ELNR) (22). For the estimation of the δ site population, [^{3}H]DADL was used in the presence of morphiceptin (10^{-5}M) to block μ sites which were thus not considered in the analysis of the [^{3}H]DADL saturation curve.

IC_{50} values were either determined graphically or by fitting the data to the logistic function: $\%B = \%B_{max} - \%B_{max} \times L/(IC_{50} + L)$ (B = specific bound tracer (d.p.m.); L = concentration of displacing ligand) with the use of the computer program MAKE Model in the Prophet-NIH system (Holford, 1982). For the morphine displacement curve against [^{3}H]naloxone in the presence of sodium ion or/and guanine nucleotide, a slope factor n was also incorporated into the logistic function as follows:

$$\%B = \%B_{max} - \%B_{max} \times L^n/(IC_{50}^n + L^n)$$

4. RESULTS

4.1 Screening of Neuroblastoma Cell Lines for Opioid Receptor Sites

The results of binding assays with [^{3}H]diprenorphine and [^{3}H]naloxone in various cell lines are shown in Table I-1. The screening procedures readily revealed the presence of opioid binding

Table I-1. Specific [^{3}H] opioid binding to neuroblastoma cell lines. Equilibrium binding assays were carried out in crude membrane homogenates. [^{3}H]Naloxone (1 nM) and [^{3}H]diprenorphine (0.25 nM) were used to detect μ and μ, δ, κ binding sites, respectively. Non-specific binding is the amount of [^{3}H] opioid binding remaining in the presence of 1 μM diprenorphine. Values represent mean ± range of one experiment. A duplicate experiment yielded similar results.

Cell lines	Origin	Specific ^{3}H-naloxone binding	Specific ^{3}H-diprenorphine binding
		CPM ± range/mg protein	
NJB 90	human	132 ± 125	13 ± 2
SK-N-MC	human	18 ± 13	111 ± 120
AG 2272	human	22 ± 11	40 ± 38
IMR 32	human	300 ± 2	1138 ± 29
NMB	human	143 ± 42	604 ± 7
Kelly	human	86 ± 23	326 ± 11
SK-N-SH	human	1681 ± 153	3277 ± 5
AG 2203	human	45 ± 25	30 ± 22
Y79 NHT C10	human	35 ± 3	224 ± 8
Neuro 2A	mouse	139 ± 100	702 ± 42
NB 41 A3	mouse	86 ± 56	117 ± 21
NG 108-15	rat x mouse	215 ± 30	3613 ± 18

sites with low affinity for [^{3}H]naloxone in NG 108-15 cells, which are known to express a homogenous population of δ receptors (West and Miller, 1983; Chang et al., 1978). Among the 11 other neuroblastoma cell lines tested, NMB, Kelly, Neuro-2A and more strongly IMR-32 showed various degrees of saturable binding for [^{3}H]diprenorphine. Because these cells did not measurably bind [^{3}H]naloxone, they are not further discussed here. Interestingly, β-endorphin specific binding sites have previously been detected in NMB, Kelly and IMR-32 cell lines (Westphal and Li, 1984). However, these reported sites appeared to recognize the COOH-terminus of the β-endorphin molecule rather than the NH_2-terminus which is associated with opioid receptor binding (Schweigerer et al., 1982).

Of all the cell lines tested, only one line, SK-N-SH, displayed avid binding for both [^{3}H]naloxone and [^{3}H]diprenorphine. This observation immediately suggested that SK-N-SH cells express μ receptor sites.

4.2 Characterization of Opioid Binding Sites in SK-N-SH Cells

The presence of μ sites was confirmed with the highly μ-selective tracer [^{3}H]DAGO (Chang et al., 1981a; Chang et al., 1981b); moreover, [^{3}H]DAGO was readily displaced by the μ ligands, naloxone, DAGO, and β-endorphin (Fig. I-1). Morphiceptin, a μ specific ligand (Biedler et al., 1973), was able to fully displace [^{3}H]DAGO at 10^{-5}M (Fig. I-1). The stereospecificity of the μ sites was demonstrated by the failure of high concentrations of (+)-naloxone to displace [^{3}H]DAGO (Fig. I-1).

Evidence for the presence of δ sites was obtained when morphiceptin and DAGO competed against [^{3}H]DADL, a μ and δ ligand with preference for

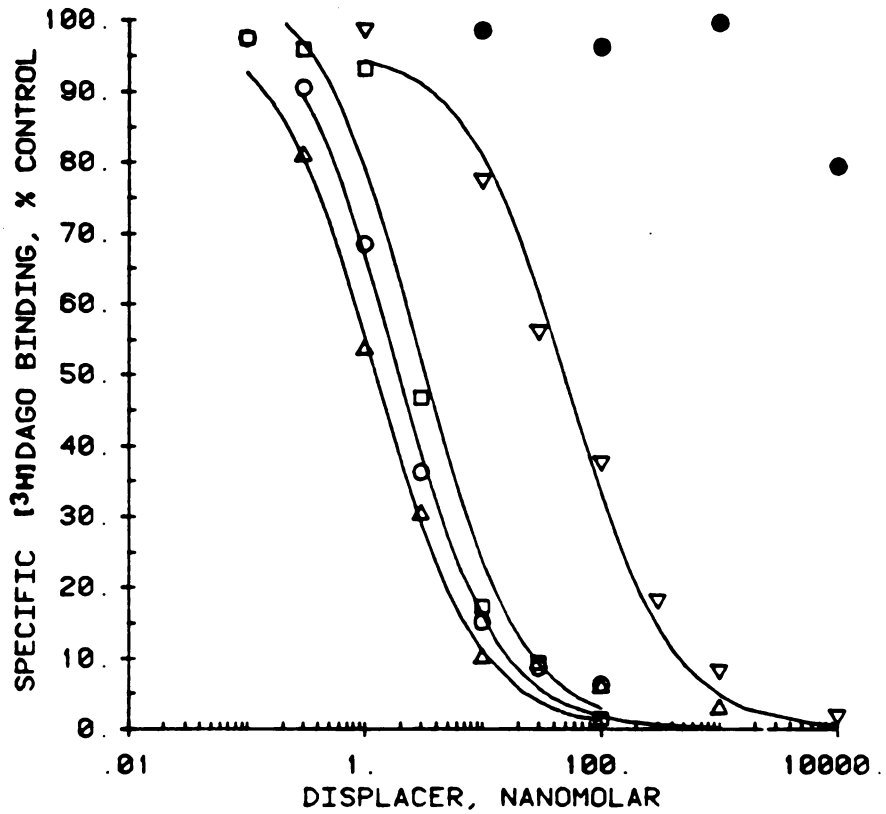

Figure I-1. Equilibrium binding competition curves of opioid ligands against [^{3}H]DAGO. The incubations were carried out in washed membrane homogenates wth increasing concentration of DAGO (O—O), naloxone (△—△) morphiceptin (▽), β-endorphin (□—□) and (+)-naloxone (●) against [^{3}H]DAGO, 1 nM. Values represent means of duplicate determinations that are within ±5% of the mean.

the δ sites (Wood, 1982) (Fig. I-2, upper panel). Morphiceptin at 10^{-5}M displaced only 65% of the specific binding of [^{3}H]DADL. The DAGO competition curve against [^{3}H]DADL, on the other hand, was biphasic, which suggested the presence of δ sites with which DAGO interacted with low affinity.

The identity of δ sites was further examined by labeling the opioid binding sites with [^{3}H]DADL in the presence of a blocking dose (10^{-5}M) of morphiceptin for the μ sites. If the residual ^{3}H-DADL binding in the presence of 10^{-5}M morphiceptin represents δ sites, DADL should be more potent in displacing the residual [^{3}H]DADL binding than displacing ^{3}H-DAGO binding. Competition curves gave DADL IC_{50} values of 11 nM against [^{3}H]DAGO and 3 nM against [^{3}H]DADL in the presence of 10^{-5} morphiceptin (Fig. I-2, lower panel). The values are in good agreement with reported μ and δ affinities of DADL (Wood, 1982; Itzhak and Simon, 1984).

To test for the presence of additional opioid binding sites, DADL was titrated in washed membrane homogenates against the μ, δ and κ tracer [^{3}H]diprenorphine (Fig. I-2, lower panel). If only μ and δ sites were present, one would expect a DADL displacement curve between those found with the μ and δ tracers. However, we observed a small but reproducible shift of the DADL versus [^{3}H]diprenorphine displacement curve to the right from that obtained with [^{3}H]DAGO. The right-shift of the [^{3}H]diprenorphine curve was more pronounced in unwashed membrane homogenates (data not shown). Several hypotheses can account for these results. First, [^{3}H]diprenorphine could bind to an additional type of opioid binding site that is partially lost during membrane washing and that displays rather low affinity for DADL. Second, the μ and δ sites may bind diprenorphine in a fashion different from that expected for a

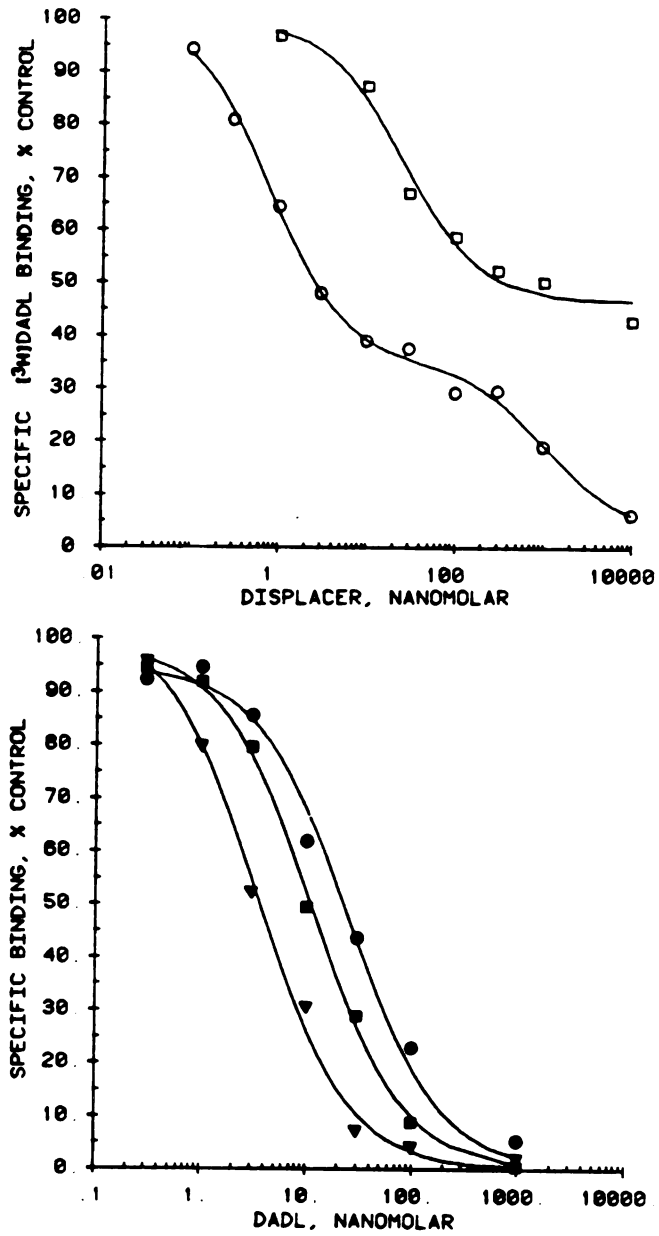

Figure I-2. Evidence for the presence of δ sites. The incubations were carried out with washed membrane homgenates.
Upper Panel: Competitive displacement of [^{3}H]DADL by DAGO (○—○) and morphiceptin (□—□).
Lower Panel: Competitive displacement by DADL of [^{3}H]DAGO (μ) (■—■), [^{3}H]DADL in the presence of 10^{-5}M morphiceptin (δ) (▼—▼), and [^{3}H]diprenorphine (●—●) are shown. Morphiceptin (10^{-5}M) effectively displaced all specific [^{3}H]DAGO binding and therefore served as a μ blocking dose. Values represent the means of duplicate determinations that are within ±5% of the mean.

binding model with two independent sites (μ-δ interactions). This possibility will be addressed in future studies. Third, binding differences may exist between the agonist tracers [^{3}H]DAGO or [^{3}H]DADL and the antagonist tracer [^{3}H]diprenorphine because of distinct agonist and antagonist conformations of the receptor sites. To address the third hypothesis, we have obtained mutual displacement curves with the μ selective antagonist naloxone and the μ-agonist DAGO against their respective tracers in crude membrane homogenates. There was only a small right-shift of the displacement curve for both ligands when the antagonist tracer was used instead of the highly μ-selective [^{3}H]DAGO (Table I-2). This small shift is readily accounted for by the small extent of [^{3}H]naloxone binding to the δ sites, and it was independent of the agonist and antagonist character of the ligand. The residual binding of [^{3}H]diprenorphine in the presence of a μ and δ blocking concentration of DADL ($3x10^{-7}$M) was too small in washed membrane homogenates to permit further study.

Further binding experiments were carried out with both crude and washed membrane homogenates, mostly with identical results (Table I-2). However, the endorphins (enkephalins, (1-13)-dynorphin, β-endorphin), were all less potent in crude homogenates despite the addition of peptidase inhibitors. The μ and δ sites were further characterized with a series of opioid ligands, and the resulting IC_{50} values are given in Table I-2. Because δ site labeling by [^{3}H]naloxone in the SK-N-SH homogenates is minimal (< 5% of total specific binding), both [^{3}H]naloxone and [^{3}H]DAGO served as the μ ligands, while [^{3}H]DADL + 10^{-5} M morphiceptin was chosen for δ-specific labeling.

Saturation binding experiments were done to estimate the total

Table I-2. IC_{50} values for different opioid ligands determined under μ and δ labeling conditions. The data represent IC_{50} values (concentration that caused a 50% inhibition of control specific binding of the tracer) for the displacement of the μ-selective ligands [^{3}H]naloxone and [^{3}H]DAGO and the δ-selective tracer [^{3}H]DADL, the latter in combination with 10^{-5} M morphiceptin to block μ sites. Incubations were performed to equilibrium (40 to 60 min) at 20°C either in crude or washed (*) membrane homogenates.

	IC_{50} values, nM[a]		
	μ Sites		δ Sites
Displacer	[^{3}H]Naloxone (0.5 nM)	[^{3}H]DAGO (1 nM)	[^{3}H]DADL (1 nM) + morphiceptin (10^{-5} M)
Naloxone	1.7	1.35±0.2[c]	30
DAGO	4	2.6±1.0 (n=7)[b]	800*
DADL	8.8±3.2 (n=2)[b]	11.5±2.1* (n=2)	2.7±1 (n=2)
Morphiceptin		49±6* (n=4)	> 10,000
Met-enkephalin		3.5*	2*
Leu-enkephalin		7*	2.1*
β-Endorphin		2.5*	7*
(1-13)Dynorphine		6*	17*
Morphine	10±5 (n=3)		200
Diprenorphine	0.3		1.0
Ethylketocyclazocine		6	100
Bremazocine		0.6	2.2

[a] IC_{50} values in washed membrane homogenates are marked with an asterisk.
[b] Results from incubations with crude and unwashed membrane homogenates did not differ and were combined.
[c] ± range (n=2) or ± S.D. (n > 3).
[d] n=number of independent experiments.

number of binding sites. Computer fitting of the [^{3}H]diprenorphine saturation curve in crude membranes to a one site model gave a Bmax value of 0.35 pmole/mg protein or approximately 70,000 sites/cell. This value represents a very rough estimate only, because [^{3}H]diprenorphine may bind to more than two binding site populations with different affinities. The saturation curve for [^{3}H]DAGO gave a μ site population of approximately 50,000 sites/cell (0.25 pmole/mg protein) in crude homogenates, suggesting that μ sites are predominant. In washed membrane homogenates, the μ site population, determined with [^{3}H]DAGO, was 0.25 pmole/mg protein, while the δ site population, calculated from a [^{3}H]DADL saturation curve in the presence of 10^{-5}M morphiceptin, was 0.056 pmole/mg protein. These values are calculated from the data shown in Fig. I-1 (DAGO-[^{3}H]DAGO) and Fig. I-2 (DADL-[^{3}H]DADL + morphiceptin), plotted as displacement curves. Hence the μ/δ ratio was approximately 4.5/1.

Trypsin (from bovine pancreas) and pronase E both effectively decreased [^{3}H]naloxone binding in a time dependent manner. Incubation carried out at 20°C with the addition of trypsin (6 u/ml) or pronase E (2300 u/ml) to the crude membrane homogenates lowered the [^{3}H]naloxone binding to 45 and 30% of control, respectively, in 10 min. This finding suggested the opioid binding sites in SK-N-SH cells are proteinaceous in character.

4.3 **Sodium and Guanine Nucleotide Modulation of Receptor Affinity in SK-N-SH Cells**

Both guanine nucleotides and NaCl were reported to be necessary for adenylate cyclase (Blume et al., 1979). In crude SK-N-SH membrane

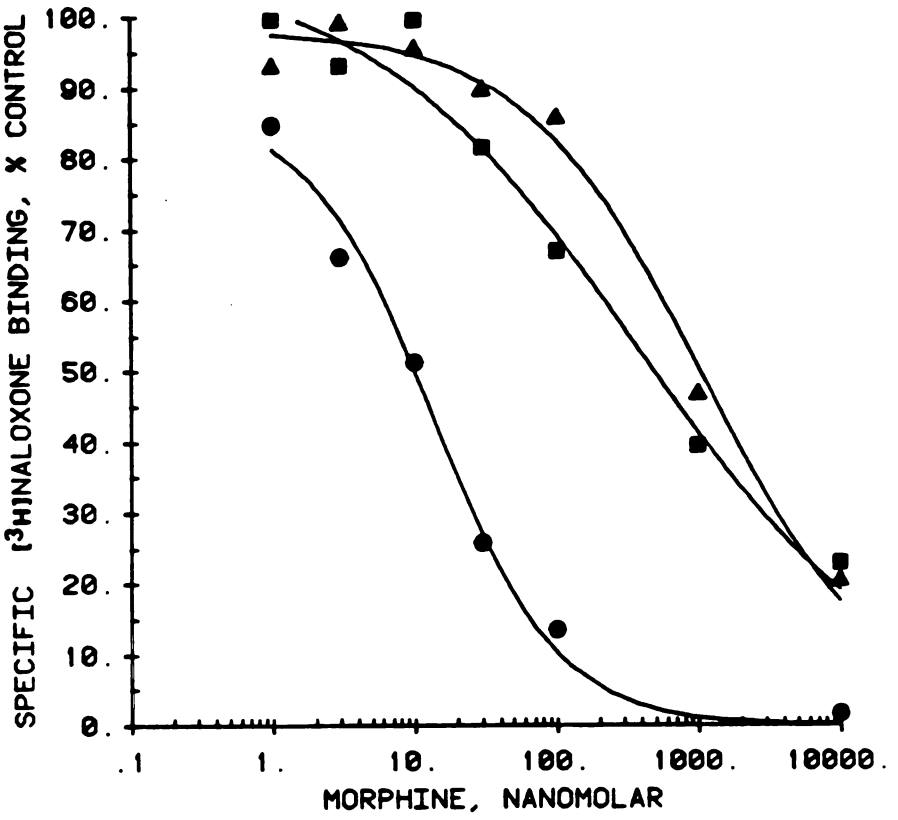

Figure I-3. Effects of NaCl and GppNHp on the potency of morphine in displacing [^{3}H]naloxone. The binding experiments were carried out in crude membrane homogenates. Competitive displacement curves of morphine against [^{3}H]naloxone under three different conditions: control (● — ●), presence of 100 mM NaCl (■ — ■) and presence of 100 mM NaCl + 50 μM GppNHp (▲ — ▲). Values represent the mean of two determinations that are within ±5% of the mean.

homogenates we have observed a reduction of specific agonist tracer binding by guanylyl imidodiphosphate (GppNHp, a GTP analog, 50 μM) and NaCl (100 mM) to 84±3% and 14±0.7% (n=3) of control, respectively, for [^{3}H]DAGO, and to 84±4% and 24±2% (n=3) of control, respectively for [^{3}H]DADL. In contrast, no effects of these regulatory factors were observed with [^{3}H]naloxone and [^{3}H]diprenorphine.

The potency of morphine, a μ agonist, in displacing [^{3}H]naloxone was assessed in the presence of sodium ions and guanine nucleotide (Fig. I-3). The competitive displacement curves for morphine against [^{3}H]naloxone in the presence of 50 μM GppNHp is essentially the same as the control curve; no difference was observed in both IC_{50} value and slope of the curve. In the presence of 100 mM NaCl, the IC_{50} value of morphine was increased from 8 nM to 350 nM, and the slope of the curve appeared shallow with the slope factor n equal to 0.5. While GppNHp (50 μM) alone did not cause a measureable shift or change in slope of the morphine displacement curve, it did shift the sodium curve to the right from an IC_{50} of 350 nM (Na^+) to ~ 1000 nM (Na^++GppNHp), with a concomitant shift of the slope factor to n=0.7.

5. DISCUSSION

Binding studies with multiple tracers and ligands revealed that SK-N-SH cells express both μ and δ opioid receptor sites. The μ sites displayed high or selective affinity for naloxone, morphine, DAGO and morphiceptin, while δ sites preferentially bound DADL and leu- and met-enkephalin (Table I-2). However, the δ preference of met-enkephalin was

small. (1-13)Dynorphin (a κ-selective ligand) (Chavkin et al., 1982), β-endorphin, and the κ-agonists ethylketocyclazocine and bremazocine showed preference for μ over δ sites, which is in agreement with previous results (Wood, 1982; Gillan and Kosterlitz, 1982). On the other hand, most previous studies reported equal affinity of diprenorphine to μ and δ sites (Chang et al., 1981), while its lower affinity to the δ sites observed here is consistent with our previous finding on the in vivo binding of diprenorphine in intact rat brain (Rosenbaum et al., 1984a; Rosenbaum et al., 1984b). The spectrum of binding affinities for the studied opioid ligands establishes the presence of μ and δ sites that closely resemble μ and δ sites observed in human (Maurer, 1982; Pfeiffer et al., 1981; Pfeiffer et al., 1982; Bonnet et al., 1981) and rodent (Wood, 1982; Chang et al., 1981; Gillan and Kosterlitz, 1982; Lord et al., 1977) brain, and the δ sites in the neuroblastoma x glioma hybrid cell line, NG 108-15 (Gilbert and Richelson, 1983).

The total number of opioid binding sites were estimated at 70,000 sites per cell on the basis of saturation binding curves with diprenorphine. However, this value represents a rough estimate only because of the uncertainties of the correct diprenorphine binding model. In particular the question remains open whether there is an additional type of binding site population that causes a small extent of residual [^{3}H]diprenorphine in the presence of saturating (μ,δ) levels of DADL, or whether deviations from the law of mass action occur from a two site binding model. The B_{max} value obtained here compares well with the δ site population on the NG 108-15 cells (300,000 sites per cell) (Klee and Nierenberg, 1974), if one considers the smaller size of the SK-N-SH

cells. Indeed, estimates expressed per mg protein are similar for the SK-N-SH cells (0.35 pmole/mg protein) and the NG 108-15 cells (0.53 pmole/mg protein) (unpublished data from a [^{3}H]DADL saturation curve).

The differential effects of sodium ions and GppNHp on opioid agonist and antagonist binding observed in SK-N-SH cells were similar to previous results in the mouse neuroblastoma x rat glioma hybrid, NG 108-15 (Blume, 1978a), and rodent brain (Blume, 1978b; Childers and Snyder, 1980). This finding suggests that the human opioid receptor of SK-N-SH cells has a similar molecular organization as the rodent receptor.

In conclusion, we have identified a human neuroblastoma cell line that expresses μ and δ opioid receptor types. Of particular interest is the fact that at least two types of binding sites are expressed, although it remains to be tested whether different sites are present on the same cell. Previous electrophysiological work in rodents has raised the possibility that a substantial fraction of the opioid responsive cells carry more than one opioid receptor type, e.g., μ and δ (Zieglgänsberger et al., 1982; Egan and North, 1981). Moreover, Barnard and Demoliou-Mason (1983) have proposed that a functional opioid receptor system may consist of a complex of the μ, δ and κ binding proteins acting in concert. Rothman and Westfall (1982) have suggested allosteric interactions among the μ and δ binding sites, while Pert and coworkers (Bowen et al., 1981) proposed biochemical interconversions among the μ and δ sites. Availability of the SK-N-SH cells should allow one to test these hypotheses under well defined conditions.

CHAPTER II

PHOSPHATIDYLINOSITOL TURNOVER IN NEUROBLASTOMA CELLS: REGULATION BY BRADYKININ, ACETYLCHOLINE, BUT NOT μ AND δ OPIOID RECEPTORS

1. SUMMARY

The effect of opioids on phosphatidylinositol (PI) turnover to release inositol triphosphate (IP_3) as second messenger was examined in mouse neuroblastoma-rat glioma hybrid cells NG108-15 (δ receptors) and human neuroblastoma cells, SK-N-SH (predominantly μ receptors). PI turnover can be stimulated in both NG108-15 and SK-N-SH cells by bradykinin and acetylcholine, respectively. In contrast, etorphine, DADL ([D-Ala^2, D-Leu^5]enkephalin), and DAGO ([D-Ala^2, MePhe^4, Gly-ol^5]enkephalin), up to 1 μM concentrations failed to affect PI turnover in both cell lines. These results suggest that IP_3 is not likely to serve as second messenger for both μ and δ opioid receptors.

2. INTRODUCTION

Electrophysiological studies on neurons of myenteric plexus and locus coerulus demonstrated that opiates and opioid peptides hyperpolarize the cell membranes by enhancement of potassium conductance (Henderson, 1983; Tokimasa et al., 1981; Williams et al., 1982). It has

been suggested that μ (Miller, 1984; Williams and North, 1983) and possibly δ (Miller, 1984) receptors are involved in this action by mobilizing intracellular calcium which eventually leads to the opening of potassium channels (Henderson, 1983; Miller, 1984; Williams and North, 1983). Recently, inositol triphosphate (IP_3) has been implicated as a second messenger for a variety of hormonal signals to release calcium from intracellular storage sites (Berridge and Irvine, 1984; Fig. II-1).

In this report, we examined the possibility that opioids affect phosphatidylinositol (PI) turnover to release IP_3 as a second messenger in two opiate receptor containing cell lines, mouse neuroblastoma-rat glioma hybrid NG108-15 (δ receptors) (Klee and Nirenberg, 1974; West and Miller, 1983) and human neuroblastoma SK-N-SH (predominantly μ receptors) (Yu et al., 1986).

3. MATERIALS AND METHODS

3.1 Materials

SK-N-SH and NG108-15 cells were kindly provided by Dr. June Biedler and Dr. Horace Loh, respectively. [^{3}H]inositol (14.2 Ci/mmol) was purchased from Amersham Corp. Bradykinin, Substance P, ATP, carbachol, lithium chloride and acetylcholine HCl were obtained from Sigma. DAGO* and DADL were puchased from Peninsula Laboratories. Dowex 1 x 8 anion

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*Abbreviations used: PI, phosphatidylinositol; IP_3, inositol triphosphate; DADL, [D-Ala^2, D-Leu^5] enkephalin; DAGO, [D-Ala^2, MePhe^4, Gly-ol^5] enkephalin.

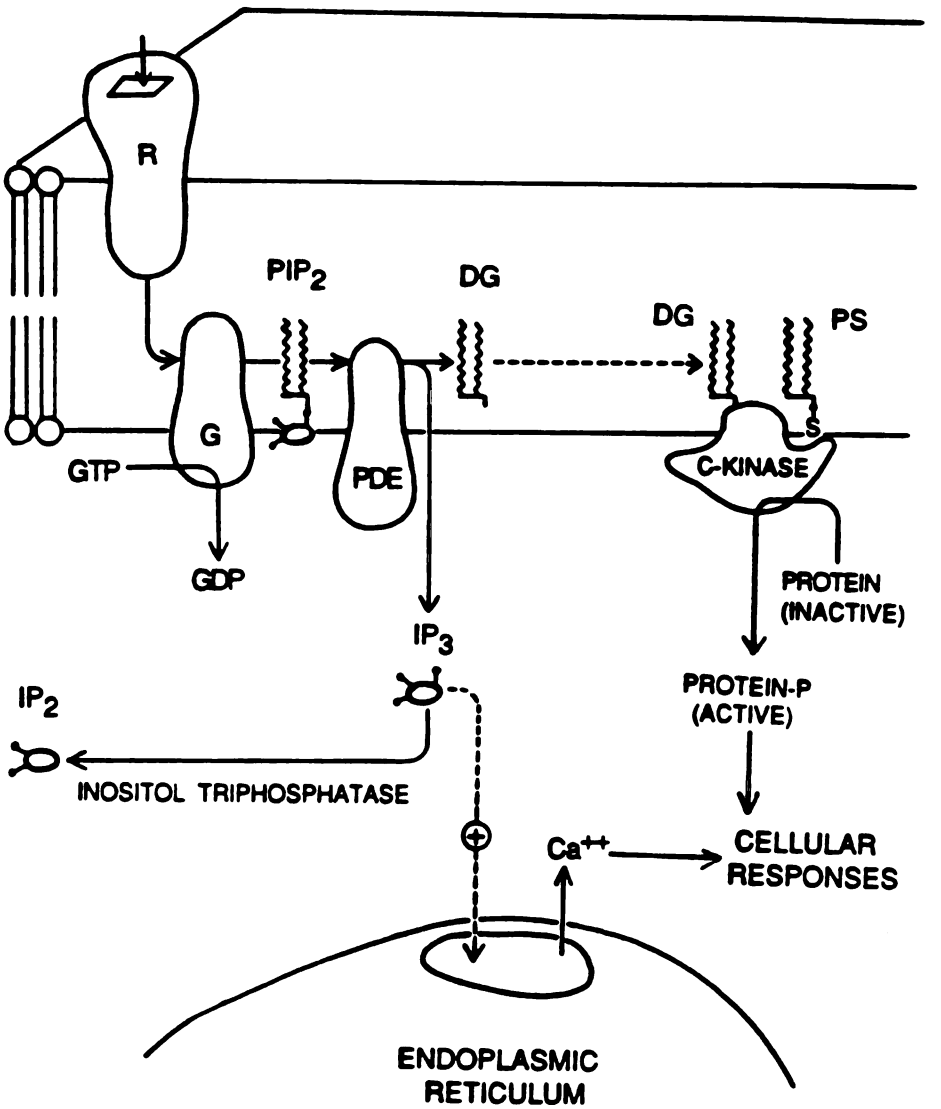

Figure II-1. Phosphatidylinositol turnover as a second messenger system for hormone receptor. Upon receptor-agonist interaction, the signal would be transmitted via a guanine nucleotide binding protein (G) to allow the phosphatidylinositol 4,5-bisphosphate (PIP_2) to be hydrolyzed by a membrane bound phosphodiesterase (PDE) to inositol triphosphate (IP_3) and diacylglycerol (DAG). Both of these hydrolyzed products appear to have a second messenger function, the former's to release calcium and the latter's to activate protein kinase C (Berridge and Irvine, 1984).

exchange columns (Cl^- form, 100-200 mesh) were obtained from Biorad, Richmond, CA. Etorphine HCl was kindly provided by the National Institute on Drug Abuse.

3.2 Methods

SH-N-SH and NG108-15 cells were grown in 150 cm^2 culture flasks under previously described conditions (Chapter I). When the cells reached approximately 80% confluency, the medium was removed, and 30 ml of fresh medium containing 1 µCi/ml of [^{3}H]inositol was added. After a prelabeling period of 2 to 3 days, cells were harvested in the same manner as described (Chapter I) and the pellet was resuspended in serum free RPMI 1640 medium to yield a protein concentration of approximately 0.6 mg/0.5 ml. Each 0.5 ml aliquot of cell suspension was incubated with 10 mM of lithium chloride (inositol monophosphatase inhibitor) at 37°C for 15 minutes before the test agents were added. The reactions were terminated by adding 0.5 ml of 20% (w/v) trichloroacetic acid (ice-cold). After 20 min at 0° C, the precipitated proteins were removed by centrifugation, and the supernatant containing the [^{3}H]inositol metabolites was collected.

For the separation of [^{3}H]inositol metabolites, we adopted the method described by Drummond *et al.* (1984). Briefly, the acid soluble fraction was extracted three times with 5 ml of water-saturated diethyl ether. After evaporating the residual ether, the extracts were placed onto a Dowex 1- X 8, Cl^- form, anion-exchange column. The [^{3}H]inositol metabolites were eluted as described by Griffin and Hawthorn (1978): [^{3}H]inositol was eluted with 6 ml double distilled water, [^{3}H]inositol monophosphate with 7 ml of 30 mM HCl, [^{3}H]inositol diphosphate with 7 ml

of 90 mM HCl, and [^{3}H]inositol triphosphate with 7 ml of 500 mM HCl.

4. RESULTS

Bradykinin (1 μM) caused a rapid accumulation of inositol phosphates in NG108-15 cells (Fig. II-2). These results agree well with those of Yano et al. that bradykinin (1 μM) induces a rapid loss of ^{32}P-phosphatidylinositol 4,5-bisphosphate label in NG108-15 cells (Yano et al., 1984).

In order to establish a positive control of drug mediated PI response in SK-N-SH cells, we tested the effects of bradykinin, acetylcholine, substance P and ATP on PI turnover. These agents are known to stimulate PI turnover in various systems (Berridge and Irvine, 1984). Of all the agents tested, only acetylcholine (10 mM) provoked a rapid and prolonged accumulation of inositol phosphate metabolites (Fig. II-3). The effects of acetylcholine on the accumulation of inositol phosphates were abolished by atropine (5×10^{-7} M) suggesting that muscarinic receptors were involved in mediating this effect (Fig. II-3).

Although PI turnover can be stimulated in both cell lines, both the opiate drug etorphine and opioid peptides with different receptor selectivity (DAGO: μ specific, DADL: δ selective with some μ affinity), at high (1 μM) and low (10 nM) concentrations, failed to elicit a response (Table II-1). Furthermore, etorphine (1 μM) had no effect on modulating the bradykinin-elicited (NG108-15) nor acetylcholine-elicited (SK-N-SH) accumulation of inositol phosphates.

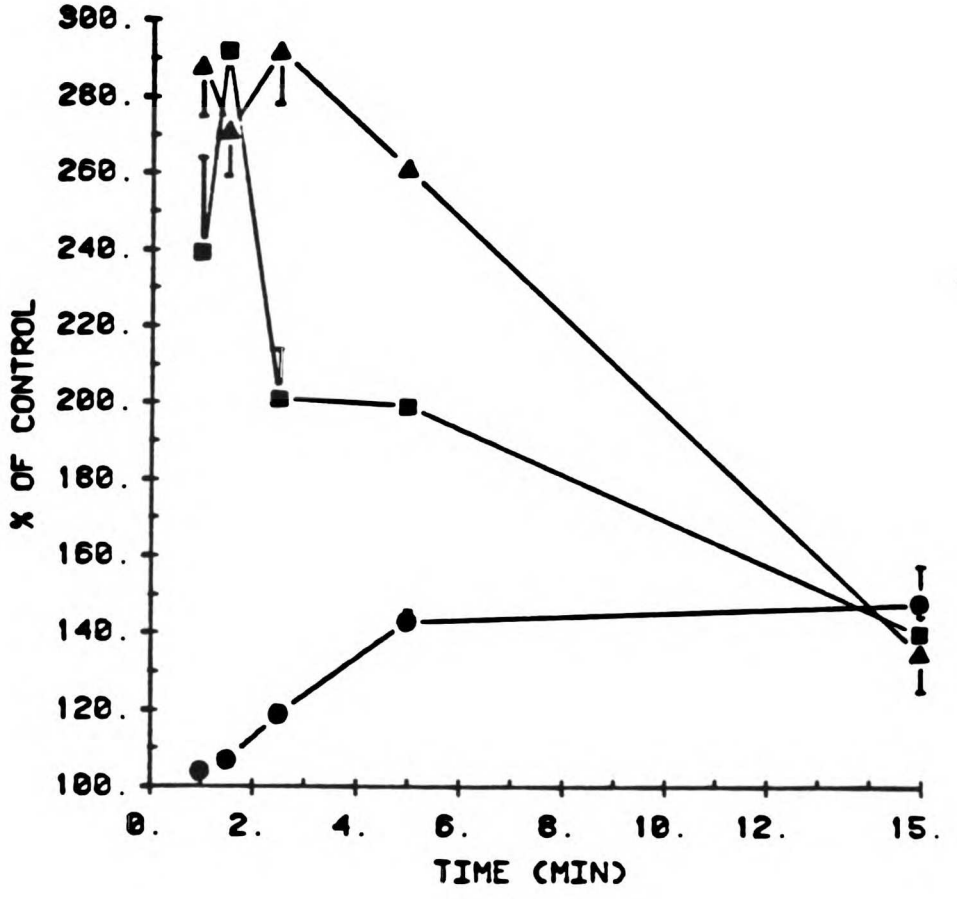

Figure II-2. Time course of accumulation of [^{3}H]inositol metabolites upon stimulation by bradykinin (1 μM) in NG108-15 cells. Values are means ± range of duplicate determinations and are one of the three experiments with similar results. Control values at 15 minutes were: inositol triphosphate (■), 224 d.p.m.; inositol diphosphate (▲), 462 d.p.m.; inositol monophosphate (●), 3602 d.p.m.

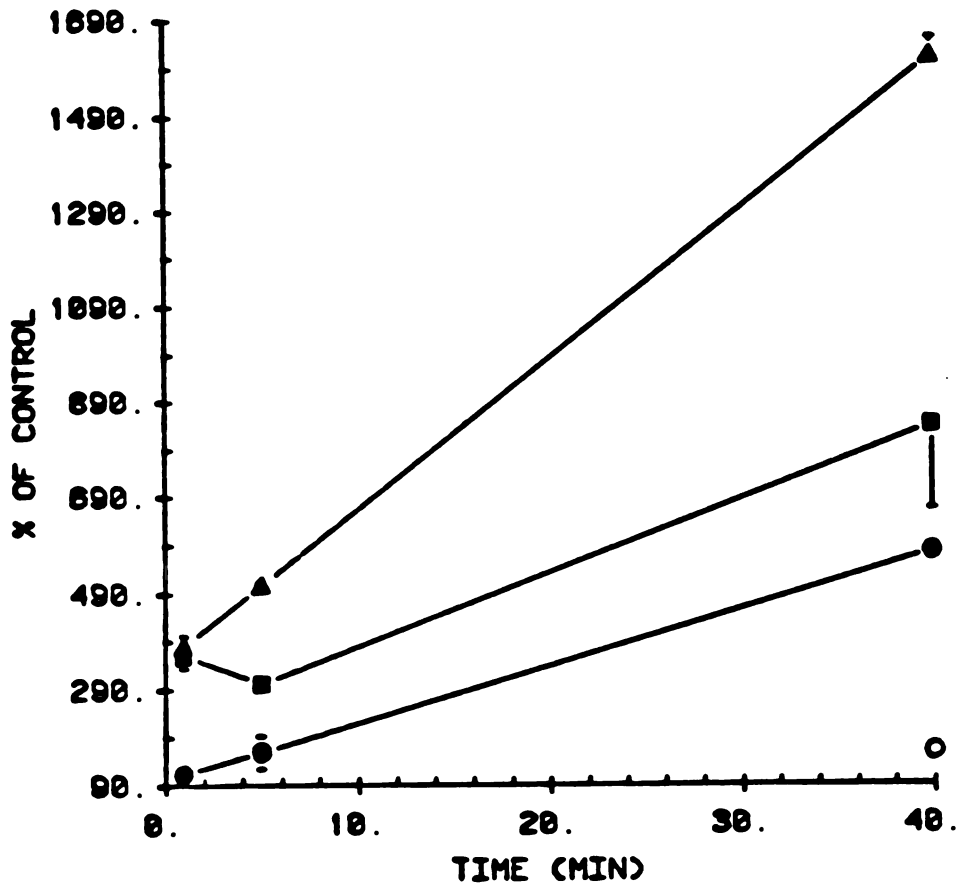

Figure II-3. Time course of accumulation of [^{3}H]inositol metabolites upon stimulation by acetylcholine (10 mM) in SK-N-SH cells. Values (inositol triphosphate (■), inositol diphosphate (▲), inositol monophosphate (● , incubated without atropine; ○ , incubated with 5 x 10^{-7}M atropine)) are means ± range of duplicate determinations and are one of three experiments with similar results. Control values at 40 minutes were: inositol triphosphate, 427 d.p.m.; inositol diphosphate, 579 d.p.m.; inositol monophosphate, 3877 d.p.m.

Table II-1. Effect of 30 min incubations with opioids on accumulation of [^{3}H]inositol metabolites in SK-N-SH and NG108-15 cells. Results are means ± range or S.E.M. for 2 or 3 experiments, respectively, each assayed in duplicate. Incubation terminated at 30s, 10 min, 15 min, 60 min, 120 min gave similar results.

		[^{3}H] radioactivity in inositol metabolites (% control)		
Cell Line	Treatment	Inositol monophosphate	Inositol diphosphate	Inositol triphosphate
SK-N-SH	DADL, 1 μM (n=3)	104±12	106±19	87±5
	Etorphine, 1 μM (n=3)	98±10	105±7	101±10
	DAGO, 1 μM (n=2)	101±5	93±10	99±7
NG108-15	DADL, 1 μM (n=2)	99.5±2.5	107±3	90±8
	Etorphine, 1 μM (n=2)	100±4	100±10	99.5±3

5. DISCUSSION

Of the three major opioid receptor subtypes (μ, δ, κ), only the δ receptor has been clearly shown to couple to adenylate cyclase (Sharma et al., 1975; West and Miller, 1983), while the second messenger of μ and κ receptors remain to be determined. This report establishes conditions under which stimulation of PI turnover by bradykinin (NG108-15) and acetylcholine (SK-N-SH) can be observed. Therefore, failure of opioids to affect the PI turnover in both cell lines under the same conditions strongly suggests that IP_3 is unlikely to serve as a direct second messenger for μ and δ receptors. However, it is unclear whether opioids have an effect on membrane hyperpolarization in SK-N-SH cells. If opioids indeed exert a similar electrophysiological effect on SK-N-SH cells as on neurons of myenteric plexus, then a mechanism other than phosphatidylinositol turnover, capable of mobilizing calcium, ought to be considered. Interestingly, upon completion of this work, Francel and Dawson (1986) reported that DADL had no effect on the PI turnover in a δ opioid receptor containing (Francel and Dawson, 1986; West and Miller, 1983) neuroblastoma hybrid cell line NCB-20. Their results are in good agreement with our findings in NG 108-15 hybrid cells.

CHAPTER III

EXPRESSION OF NEUROTRANSMITTER RECEPTORS IN SUBCLONES OF A HUMAN NEUROBLASTOMA CELL LINE

1. SUMMARY

Phenotypic variability of neurotransmitter receptors in the human neuroblastoma cell line SK-N-SH was studied with the use of three subclones that interconvert at a slower rate than the parent cell line, i.e., a neuroblast-type subclone (SH-SY5Y), a non-neuronal, strongly substrate adherant epithelial-like subclone (SH-EP), and an intermediate-type subclone (SH-IN). Dramatic differences were observed for the expression of neurotransmitter systems, i.e., the μ and δ opioid receptor, the muscarinic cholinergic receptor and its effect on phosphatidylinositol turnover, and the uptake$_1$ transporter for catecholamines. While these systems were strongly expressed in the neuroblast-like clones SH-SY5Y and SH-IN, they were absent or barely detectable in the non-neuronal EP clone. Furthermore, the μ and δ opioid receptors expressed in the SH-SY5Y and SH-IN clones were in a similar ratio as in the parent cells.

2. INTRODUCTION

Neuroblastoma cells are thought to represent primitive sympathoblasts which posess morphological and biological properties *in vivo* and *in vitro* (Biedler *et al.*, 1973; Ross and Biedler, 1985). Neuroblastoma cell lines established *in vitro* have been shown to consist of two morphologically distinct types of cells: neuroblast-like and epithelial-like (Tumilowicz *et al.*, 1970; Weston, 1970; Biedler *et al.*, 1973; Bernal *et al.*, 1983).

In order to facilitate study of the phenotypic variants, Biedler and colleagues isolated cloned cell lines from SK-N-SH that were relatively homogenous morphologically, although they could interconvert at a very slow rate (Ross *et al.*, 1983). Subclones include SH-SY5Y (neuroblast-like), SH-IN (intermediate), and SH-EP (epithelial-like).

The SK-N-SH cell line has recently been shown to express μ and δ opioid receptors, a catechol uptake$_1$ system (Richards and Sadée, 1986), and a acetylcholine-responsive phosphatidylinositol turnover system (Yu and Sadée, 1986). The acetylcholine-stimulated phosphatidylinositol turnover was blocked by atropine, suggesting that these cells also express muscarinic cholinergic receptors (Chapter II). Since the SK-N-SH cell line consists of two or more morphologically distinct cell types, it is unclear which of these cell types do or do not express any of these reported neurotransmitter receptor systems.

The purpose of this study was to investigate coordinate neurotransmitter receptor system expression among phenotypically stable subclones of SK-N-SH cells.

3. MATERIALS AND METHODS

3.1 Materials

The SH-SY5Y, SH-EP and SH-IN cell lines were provided by Dr. June Biedler of Sloan-Kettering Institute for Cancer Research, Rye, NY. All [^{3}H] chemicals were purchased from Amersham, Inc., Arlington, IL. Quinudidinyl benzylate (QNB)* was purchased from Research Biochemicals, Inc., Wayland, MA. All other general chemicals were purchased from Sigma, St. Louis, MO. The diprenorphine was donated by the National Institute on Drug Abuse, Bethesda, MD.

3.2 Methods

3.2a Cell Cultures

SH-SY5Y, SH-IN and SH-EP cells were grown at 37°C in monolayer in RPMI-164 medium supplemented with 10% fetal calf serum containing 100 µg streptomycin/ml and 100 IU penicillin/ml. Confluent cells were harvested with 0.04% EDTA in CA^{2+}/Mg^{2+} free phosphate-buffered saline with mechanical agitation. For receptor binding assays, freshly prepared membrane homogenates (Chapter I) were used.

3.2b Receptor Assays

The assay for µ and δ opiate receptors is similar to the previously described procedure (Chapter I). [^{3}H]DAGO (1 nM, 60 Ci/mmol); [^{3}H]diprenorphine (0.25 nM, 41 Ci/mmol) and [^{3}H]DADLE (1 nM, 50

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*Abbreviations used: QNB, quinudidinyl benzylate; DAGO, [D-Ala^2,MePhe^4,Gly-ol^5] enkephalin; DADL, [D-Ala^2,D-Leu^5] enkephalin.

Ci/mmol), the latter in the absence or presence of 10^{-5} M morphiceptin to block μ sites, were incubated with washed membrane homogenates (50 mM) Tris buffer, pH 7.4) at 20°C for 1 hr. The bound radioactivity was determined by filtering the homogenate mixture through Whatman GF/B glass fiber filters, after which the filter was washed three times with 1 ml ice-cold Tris-HCl buffer (50 mM). The glass fiber filter was then collected and soaked in 10 ml Scinti Verse II (Fischer Scientific Co., NJ) overnight, before the [^{3}H] activity was measured by liquid scintillation counting. Nonspecific tracer binding, measured in the presence of 1 μM unlabeled diprenorphine, accounted for less than 10% of total [^{3}H] binding.

The assay for muscarinic cholinergic receptors was derived from previously established methods (Hedlund et al., 1982; Richelson and Divinetz, 1977). One milliliter aliquots (~80-120 μg protein) of washed membrane homogenates were incubated for 60 min at 20°C in 1.5 ml polypropylene tubes containing [^{3}H](-)QNB (0.25 ± 0.4 nM; 30 Ci/mmol) and competing ligand. A filtration assay identical to the one described above for the opioid receptor assay was used to separate the bound from unbound [^{3}H] activity. Nonspecific tracer binding, determined in the presence of 10^{-6} M atropine, accounted for less than 10% of total [^{3}H] binding.

3.2c Measurement of Phosphatidylinositol Turnover

The procedure and method for [^{3}H]inositol labeling and [^{3}H]inositol phosphate metabolites separation was identical to the previously described method (Chapter II).

3.2d [^{3}H]Catecholamine Uptake

The accumulation of [2,5,6-^{3}H]dopamine (10 nM, 12 Ci-mmol) into SH-SY5Y cells via noradrenergic uptake sites (uptake$_1$ was measured in 17 mm culture wells as described (Richards and Sadée, 1986). Data were normalized to protein content (~0.2 mg/sample). Nonspecific uptake was defined in the presence of 10^{-6} M desipramine, representing ~10% or less of total cell-associated radioactivity. It was subtracted from all uptake data.

4. RESULTS

The three SK-N-SH subclones were tested for the presence of μ and δ opioid receptors and muscarinic cholinergic receptors (Table II-1), for carbachol effects on phosphatidylinositol turnover (Table III-2), and for the expression of the uptake$_1$ system for catecholamine transport (Table III-3). The tracers employed for the opioid receptors are either selective for μ sites only ([^{3}H]DAGO, for μ and δ sites ([^{3}H]DADL) or for μ, δ and κ sites ([^{3}H]diprenorphine). In the presence of 10^{-5} M morphiceptin, [^{3}H]DADL labeled only δ sites in the SK-N-SH cells (Yu et al., 1986). Whereas μ and δ tracer binding was indistinguishable between the neuroblast (SY-5Y) and intermediate (SH-IN) cells, [^{3}H]diprenorphine binding was barely detectable above background in the substrate-adherent SH-EP cells. In the latter cells, only [^{3}H]diprenorphine was employed because of its high affinity for all sites.

A similar expression pattern, i.e., positive for SH-SY5Y and SH-IN

Table III-1. Expression of μ and δ opioid receptors and of muscarinic cholinergic receptors in subclones of SK-N-SH. Nonspecific tracer binding was subtracted. The experiments were repeated with similar results.

	Specific binding (dpm/mg protein)				
	[^{3}H]DAGO (μ sites)	[^{3}H]DADL (μ+δ sites)	[^{3}H]DADL + morphiceptin (δ sites)	[^{3}H]diprenorphine (μ+δ sites)	[^{3}H]QNB
SH-SY5Y	8670 ± 380[c]	7620 ± 110[c]	2720 ± 810[c]	7980 ± 590[c]	13,410 ± 430
SH-IN	9280 ± 300	5470 ± 36	2340 ± 60	6690 ± 180	16,020 ± 920
SH-EP	*[a]	*	*	428 ± 50	N.D.[b]

[a]Not determined.

[b]Not detectable above background (~400 dpm).

[c]± range, n=2.

[d]S.D., n=3.

Table III-2. Stimulation of the accumulation of [^{3}H]inositol monophosphate ([^{3}H](IP)) by carbachol in SK-N-SH subclones (in the presence of Li^+, over 40 min).

	[^{3}H]IP (dpm/sample) (N=2, ± range)	
	Control	Carbachol (1 mM)
SH-SY5Y	2590 ± 360	5950 ± 690
SH-IN	2800 ± 40	9330 ± 50
SH-EP	1080 ± 30	1480 ± 160
	604 ± 30[a]	698 ± 50[a]

[a]Independent experiment; n=3, ± S.D. Further experiments with SH-IN and SH-SY5Y repeatedly yielded similar results.

Table III-3. Expression of the catechol uptake$_1$ system in SK-N-SH subclones. [^{3}H]Dopamine (10 nM) uptake was measured in the absence or presence of 10^{-6} M desipramine (nonspecific uptake ~6000 dpm/sample).

	[^{3}H]Dopamine (dpm/mg protein)[a]
SH-SY5Y	132,300 ± 13,600
SH-IN	153,400 ± 64,721
SH-EP	N.D.[b]

[a]± S.D., n=8, from two independent experiments.

[b]Not detectable above background (nonspecific uptake).

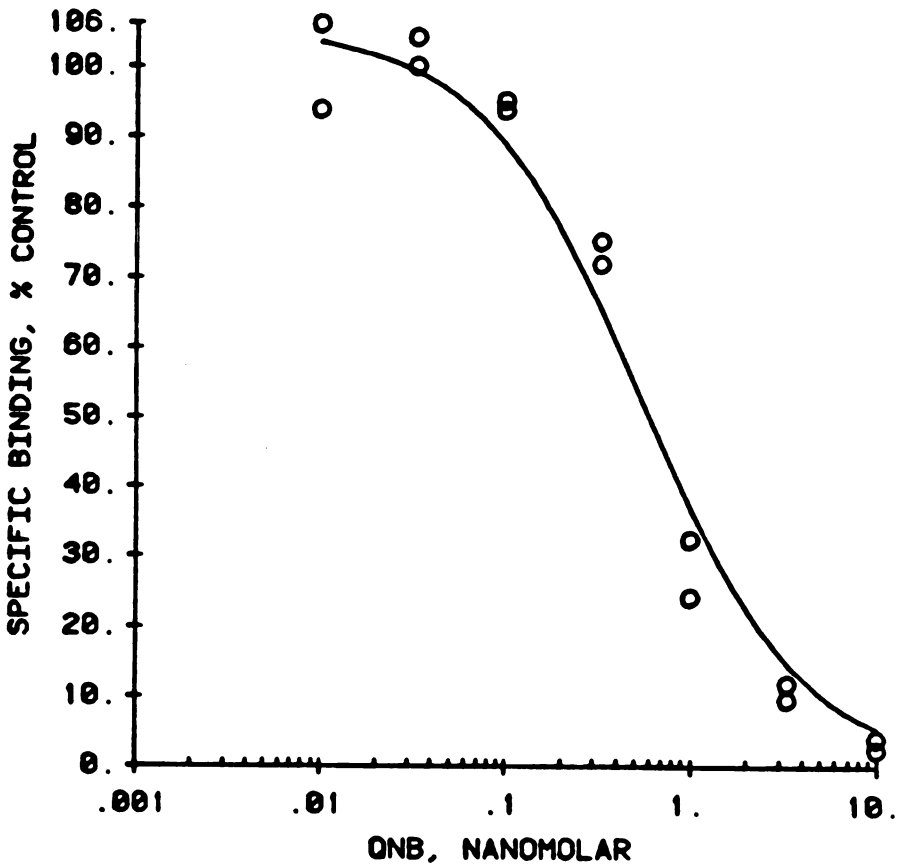

Figure III-1. Equilibrium displacement curve of QNB against [^{3}H]QNB. Binding experiments were done in SH-SY5Y cell membrane homogenates. The solid line represents the computer fit to a one-site binding model, yielding KD = 0.16 nM and B_{max} = 0.46 pmol/mg protein.

and negative for SH-EP, was found for the muscarinic cholinergic receptors (Table II-1) and the carbachol-mediated stimulation of [^{3}H]inositol monophosphate relase (Table II-2) (as well as inositol di- and triphosphate release; data not shown). [^{3}H]QNB labeling of the SH-IN cells was consistently higher than that of the SH-SY5Y cells, as reflected also by a greater stimulation of inositol phosphate release in the SH-IN cells. Titration of the [^{3}H]QNB binding sites on SH-SY5Y cells with unlabeled QNB revealed a receptor density of 0.46 pmol/mg protein (Fig. III-1). By contrast, only a small stimulation of [^{3}H]inositol phosphate release was observed for SH-EP cells, in two experiments. It was too small for quantitative evaluation, but was much smaller than for the other two SK-N-SH clones.

Finally, the uptake of [^{3}H]dopamine into the SH-SY5Y and SH-IN clones was much greater than that into the SH-EP clone (Table III-3). In the former two clones, desipramine (10^{-6} M) greatly reduced the uptake of [^{3}H]dopamine which is consistent with the uptake$_1$ system previously described for SK-N-SH cells (Richards and Sadée, 1986).

5. DISCUSSION

The three subclones of SK-N-SH (SH-SY5Y, SH-IN, and SH-EP) are suitable for the study of phenotypic interconversion within the neural crest lineage, as they are relatively stable and interconvert at a very slow rate (Ross et al., 1983).

A striking difference was observed with the expression of neurotransmitter systems among the SK-N-SH clones. The μ and δ opioid

receptors, the muscarinic cholinergic receptor, and the uptake$_1$ system were all expressed to similar degrees in the neuroblast-like SH-SY5Y and SH-IN clones, while they were either absent or at the detection threshold in the non-neuronal SH-EP cells. The expression of μ and δ opioid receptors in SH-SY5Y cells was recently confirmed (Kazmi and Mishra, 1986). Together with the same result for catecholamine-synthesizing enzymes (Ross et al., 1983), it appears that these neurotransmitter systems are under coordinate control that parallels observed morphological changes between the non-neuronal and neuronal cells.

Although results from electrophysiological studies have suggested that a substantial fraction of the opioid-responsive cells carry more than one opioid receptor type, e.g., μ and δ (Zieglgansberger et al., 1982; Egan and North, 1981), almost all cell lines that have been shown to carry opioid receptors were found to express a homogenous population of δ receptors (West and Miller, 1983; Gunther et al., 1986). Therefore, some uncertainty remains as to whether the μ and δ opioid receptors in SK-N-SH cells were expressed together in a single cell population or separately in two sub-populations of SK-N-SH cells. This study provides indirect evidence that μ and δ opioid receptors were indeed co-expressed in a single cell.

CHAPTER IV

DIFFERENTIATION OF HUMAN NEUROBLASTOMA CELLS: MARKED POTENTIATION OF PGE_1-STIMULATED ACCUMULATION OF CYCLIC AMP BY RETINOIC ACID

1. SUMMARY

Human neuroblastoma cells, SH-SY5Y, were induced to differentiate by treatment with either nerve growth factor (NGF, 50 ng/ml), retinoic acid (RA, 10 μM), dibutyryl cyclic AMP (dBcAMP, 1 mM) or 12-0-tetradecanoyl-phorbol-13-acetate (TPA, 0.1 μM), and changes of several neurotransmitter receptor systems were determined. Muscarinic cholinergic receptor density and the effect of carbachol (1 mM) on phosphatidylinositol (PI) turnover were not affected. RA up-regulated while dBcAMP down-regulated μ opioid receptor, each by a factor of two or less. [^{3}H]Dopamine uptake was enhanced twofold in RA-treated cells. However, striking differences were observed among the effects of differentiating agents on PGE_1 (1 μM) stimulated cAMP accumulation, which was drastically increased only by retinoic acid. Similar results were obtained with PGE_2, although PGE_2 was less efficacious than PGE_1 at 1 μM concentration. The increased sensitivity to PGE_1 was associated with an effect on PGE_1 receptors, while the Gs coupling proteins and adenylate cyclase were not measurably affected. A similar enhancement of the PGE_1-cAMP response by retinoic acid was also observed in two additional human neuroblastoma cell lines tested, Kelly and IMR-32, suggesting that up-regulation of the prostaglandin response by retinoic

acid is common among neuroblastoma cells.

2. INTRODUCTION

Neuroblastoma cell lines represent immature peripheral neurons that frequently express several interconverting morphological phenotypes when grown in culture, i.e., neuroblast cells and non-neuronal cells (Ross et al., 1983). To study differentiation of neuroblastoma cells into mature neurons, we have selected a phenotypically stable neuroblast subclone (SH-SY5Y) derived by Biedler and colleagues from the parent SK-N-SH neuroblastoma cell line (Ross et al., 1983). SH-SY5Y cells differentiate in the presence of retinoic acid (RA) (Påhlman et al., 1984), nerve growth factor (NGF) (Påhlman et al., 1981; Spinelli et al., 1982; Sonnenfeld and Ishii, 1982; Perez-Polo et al., 1979; Schulze and Perez-Polo, 1982), 12-0-tetradecanoyl-phorbol-13-acetate (TPA) (Påhlman et al., 1984; Påhlman et al., 1981; Spinelli et al., 1982), dibutyryl cyclic AMP (dBcAMP) (Schulze and Perez-Polo, 1982; Kuramoto et al., 1977; Kuramoto et al., 1981), and deoxybromouridine (Kuramoto et al., 1981). Morphological differentiation marked by neurite extension is associated with the appearance of electrical excitability (Perez-Polo et al.. 1979; Kuramoto et al., 1977; Kuramoto et al., 1981), enhancement of neuron specific enolase activity (Påhlman et al., 1984) and increase of neurotransmitter concentrations and neurosecretory granules (Påhlman et al., 1981). The enhancement of neuronal markers after differentiation treatment may suggest that the differentiated SH-SY5Y cell is a better model for neurotransmitter-receptor coupling studies.

The SH-SY5Y cell line, like the parent cell line SK-N-SH expresses μ and δ opioid receptor in a ratio of approximately 5 to 1 (Yu et al., 1986; Kazmi and Mishra, 1986), muscarinic cholinergic receptors and the catecholamine $uptake_1$ system (Richards and Sadée, 1986). We have further established that the muscarinic cholinergic receptors in these cells are positively coupled to phosphatidylinositol (PI) turnover (Yu and Sadée, 1986). While opioids did not affect phosphatidylinositol turnover (Yu and Sadée, 1986), they reduced PGE_1 stimulated accumulation of cAMP (Yu et al., 1986).

The goal of the present study was to examine the fate of several neurotransmitter systems during differentiation with various agents. The results show differential effects of several inducing agents on the neurotransmitter systems, most notably a drastic enhancement by retinoic acid of the PGE_1 stimulated cAMP accumulation.

3. MATERIALS AND METHODS

3.1 Materials

The SH-SY5Y cell line was kindly provided by Dr. June L. Biedler of the Sloan-Kettering Institute for Cancer Research, Rye, NY. The IMR-32 cell line was obtained from the American Type Culture Collection, Rockville, MD, and the Kelly cells were a gift from Dr. Fred Gilbert of Mount Zion School of Medicine, NY. The cyclic AMP assay kit and other [^{3}H] chemicals were purchased from Amersham, Inc., Arlington Heights, IL. The following compounds were purchased from Sigma, St. Louis, MO: dBcAMP, all-trans-retinoic acid, NGF (2.5 S form), TPA, atropine, PIA,

desipramine, TRH and morphiceptin. DAGO,* DADLE and VIP were purchased from Peninsula Laboratories, Palo Alto, CA; cholera toxin and pertussis toxin from List Biological, Campbell, CA, and quinuclidinyl benzilate (QNB) from Research Biochemicals Inc., Wayland, MA. Diprenorphine was donated by the National Institute on Drug Abuse, Bethesda, MD.

3.2 Methods

3.2a Cell Culture

SH-SY5Y, IMR-32 and Kelly cells were grown at 37°C in monolayer in RPMI-1640 medium supplemented with 10% fetal calf serum containing 100 µg streptomycin/ml and 100 IU penicillin/ml. Confluent cells were harvested with 0.04% EDTA in Ca^{2+}/Mg^{2+} free phosphate-buffered saline with mechanical agitation. For receptor binding assays, freshly prepared membrane homogenates (Yu et al., 1986) were used, while for cyclic AMP production, catecholamine uptake and PI turnover experiments, intact cells were used.

3.2b Differentiation

Cells were induced to differentiate by culturing with either NGF (50 ng/ml), RA (10 µM), dBcAMP (1 mM) or TPA (0.1 µM) for 6 days. Medium was replaced every 2 days. In the case of RA- or TPA-treated cells, equal ethanol concentrations were present in the control cells.

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*Abbreviations used: RA, retinoic acid; NGF, nerve growth factor; dBcAMP, dibutyryl cyclic AMP; TPA, 12-0-tetradecanoyl-phorbol-13-acetate; PGE_1, prostaglandin E_1; IBMX, 3-isobutyl-1-methyl-xanthine; PI, phosphatidylinositol; DAGO, [D-Ala^2, MePhe^4, Gly-ol^5] enkephalin; DADLE, [D-Ala^2, D-Leu^5] enkephalin; QNB, quinuclidinyl benzilate; VIP, vasoactive intestinal peptide; PIA, phenylisopropyl-adenosine; TRH, thyrotropin releasing hormone.

For cells differentiated in 17 mm wells, the inducing agent was added no earlier than 24 hrs after the transfer of the cells to allow sufficient time for seeding.

3.2c Opiate Receptors

The assay of μ and δ opiate receptors on SK-N-SH cells has been previously described (Chapter III). [^{3}H]DAGO (1 nM, 60 Ci/mmol) and [^{3}H]DADLE (1 nM, 50 Ci/mmol), the latter in the absence or presence of 10^{-5} M morphiceptin to block μ sites, were incubated with washed cell membrane homogenates (50 mM Tris buffer, pH 7.4) at 20°C for 1 hr. The bound radioactivity was determined by filtering the homogenate mixture through Whatman GF/B glass fiber filters (Chapter III). Tracer binding in the presence of 10^{-6} M diprenorphine served as the value for non-specific binding.

3.2d Muscarinic Cholinergic Receptors

The receptor assay has been described previously (Chapter III). Briefly, 1 ml aliquots (~ 80-120 μg protein) of washed membrane homogenates were incubated for 60 min at 20°C in 1.5 ml polypropylene tubes containing [^{3}H](-)QNB (0.25 to 0.4 nM; 30 Ci/mmol) and competing ligand. [^{3}H](-)QNB labeling reached a plateau at 60 min at which time bound [^{3}H] activity was determined by the filtration assay as described (Chapter III). Nonspecific binding was determined in the presence of 10^{-6} M atropine.

3.2e Measurement of Phosphatidylinositol Turnover

The procedure and method for [^{3}H]inositol labelling and [^{3}H]-

inositol phosphate metabolites separation were identical to the previously published method (Chapter II).

3.2f [^{3}H]Catecholamine Uptake

The accumulation of [2,5,6-^{3}H]-dopamine (10 nM, 12 Ci/mmol) into SH-SY5Y cells via noradrenergic uptake sites (uptake$_1$) was measured in 17 mm culture wells as decribed (Richards and Sadée, 1986). Data were normalized to protein content (~ 0.2 mg/sample). Nonspecific uptake was defined in the presence of 10^{-6} M desipramine, representing ~ 10% or less of total cell associated radioactivity. It was subtracted from all uptake data.

3.2g cAMP Production

These studies were done in 17 mm culture wells with slight modification of a previously described procedure (Yu et al., 1986). Briefly, intact attached monolayer cells in each well were rinsed once with 1 ml serum free RPMI-1640 medium after aspiration of culture medium. PGE$_1$ was added after a preincubation period of 10 min with 0.5 mM IBMX in 300 µl serum free medium at 37°C. The reaction was terminated by adding 100 µl ice-cold 20% w/v trichloroacetic acid directly to the cell culture medium. Since replacement with fresh medium before adding trichloroacetic acid had no significant effect on the cAMP level in each well. The cells were then scraped from the well and transferred to 1.5 ml polypropylene microcentrifuge tubes and centrifuged (500 x g, 15 min, 0°C). The supernatants were extracted three times with 2 ml of water-saturated diethyl ether. After evaporation of the residual ether, the cAMP content was determined with

the use of a competitive protein binding assay kit (Amersham). Protein concentration was determined with the method of Lowry et al. (1951), and the data were normalized to protein content (~ 0.25-0.3 mg/well).

3.2h Data Analysis

IC_{50} values of receptor binding were determined by fitting the data to the logistic function

$$\% B = \% Bmax - \% Bmax * L^n/(IC_{50}{}^n + L^n)$$

(B = specific bound tracer (d.p.m.); L = concentration of displacing ligand) with the use of the computer program MAKE Model in the Prophet-NIH system as previously described (Chapter I).

4. RESULTS

Table IV-1 summarizes the effects of six-day treatments with three differentiating agents on several neurotransmitter receptor systems in SH-SY5Y cells. Specific μ and δ receptor binding increased in RA- and NGF-treated but decreased in dBcAMP-treated cells (Table IV-1). Specific muscarinic cholinergic receptor binding, on the other hand, was not affected by any of the differentiation inducers (Table IV-1). Analysis of equilibrium binding isotherms gave the following IC_{50} values and slope factors (n), for muscarinic cholinergic receptors (QNB against [^{3}H](-)QNB): control (0.51 ± 0.13 nM, n=1.53), RA (0.68 ± 0.17 nM, n=1.65) and dBcAMP (0.52 ± 0.21, n=1.51). Similarly, the μ receptor

Table IV-1. Effects of differentiation on the neurotransmitter receptor systems. Equilibrium binding experiments were carried out with fresh washed membrane homogenates at 20°C while carbachol-induced PI turnover and [^{3}H]dopamine experiments were carried out with intact cells at 37°C. All experiments were done in parallel with treated (6 days) and untreated cells and the results are expressed as percentage of the values obtained from untreated (control) cells. Data shown are either mean ± S.D. ($n \geq 3$) or mean ± range ($n = 2$).

	% Control (untreated) cells		
	RA	dBcAMP	NGF
Specific μ binding	182 ± 38 (n = 6)	70 ± 8 (n = 7)	167 ± 36 (n = 3)
Specific δ binding	156 ± 10 (n = 3)	49 ± 28 (n = 4)	116 ± 7 (n = 3)
Specific [^{3}H]QNB binding	96 ± 12 (n = 3)	117 ± 12 (n = 3)	
Carbachol (1 mM) induced release of [^{3}H]inositol triphosphate	91 ± 9 (n = 2)	116 ± 10 (n = 2)	
Specific [^{3}H]dopamine uptake	231 ± 58 (n = 2)	111 ± 15 (n = 2)	

affinities toward its ligands also remained unaltered after differentiation treatment. These results indicate that the receptor affinities toward their respective ligands remain unchanged after RA and dBcAMP treatment; therefore, a change in specific binding of [^{3}H] ligand directly reflects a change in receptor concentration on these cells.

The effect of carbachol (1 mM) on inositol triphosphate release was not affected by differentiation (Table IV-1). The catecholamine uptake$_1$ system responded with a twofold increase only after RA-treatment (Table IV-1). This system was tested here with [^{3}H]dopamine rather than norepinephrine, because [^{3}H]dopamine was more efficiently concentrated into the cells (Richards and Sadée, 1986). However, none of these differentiation effects caused larger than two- to threefold changes.

The basal level of cAMP accumulation determined in the presence of IBMX (0.5 mM) (a phosphodiesterase inhibitor) was not affected by NGF nor TPA treatment, whereas there was a 30% increase in RA-treated cells (Fig. IV-1, left panel). However, upon PGE$_1$ (1 µM) stimulation, the cAMP level accumulated over 15 min reached 30-fold higher level in RA-treated cells than in control cells (Fig. IV-1, right panel).

TPA-differentiated cells also displayed an enhanced PGE$_1$-cAMP response, but the increase was only two- to threefold (Fig. IV-1, right panel), while the basal level was not affected (Fig. IV-1, left panel).

The dBcAMP differentiated cells could not be directly evaluated because of the interference by dBcAMP with intracellular cAMP levels. However, the cAMP level in response to PGE$_1$ stimulation did not exceed the control by more than fivefold, suggesting that dBcAMP-differentiation failed to mimic RA in the PGE$_1$-cAMP response.

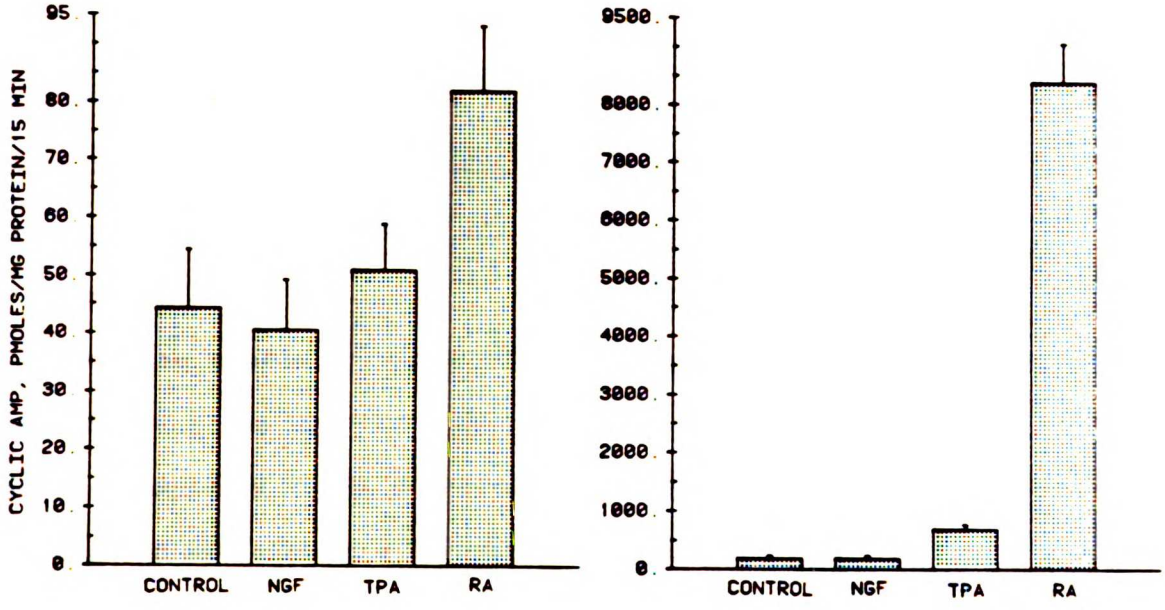

Figure IV-1. cAMP accumulation in intact cells. Cells were treated with differentiating agents for six days.
Left panel: basal level of cAMP.
Right panel: PGE_1 (1 μM) stimulated accumulation of cAMP.
Results shown are mean ± S.D. of at least three experiments, measured in the presence of 0.5 mM IBMX.

The time course of PGE_1-stimulated accumulation of cAMP in both control and RA-treated cells is shown in Fig. IV-2. Accumulation of cAMP in control and RA-treated cells peaked at 5 and 30 min, respectively (Fig. IV-2). While partial refractoriness to PGE_1 stimulation occurred after 5 min in control cells (Fig. IV-2, left level), tachyphylaxis was much less apparent in the RA-treated cells (Fig. IV-2, right panel).

The 15 min PGE_1 (1 µM)-stimulated accumulation of cAMP in cells treated for six days with 0.1, 1 and 10 µM RA was 1931 ± 78, 3132 ± 49 and 7447 ± 186 pmol cAMP per mg protein (control values: 392 ± 10.5 pmol/mg). These results indicate that the effect of RA is concentration dependent, and that a large effect occurs already with nanomolar RA concentration.

The time course of the RA-induced sensitization of the PGE_1-cAMP response is shown in Fig. IV-3. While neurite outgrowth required continued exposure to RA for at least 48 hrs, the potentiation of cAMP accumulation was already substantial at 24 hrs (Fig. IV-3).

RA treatment did not result in uncoupling of inhibitory control of adenylate cyclase as a possible explanation of the enhanced PGE_1-cAMP response since the inhibitory opioid effect on PGE_1-cAMP accumulation was actually enhanced. In control cells, morphine (10 µM) caused only a small inhibition (19.5 ± 6.1%, n = 5), but in RA-treated cells, the inhibition was large (49.3 ± 5.7%, n = 7). Hence, both stimulatory and inhibitory adenylate cyclase-receptor coupling were enhanced by RA treatment. The enhanced inhibitory response of opiates on cAMP accumulation in RA-treated SH-SY5Y cells provides an excellent model for studying the molecular pharmacology of opiates, and specifically

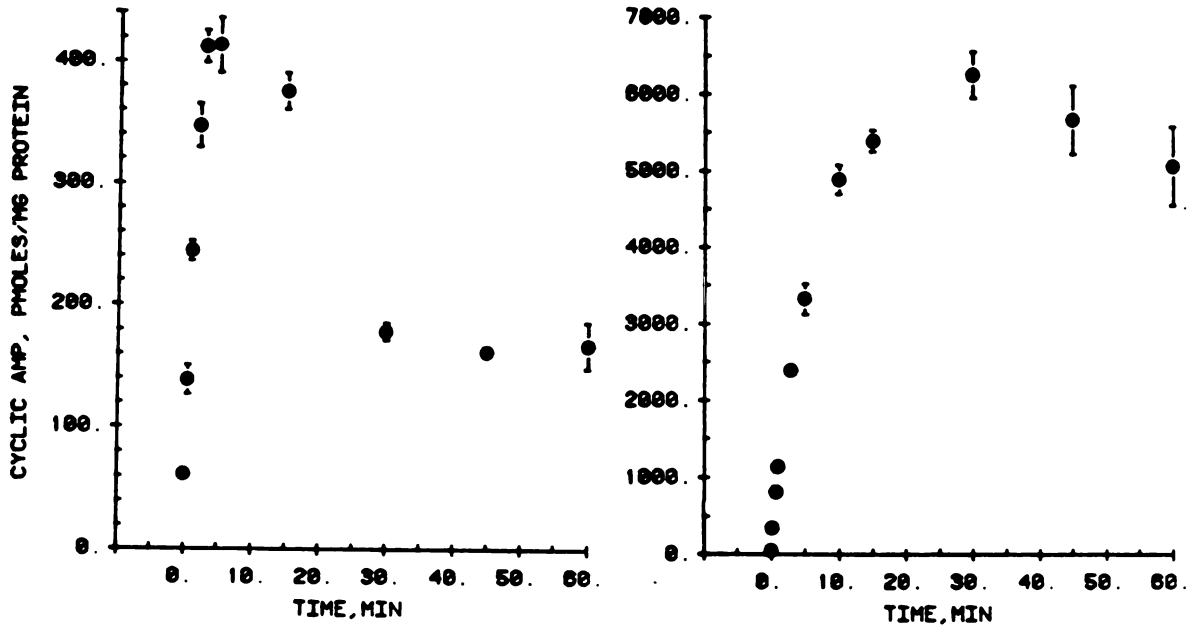

Figure IV-2. Time-course of cAMP accumulation upon PGE_1 stimulation. Intact cells were stimulated with 1 μM PGE_1 at 37°C and the accumulation of cAMP was measured in both control (left panel) and RA-treated (right panel) cells. Values shown are mean ± range of duplicate samples measured in the presence of 0.5 mM IBMX. A duplicate experiment gave similar results.

narcotic analgesics, at the μ receptors.

In order to determine whether the increased PGE_1-cAMP response is caused by an increase of adenylate cyclase itself, we also tested the effects of RA on forskolin-stimulated accumulation of cAMP in intact cells. Forskolin has been reported to activate adenylate cyclase directly in a guanyl nucleotide-independent manner (Seamon et al., 1981). In control cells, forskolin (100 μM) was more potent that PGE_1 (1μM) in causing cAMP accumulation (Fig. IV-3); however, the forskolin-cAMP response increased by only 80% over the 6 day treatment with RA, in contrast to a 15-to 30-fold increase of the PGE_1-cAMP response.

Dose-response curves of PGE_1 in stimulating cAMP accumulation in control and RA-treated SH-SY5Y cells are shown in Fig. IV-4. At 1 μM concentration of PGE_1, which was the concentration commonly used here, cAMP accumulation was maximally stimulated in RA-treated cells and was near maximum (~80%) in control cells (Fig. IV-4). While forskolin dose-response curves were superimposable (data not shown), there was a large shift of the EC_{50} values of the PGE_1 stimulation from control (236 ± 46 nM) to RA-treated cells (13.5 ± 3 nM) (Fig. IV-4). Maximum cAMP accumulation (E_{max}) was increased from 486 ± 22 pmol cAMP/mg protein/15 min in control cells to 5822 ± 222 pmol cAMP/mg protein/15 min in RA-treated cells. The slope factor, n, however, was unaffected (control, n=0.72 ± 0.06; RA treatment, n=0.81 ± 0.1).

Since PGE_2 predominates over PGE_1 in vivo, the adenylate cyclase response to PGE_2 was also tested. In intact SH-SY5Y cells, PGE_2 (1 μM) stimulation resulted in 294 ± 6 pmol cAMP/mg protein/15 min in control, and 2364 ± 148 pmol cAMP/mg protein/15 min in RA-treated cells.

To investigate whether the observed effect on cAMP response by RA

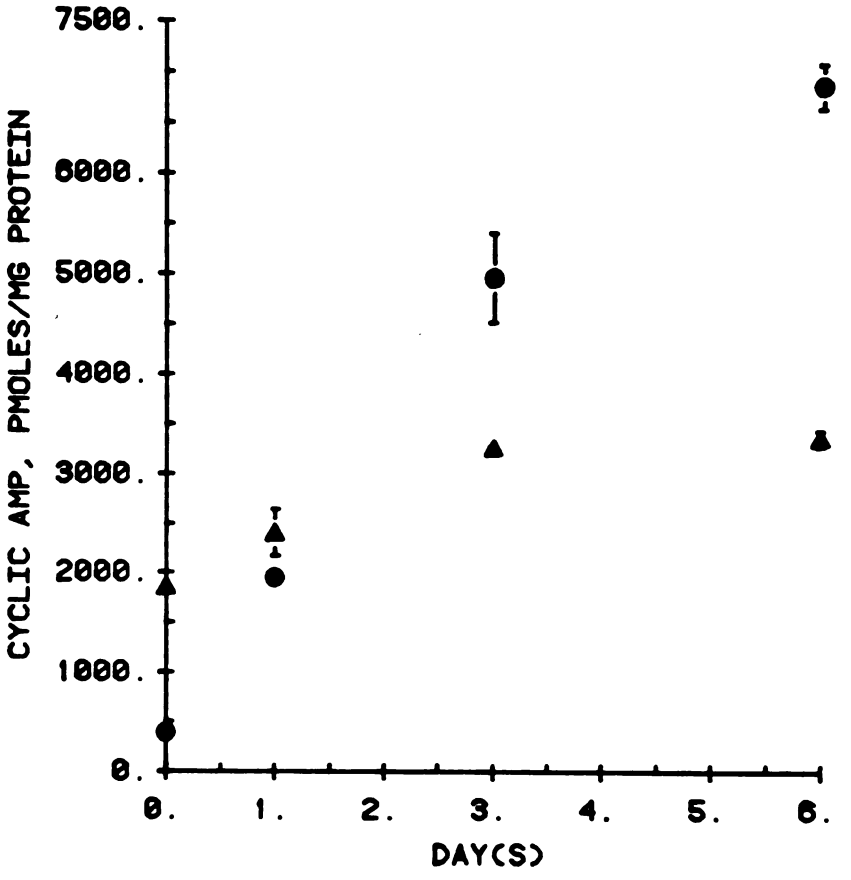

Figure IV-3. Time dependence of the RA effect on stimulated cAMP accumulation. Cells were treated with 10 μM RA for up to six days. cAMP accumulation was measured in the presence of IBMX (0.5 mM) 15 min after the addition of PGE_1 (1 μM) (●) or forskolin (100 μM) (▲). Values shown are mean ± range of duplicate samples. A duplicate experiment gave similar results.

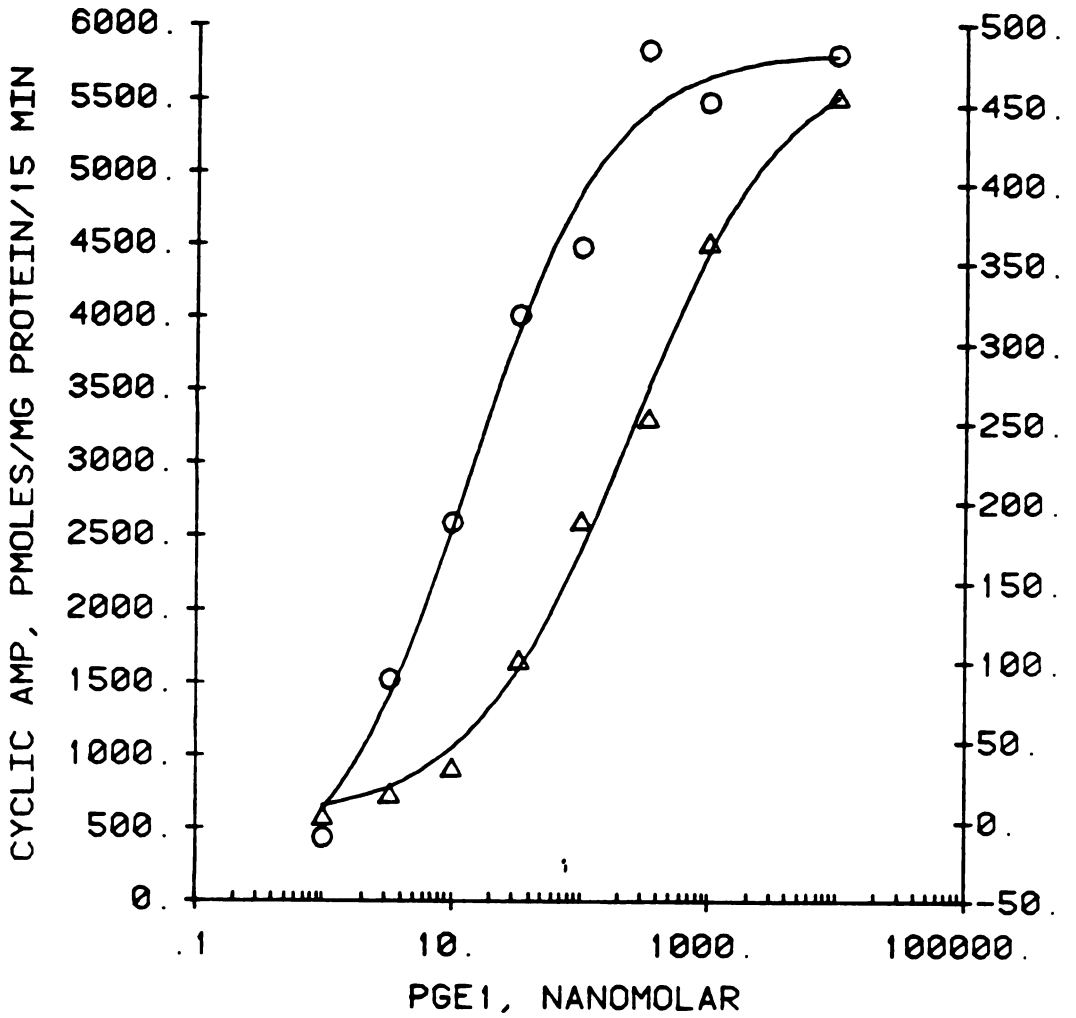

Figure IV-4. Dose-response curves of PGE_1 in stimulating cAMP accumulation. Intact SH-SY5Y cells were stimulated with increasing concentrations of PGE_1 and intracellular cAMP content was measured (in the presence of 0.5 mM IBMX) in control (△ , right y-axis) and RA-treated (○ , left y-axis) cells. Values represent mean of duplicate samples that are within ± 10% of the mean. A repeated experiment gave similar results.

is also applicable to other agents that stimulate adenylate cyclase via receptor-Gs coupling unit, various concentrations of isoproternol, dopamine, VIP, TRH, vasopressin, and the adenosine receptor agonist PIA (in the absence of IBMX) were tested in intact SH-SY5Y cells. Of all these agents tested, only PIA and VIP generated a stimulatory response of cAMP accumulation, which was not drastically changed by RA treatment (<3-fold change in several experiments). Further, RA did not uncover a response to the other stimulatory agents.

To determine whether the enhancement of the PGE_1-cAMP response by RA in SH-SY5Y cells is common among neuroblastoma cell lines, two additional human neuroblastoma cell lines, IMR-32 and Kelly, were tested. The effects of PGE_1 (1 μM) were compared in control (untreated) and RA-treated (10 μM X 6 days) cells (Table IV-2). The basal level of cAMP in Kelly cells was relatively high (181 pmol/mg protein/15 min) in comparison to SH-SY5Y (38 pmol/mg protein/15 min) or IMR-32 cells (30 pmol/mg protein/15 min). However, upon RA treatment, the basal level of cAMP in Kelly cells was reduced (28 pmol/mg protein/15 min) in contrast to a slight increase in SH-SY5Y cells (47 pmol/mg protein/15 min) or no change in IMR-32 cells (31 pmol/mg protein/15 min). Despite these differences, RA greatly enhanced the ability of PGE_1 to elevate cAMP above basal levels in all three cell lines tested (Table IV-2).

5. DISCUSSION

The differentiation agents RA, dBcAMP and NGF all induce similar morphological changes in SH-SY5Y cells (Påhlman et al., 1984; Påhlman et

Table IV-2. PGE_1 (1 μM)-stimulated cAMP accumulation.

Cell lines	% of basal level[a]	
	Control	RA-treated
SH-SY5Y	1180 ± 17	15610 ± 505
Kelly	144 ± 29	1807 ± 31
IMR-32	380 ± 44	1711 ± 57

[a]Measured (in the presence of 0.5 mM IBMX) as pmol cAMP/mg protein/15 min.

al., 1981; Spinelli et al., 1982; Sonnenfeld and Ishii, 1982; Perez-Polo et al., 1979; Schulze and Perez-Polo, 1982; Kuramoto et al., 1977; Kuramoto et al., 1981); however, their effects on the neurotransmitter systems were diverse. RA and NGF both up-regulated opioid receptors in SH-SY5Y cells. Up-regulation of opioid receptor sites by NGF has previously been reported in the rat pheochromocytoma cell line PC12 (Inoue and Hatanaka, 1982).

High concentrations of opioid receptors localized at neuritic processes may account for the effect of RA or NGF on opioid receptor density in these cells. The unexpected down-regulation of the μ and δ opioid receptors by dBcAMP points to the presence of multiple regulatory mechanisms that determine the level of each of the neurotransmitter systems. Noronha-Blob et al. (1986) also reported that dBcAMP down-regulates opioid, muscarinic and α_2-adrenergic receptors simultaneously in a clonal hybrid neuroblastoma cell line, NCB-20.

In contrast, dBcAMP did not affect the muscarinic receptor in SH-SY5Y cells. Further, the time course of opioid receptor down-regulation in SH-SY5Y cells was gradual and paralleled the time course of morphological differentiation (data not shown), whereas in NCB-20 cells, down-regulation occurred rapidly and peaked at 2 days (Noronha-Blob et al., 1986). Down-regulation of opioid receptors by dBcAMP may, therefore, proceed via different pathways in these two cell lines.

The most dramatic change observed after differentiation was the enhanced PGE_1-cAMP response after RA treatment. This enhancement was observed in three human neuroblastoma lines tested, SH-SY5Y, Kelly and IMR-32. Similarly, RA was previously found to increase the stimulatory control of adenylate cyclase by calcitonin (7.5-fold) (Liapi et al.,

1986) and parathyroid hormone (two- to fourfold) (Evain et al., 1981; Evain et al., 1982) in F9 embryonal carcinoma cells. However, in the neuroblastoma cell line SH-SY5Y, the RA effects were specific to the PGE_1-cAMP response, since none of the other cAMP-stimulatory agents tested was significantly altered by RA. A similar enhancement of the PGE_1-cAMP response in two additional human neuroblastoma lines tested, IMR-32 and Kelly, suggest that this RA effect is common among neuroblastoma cells. PGE_2 stimulation was also enhanced by RA, but PGE_2 was less efficacious than PGE_1 under comparable conditions.

These findings in different cell types suggest that the cAMP response is a primary target of RA-induced differentiation and that the dramatic increase of the PGE_1-cAMP response in SH-SY5Y cells can serve as a model to elucidate the responsible molecular mechanisms. TPA-differentiated cells also displayed an enhanced PGE_1-cAMP response, but the increase was only two- to threefold. This result parallels findings in S-49 lymphoma cells that TPA acutely enhances by twofold the isoproterenol stimulated, but not basal cAMP level, presumably via an action on protein kinase C (Bell et al., 1985). The much greater cAMP response after RA differentiation in SH-SY5Y suggests that activation of protein kinase C does not serve as a major mechanism in mediating the RA effect.

The failure of RA to cause large changes of forskolin stimulation of adenylate cyclase, which is thought to occur directly without involving hormone receptors or Gs (Seamon et al., 1981), suggested that the enzyme did not change significantly. The forskolin response was increased by only a small factor (1.8-fold) in intact cells (Fig. IV-3). Further, ^{32}P-ADP ribosylation by cholera toxin and northern-blot

analysis of mRNA for the α chains of Gs experiments did not reveal any quantitative changes resulting from RA treatment (F.H. Chang and H.H. Bourne, personal communication). On the other hand, a three- to five-fold increase of cAMP accumulation (in the presence of IBMX) was caused by cholera toxin in intact cells after RA treatment (data not shown). Therefore, some alteration of the coupling protein Gs cannot be entirely ruled out. In contrast, a significant increase of the PGE_1 receptor activity was observed after RA treatment, both with $[^3H]PGE_1$ receptor binding studies (G. Hochhaus and W. Sadée, personal communication), and with the PGE_1-cAMP response in intact cells. These results suggest that RA selectively up-regulates the PGE_1 receptor, with little or no effect on Gs and adenylate cyclase. The previously reported RA-induced enhancement of the calcitonin-cAMP response in F9 embryonal carcinoma cells was also associated with an up-regulation of the calcitonin receptor (Liapi et al., 1986).

Dose-response curves with PGE_1 before and after RA treatment revealed not only a dramatic increase of the maximum response, but also a shift to higher PGE_1 sensitivity by over one order of magnitude (Fig. IV-4). Our results suggest that these quantitative changes are primarily a result of PGE_1 receptor up-regulation; however, concomitant changes of the receptor-effective coupling mechanism cannot be ruled out.

In conclusion, RA treatment of neuroblastoma cells caused increased sensitivity towards PGE_1 stimulation of adenylate cyclase, which was associated with an up-regulation of the PGE_1 receptor in SH-SY5Y cells. The RA effect was rather specific for PGE_1 in neuroblastoma cells, as other cAMP-stimulatory hormones-neurotransmitters did not respond in the SH-SY5Y cell line. The molecular mechanisms by which RA

caused these receptor changes remains to be studied, but the results suggest that activation of protein kinase C may not play a major role.

Our results document that the regulation of the neurotransmitter receptor systems studied are under multiple control, with either up- or down-regulation occurring with the various differentiation agents tested. While the enhancement of the PGE_1-cAMP response by RA was the most pronounced effect, RA also up-regulated several other neurotransmitter systems, e.g., the μ and δ opioid receptor and the $uptake_1$ mechanism (Table IV-1). This latter finding is of potential therapeutic interest because of the use of ^{125}I-metaiodobenzoylguanidine in the treatment of neuroblastoma (Buck et al., 1985), a drug that is concentrated into neuroblastoma cells via the $uptake_1$ mechanism. The role of retinoids in cell differentiation and proliferation has long been recognized (see Sporn and Roberts, 1983), along with its potential as a cancer chemotherapeutic and chemopreventive agent (see Bollag, 1979; Sporn and Newton, 1979; Sporn and Newton, 1981). The current findings may shed new light on the molecular mechanism of RA effects in tumor cells.

CHAPTER V

EFFICACY AND TOLERANCE OF NARCOTIC ANALGESICS AT THE μ OPIOID RECEPTOR IN DIFFERENTIATED HUMAN NEUROBLASTOMA CELLS

1. SUMMARY

Upon differentiation with retinoic acid of the human neuroblastoma cells SH-SY5Y into mature neurons, opioid drugs become highly effective in suppressing prostaglandin E_1- (50% inhibition) and forskolin- (70% inhibition) stimulated adenylate cyclase activity, which was assessed by measuring cAMP accumulation in intact cells. While the SH-SY5Y cells carry both μ and δ receptors in a ratio of $\mu/\delta \cong 5/1$, the response is predominantly mediated by the μ receptor. Morphine acts as a full agonist with an EC_{50} of 50 to 100 nM which falls into the therapeutic range expected for narcotic analgesic effects mediated by the μ receptor. Narcotic analgesic drugs with only partial agonism fail to evoke full response, which suggests that this cell model could provide a rapid screening assay for narcotic analgesic efficacy. Continued exposure of the cells to morphine resulted in partial tolerance within 12 hours with a fourfold shift of morphine's EC_{50} to higher concentrations, while longer morphine exposure did not cause any further shift. Thus the differentiated SH-SY5Y cells provide a suitable system for studying the molecular mechanisms of the narcotic analgesics.

2. INTRODUCTION

Pharmacological and biochemical evidence supports the existence of multiple opioid receptor subtypes (e.g., μ, δ, κ) (Robson et al., 1983). Although considerable evidence suggests that the μ opioid receptors play a key role in mediating the analgesic effect of narcotics (Gacel et al., 1981; Rosenbaum et al., 1984; Goodman et al., 1980; Carr et al., 1984), the cellular mechanisms that underlie narcotic analgesia, tolerance and dependence remain unclear because of a lack of well defined experimental models.

Recently, we have identified a human neuroblastoma cell line, SK-N-SH, which expresses μ and δ opioid receptors in a ratio of approximately 5 to 1 (Yu et al., 1986). Phosphatidylinositol turnover was not affected by opioids in these cells (Yu and Sadée, 1986) while both etorphine and morphine inhibited PGE_1-stimulated cAMP accumulation to about 80% of control (Yu et al., 1986). However, the magnitude of the inhibition was insufficient to determine which receptor type (μ or δ) was responsible for the effect (Yu et al., 1986). In this study, we define experimental conditions that render these neuroblastoma cells into a reproducible cell model to study the biochemical correlates of opiate efficacy and tolerance.

Neuroblastoma cells express several distinct phenotypes, including immature neuroblast forms that can be induced to differentiate into mature neurons marked by extensive neurite outgrowth (Ross et al., 1983). To study the opioid receptor system in the differentiated cells, we have selected a phenotypically stable neuroblast subclone (SH-SY5Y) derived by Biedler and colleagues from the parent SK-N-SH neuroblastoma

cell line (Ross et al., 1983; Biedler et al., 1973). The opioid receptor system in SH-SY5Y cells (Kazmi and Mishra, 1986; Sonnenfeld et al., 1984) is essentially identical to that in the parent cell line, SK-N-SH (Yu et al., 1986). Moreover, it has been shown that SH-SY5Y cells differentiate in the presence of RA (Påhlman et al., 1984) dBcAMP (Schulze and Perez-Polo, 1982; Kuramoto et al., 1977; Kuramoto et al., 1981), and NGF (Schulze and Perez-Polo, 1982; Perez-Polo et al., 1979; Spinelli et al., 1982; Påhlman et al., 1981). Morphological differentiation is associated with the appearance of electrical excitability (Kuramoto et al., 1977; Kuramoto et al., 1981; Perez-Polo et al., 1979), enhancement of neuron specific enolase activity (Påhlman et al., 1984) and increase of neurotransmitter concentrations and neurosecretory granules (Påhlman et al., 1981).

In this chapter, a significant increase in opiate inhibition of adenylate cyclase in RA differentiated SH-SY5Y cells was demonstrated. The efficacy and tolerance of μ agonists including the narcotic analgesics were studied in this cell model.

3. MATERIALS AND METHODS

3.1 Materials

The SH-SY5Y cell line was kindly provided by Dr. June L. Biedler of the Sloan-Kettering Institute for Cancer Research, Rye, NY. The cyclic AMP assay kit and other [^{3}H] chemicals were purchased from Amersham, Inc., Arlington Heights, IL. The following compounds were purchased from Sigma, St. Louis, MO: dBcAMP, all-trans-retinoic acid (RA), NGF

(2.5 S form), forskolin, PGE_1, bestatin and morphiceptin. DAGO,* DPDPE and DADLE were purchased from Peninsula Laboratories, Palo Alto, CA. Diprenorphine was donated by the National Institute on Drug Abuse, Bethesda, MD.

3.2 Methods

3.2a Cell Culture

SH-SY5Y cells were grown at 37°C in monolayer in RPMI-1640 medium supplemented with 10% fetal calf serum containing 100 µg streptomycin/ml and 100 IU penicillin/ml. Confluent cells were harvested with 0.04% EDTA in Ca^{2+}/Mg^{2+} free phosphate-buffered saline with mechanical agitation. For receptor binding assays, freshly prepared membrane homogenates (Chapter I) were used, while for the cyclic AMP experiments, intact cells were used.

3.2b Differentiation

Cells were induced to differentiate by culturing with either RA (10 µM), dBcAMP (1 mM) or NGF (100 ng/ml) for 6 days. Medium was replaced every 2 days. In the case of RA-treated cells, equal ethanol (solvent for RA) concentrations were present in the control cells. For cells differentiated in 17 mm wells, the inducing agent was added no earlier than 24 hrs after the transfer of the cells to allow sufficient time for seeding.

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*Abbreviations used: RA, retinoic acid; dBcAMP, dibutyryl cyclic AMP; NGF, nerve growth factor; PGE_1, prostaglandin E_1; IBMX, 3-isobutyl-1-methyl-xanthine; DAGO, [D-Ala^2, $MePhe^4$, $Gly\text{-}ol^5$] enkephalin; DADLE, [D-Ala^2, D-Leu^5] enkephalin; DPDPE, [D-Pen^2, D-Pen^5] enkephalin.

3.2c Opiate Receptors

The assay of μ and δ opiate receptors on SK-N-SH cells has been previously described (Chapter III). [^{3}H]DAGO (1 nM, 60 Ci/mmol) and [^{3}H]DADLE (1 nM, 50 Ci/mmol), the latter in the absence or presence of 10^{-5} M morphiceptin to block μ sites, were incubated with washed cell membrane homogenates (50 mM Tris buffer, pH 7.4) at 20°C for 1 hr. The bound radioactivity was determined by filtering the homogenate mixture through Whatman GF/B glass fiber filters. Tracer binding in the presence of 10^{-6} M diprenorphine served as the value for non-specific binding.

3.2d Chronic Morphine Treatment

SH-SY5Y cells were exposed to 10μM morphine for 12 hrs to 6 days during the RA treatment over 6 days. Immediately before the cAMP experiments, identical concentrations of morphine were added to naive control (RA-treated) cells. After 5 min incubation at 37°C, the medium in the culture wells of both naive control and morphine-treated cells was removed, and the cells were rinsed 3 times with 1 ml serum free RPMI-1640 medium to remove the morphine. The 5 min preincubation of control cells with 10 μM morphine was performed to rule out any effects that may have arisen from residual morphine in the cells after washing. However, no differences were noted whether or not morphine was added to control cells 5 min prior to the experiments.

3.2e cAMP Production

These studies were done in 17 mm culture wells with slight modification of a previously described procedure (Yu *et al.*, 1986).

Briefly, intact attached monolayer cells in each well were rinsed once with 1 ml serum free RPMI-1640 medium after aspiration of culture medium. Opioids and PGE_1 (1 µM) or forskolin (100 µM) were added after a preincubation period of 10 min with 0.5 mM IBMX and 30µM bestatin, if morphiceptin was used, in a final volume of 300 µl serum free medium at 33°C. The reaction was terminated 15 min after PGE_1 or forskolin was added by adding 100 µl ice-cold 20% w/v trichloroacetic acid directly to the cell culture medium, as replacement with fresh medium before adding trichloroacetic acid had no significant effect on the cAMP level in each well. The cells were then scraped from the well and transferred to 1.5 ml polypropylene microcentrifuge tubes and centrifuged (500 x g, 15 min, 0°C). The supernatants were extracted three times with 2 ml of water-saturated diethyl ether. After evaporation of the residual ether, the cAMP content was determined with the use of a competitive protein binding assay kit (Amersham). Protein concentration was determined with the method of Lowry et al. (1951), and the data were normalized to protein content (~ 0.25-0.3 mg/well).

3.2f Data Analysis

IC_{50} values of receptor binding were determined by fitting the data to the logistic function:

$$\% B = \% B_{max} - \% B_{max} * L^n/(IC_{50}{}^n + L^n)$$

(B = specific bound tracer (d.p.m.); L = concentration of displacing ligand; n = slope factor). The EC_{50} values for the dose-response experiments were determined by fitting the data to the pharmacodynamic

equation:

$$E = (E_{max} \cdot C^n)/(EC_{50}^n + C^n)$$

(E = % inhibition; C = opioid concentration present; n = slope factor). The data were fitted with the use of the computer program MAKE Model in the Prophet-NIH System as previously described (Chapter I).

4. RESULTS

4.1 Opioid Receptor Regulation During Cell Differentiation

After a 6 day treatment of SH-SY5Y cells with differentiating agents, specific μ and δ receptor binding per mg protein was increased by RA (% control binding: 182 ± 36%, n = 6 (μ); 156 ± 10%, n = 3 (δ)) and by NGF (% control binding: 167 ± 36%, n = 3 (μ); 116 ± 7%, n = 3 (δ)), while it decreased in dBcAMP-treated cells (% control binding: 70 ± 8%, n = 7 (μ); 49 ± 28%, n = 4 (δ)). The changes of opioid receptor binding occurred gradually over the treatment period (Fig. V-1). Equilibrium binding competition curves of DAGO against [^{3}H]DAGO, obtained in cell membrane homogenates of RA- and dBcAMP-differentiated cells, were superimposable to those in control cell membranes (Fig. V-2). Similiarly, the calculated IC_{50} values and slope factors (n) for DADLE binding at the δ site, measured against [^{3}H]DADLE in the presence of 10^{-5} M morphiceptin was unchanged by RA treatment (control $IC_{50\delta}$ = 1.5 ± 0.21 nM, n = 0.75; $IC_{50\delta}$ = 1.42 ± 0.31 nM, n = 0.96). The DADLE affinity toward the δ receptor in dBcAMP-treated cells was not

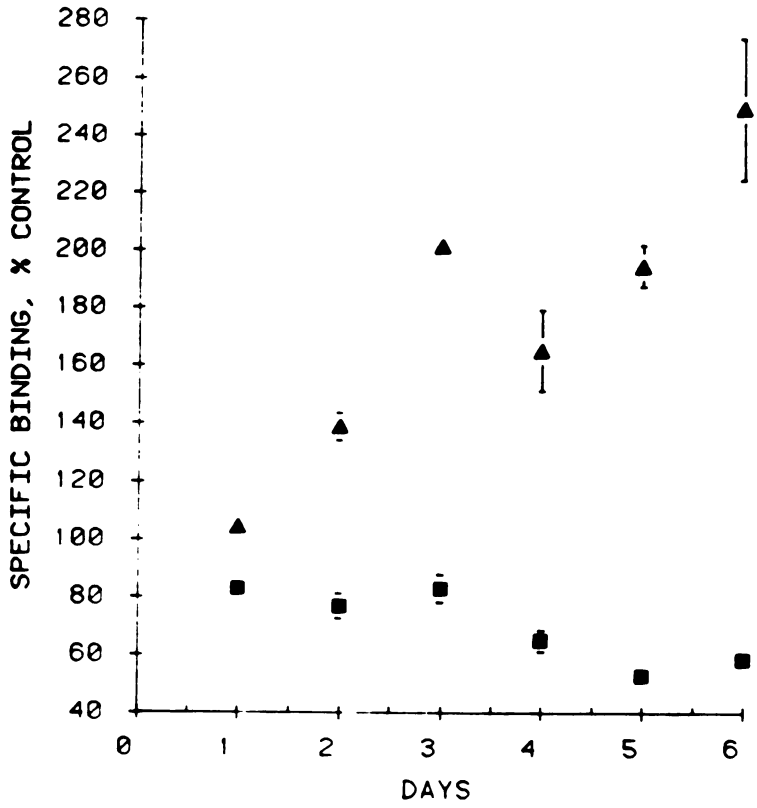

Figure V-1. Time-course of specific μ receptor binding during cell differentiation. Cells were treated with either 10 μM RA (▲) or 1 mM dBcAMP (■). Equilibrium binding experiments were carried out with fresh washed membrane homogenates in the presence of 1 nM [^{3}H]DAGO ± 1 μM diprenorphine to define non-specific binding. Values represent means ± S.D. of triplicate determinations from one representative experiment. Control cells express approximately 50,000 μ sites and 10,000 δ sites per cell.

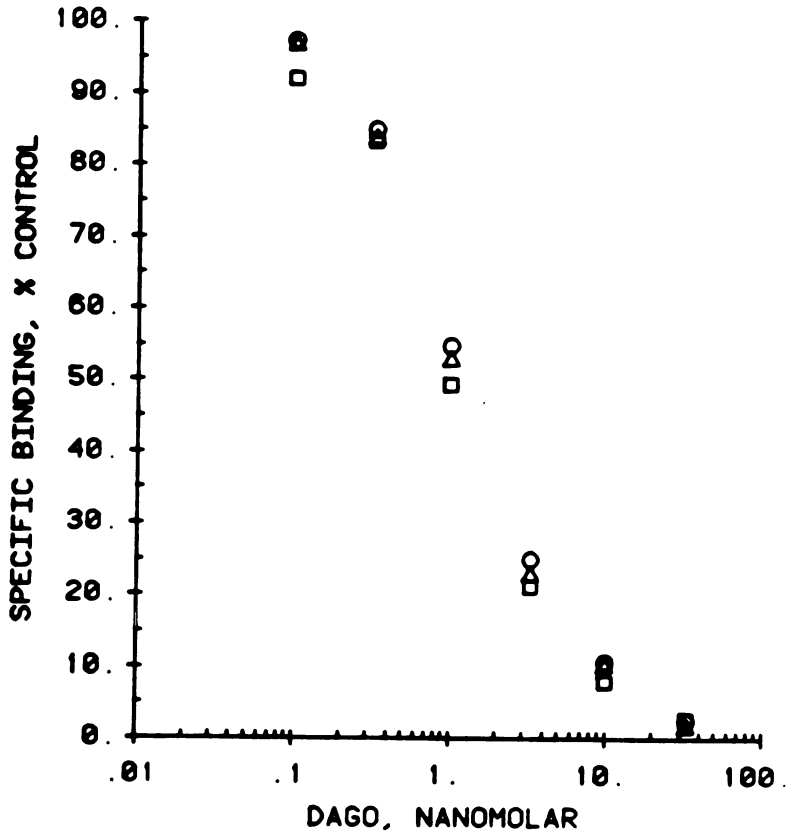

Figure V-2. Equilibrium binding competition curves of DAGO against [^{3}H]DAGO. Equilibrium binding competition experiments were performed in control (○), RA-treated (△) and dBcAMP-treated (□) cell membrane homogenates. Values represent means of duplicate determinations that are within ± 5% of the mean.

determined because of the low level of specific [^{3}H]DADLE binding remaining in the presence of 10^{-5} M morphiceptin. These results indicate that the receptor affinities toward their respective ligands remained unchanged after RA and dBcAMP treatment; therefore, a change in specific binding of [^{3}H] ligand directly reflects a change in receptor concentration on these cells.

4.2 **Stimulation of cAMP Production**

To study adenylate cyclase activity in intact cells, incubations were performed in the presence of the phosphodiesterase inhibitor IBMX (0.5 mM). Forskolin was considerably more potent than PGE_1 in undifferentiated cells (cAMP accumulation in pmol/mg protein/15 min; basal: 59 ± 15, n = 3; forskolin (100 µM): 1825 ± 105, n = 3; PGE_1 (1 µM): 280 ± 60, n = 3). Treatment of the SH-SY5Y cells with RA dramatically increased the ability of PGE_1 (1 µM) to stimulate cAMP (8236 ± 250 (n = 3) pmoles/mg protein/15 min), while forskolin (100 µM) was only 1.8 fold more potent after RA treatment (3125 ± 132 (n = 3) pmol/mg protein/15 min). The dramatic increase in PGE_1 stimulation was unique to RA, since it was not observed with either dBcAMP or nerve growth factor (100 ng/ml) induced differentiation (Chapter IV). The selected concentrations of PGE_1 and forskolin gave maximal stimulation of cAMP production.

4.3 **Opioid Inhibition of cAMP Production**

In control SH-SY5Y cells, morphine (10 µM) lowered cAMP accumulation by 23 and 35% in PGE_1 (1 µM)- and forskolin (100 µM)-

stimulated cells, respectively (Fig. V-3). However, upon differentiation with RA for 6 days, morphine's inhibitory effects were significantly increased to ~50% in PGE_1- and to ~70% in forskolin-stimulated cells (Fig. V-3). In all experiments, morphine was more effective in suppressing the forskolin-stimulated than the corresponding PGE_1-stimulated response. The inhibitory effects were naloxone reversible (Table V-I), and incubation with pertussis toxin (100 ng/ml) for 48 hrs abolished the inhibitory effect of morphine (Table V-I).

4.4 Effect of μ and δ Selective Opioids on cAMP Production

In order to determine the contribution of μ and δ sites in the regulation of adenylate cyclase, the efficacies of various opioids in inhibiting PGE_1 (1 μM) or forskolin (100 μM) stimulated cAMP accumulation were compared in RA-differentiated SH-SY5Y cells (Table V-1). The μ specific or selective agonists morphiceptin, DAGO and morphine, the general agonist etorphine, and the μ,δ agonist DADLE were equally effective. In contrast, we confirmed previous results that morphiceptin (10 μM) was without any effect on PGE_1-stimulated cAMP accumulation at the δ receptors in NG108-15 neuroblastoma x glioma hybrids (Law et al., 1983). The highly μ selective ligand DAGO displayed an IC_{50} value of 9.6 ± 1.6 nM when a full dose response curve was run. In contrast, the δ selective peptide DPDPE was only partially effective at 10μM (Table V-I); however, at this high concentration DPDPE is expected to also interact at the μ receptors (Corbett et al., 1984).

At the lowest concentrations of DPDPE tested (30 nM), the cAMP inhibition was only 23% of the maximum effect. To determine the potency of morphine in suppressing cAMP accumulation, dose-response curves were

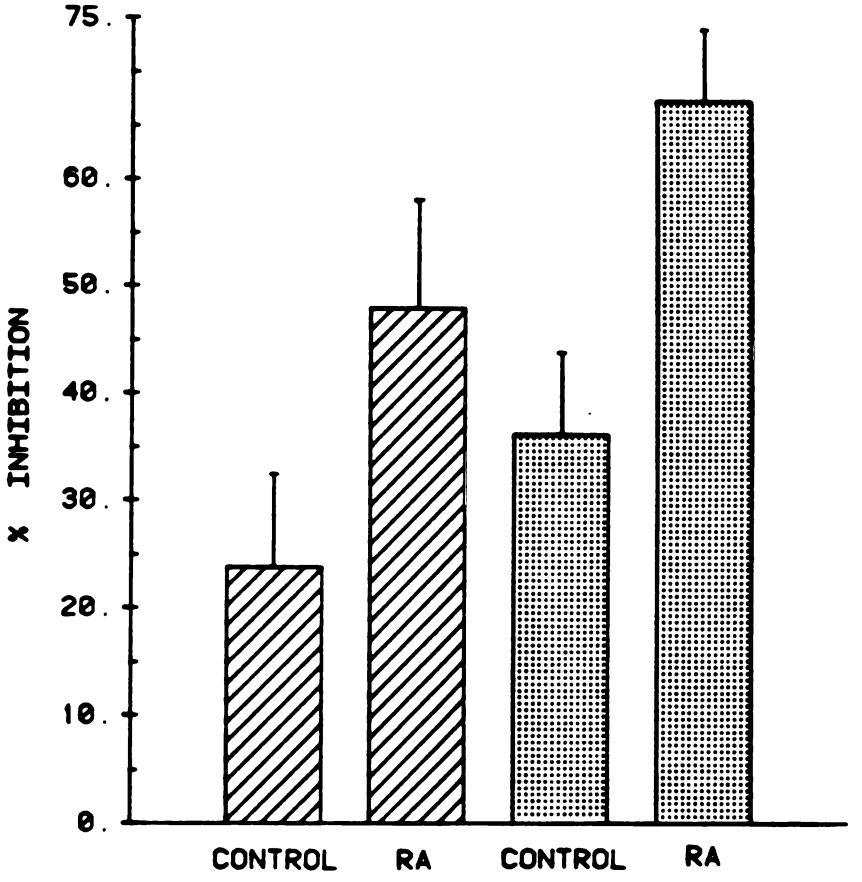

Figure V-3. Inhibitory effects of morphine on stimulated cAMP accumulation. Inhibitory effects of morphine (10 μM) on PGE_1 (hatched bars) and forskolin (dotted bars) stimulated cAMP accumulation in intact cells were measured. Values represent means ± S.E.M. of at least four experiments with triplicate determinations.

Table V-I. Efficacy of opioids in inhibiting stimulated cAMP accumulation. SH-SY5Y cells were treated with 10 μM RA for 6 days, and the inhibition of the stimulated cAMP accumulation was measured. Adenylate cyclase was stimulated with either 1 μM PGE_1, or 100 μM forskolin. Values represent mean ± S.D. of 3 to 6 determinations.

	cAMP Production, % maximal inhibition	
Treatment	PGE_1-Stimulated	Forskolin-Stimulated
Morphine, 10 μM	100 ± 5	100 ± 3
Morphiceptin, 10 μM	93 ± 2	104 ± 4
DAGO, 1 μM	112 ± 7	83 ± 9
Etorphine, 0.1 μM	105 ± 4	92 ± 3
DADLE, 1 μM	100 ± 3	104 ± 3
DPDPE, 0.03 μM		23 ± 2
DPDPE, 0.1 μM		47 ± 1
Ethylketocyclazocine, 1 μM	104 ± 5	
Pentazocine, 10 μM	64 ± 4	54 ± 12
Cyclazocine, 0.1 μM	74 ± 5	
Levallorphan, 0.1 μM	45 ± 12	
Nalorphine, 0.1 μM	43 ± 3	52 ± 6
Naloxone	-0-	-0-
Naloxone (10μM) + Morphine (1 μM)	10 ± 6	10 ± 2
Pertussis Toxin[a] + Morphine (10 μM)	14 ± 3	

[a]Cells were pretreated with 100 ng/ml pertussis toxin for 48 hr.

obtained in RA-treated SH-SY5Y cells stimulated with either 1 μM PGE_1 or 100 μM forskolin (Fig. V-4). The IC_{50} value for morphine obtained in one experiment with PGE_1-stimulated cells was 60 ± 9 nM (± SE) (slope factors fixed at n = 1) or 32 ± 14 nM (fitted slope factor n = 0.85), with maximum responses ranging from 45 to 62% inhibition. Because of the lower percentage inhibition agaiinst PGE_1, there is an increased error associated with estimating morphine's IC_{50}. Forskolin-stimulated cells yielded similar IC_{50} values for morphine from 62 to 107 nM with maximum effects from 65 to 79% inhibition. The fitted slope factors, N, were generally in the range of 0.7 to 0.85.

4.5 **Efficacy of Narcotic Analgesics and Partial Agonists**

The efficacy of several commonly used narcotic analgesics in the RA treated SH-SY5Y cell model was determined at high drug concentrations that are expected to yield maximum responses on the basis of their known μ receptor affinities (Table V-I). Among these, only ethylketo-cyclazocine reaches a response equivalent to that of the other full agonists listed in Table V-I, while pentazocine, cyclazocine, levallorphan and nalorphine produced only partial responses at the indicated concentrations. No substantial differences were noted between PGE_1 and forskolin stimulated cells. A dose-response curve was obtained with the clinically used partial narcotic analgesic agonist, pentazocine, which yielded an estimated E_{max} value of 37 ± 4% inhibition, or approximately 60% of the morphine response, and an IC_{50} value of 800 ± 300 nM (Fig. V-4).

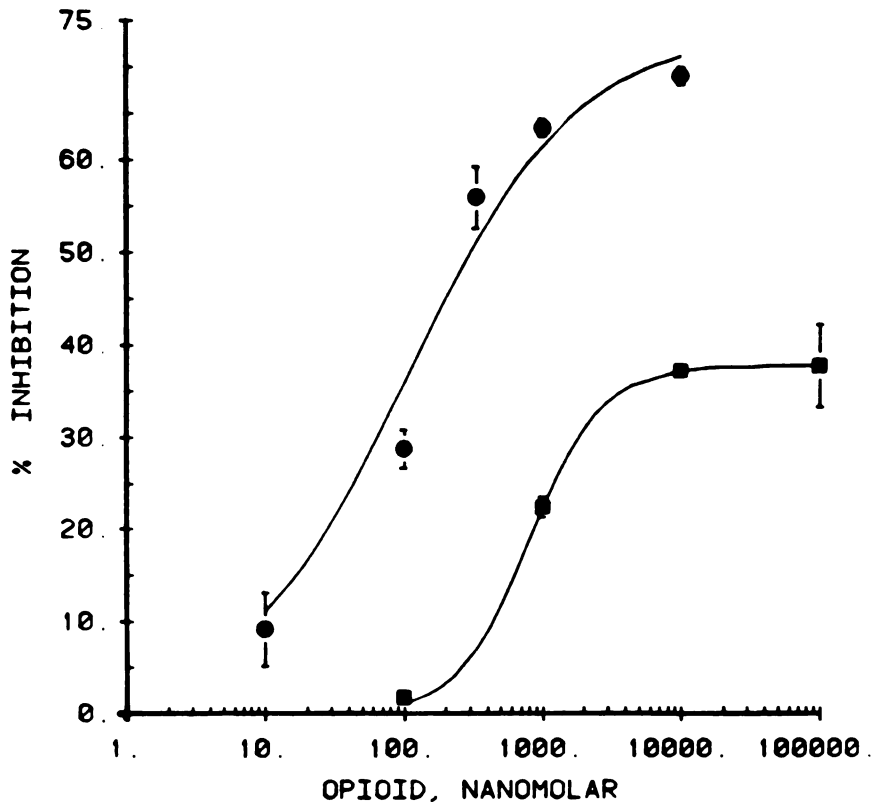

Figure V-4. Dose-response curves of morphine and pentazocine in inhibiting forskolin-stimulated cAMP accumulation. Dose-response curves of morphine (●) and pentazocine (■) were determined in RA-treated cells. Values represent means ± S.D. of triplicate determinations from one representative experiment.

4.6 **Morphine Tolerance**

RA-treated cells were exposed for varying time periods to morphine (10 μM), and the sensitivity of forskolin stimulated cAMP accumulation to inhibition by 300 nM morphine was tested. There was a significant decrease in morphine's efficacy from 61 ± 4% (n = 3) to 38 ± 2.5% (n = 3) inhibition after 12 hours of exposure to morphine, with no further change noted for up to 6 days of morphine exposure. A full morphine dose-response curve was obtained with a 2 day morphine exposure prior to the experiment, in direct comparison with an untreated control (Fig. V-5). The IC_{50} of morphine shifted from 78 ± 24 nM (control) to 316 ± 145 nM, while the maximum inhibition decreased from 68 ± 5% to 50 ± 5%. Slope factors were n = 0.62 ± 0.08 (control) and 0.89 ± 0.31.

5. **DISCUSSION**

This study establishes the subclone SH-SY5Y (Ross et al., 1983) of the parent human neuroblastoma cell line SK-N-SH (Biedler et al., 1973) as a suitable model for studying the biochemical correlates of narcotic analgesic efficacy and tolerance. Like most established neuroblastomas, SK-N-SH expresses several interchangeable phenotypes, i.e., strongly substrate adherent non-neuronal cells (S cells) and neuroblast-type cells (N cells). We have established that only the N cells carry opioid receptors. Therefore, we have selected the phenotypically stable neuroblastic clone SH-SY5Y, which also expresses predominantly μ receptors and fewer δ opioid receptors (Kazmi and Mishra, 1986). However, opioid agonists were no more effective in suppressing the PGE_1

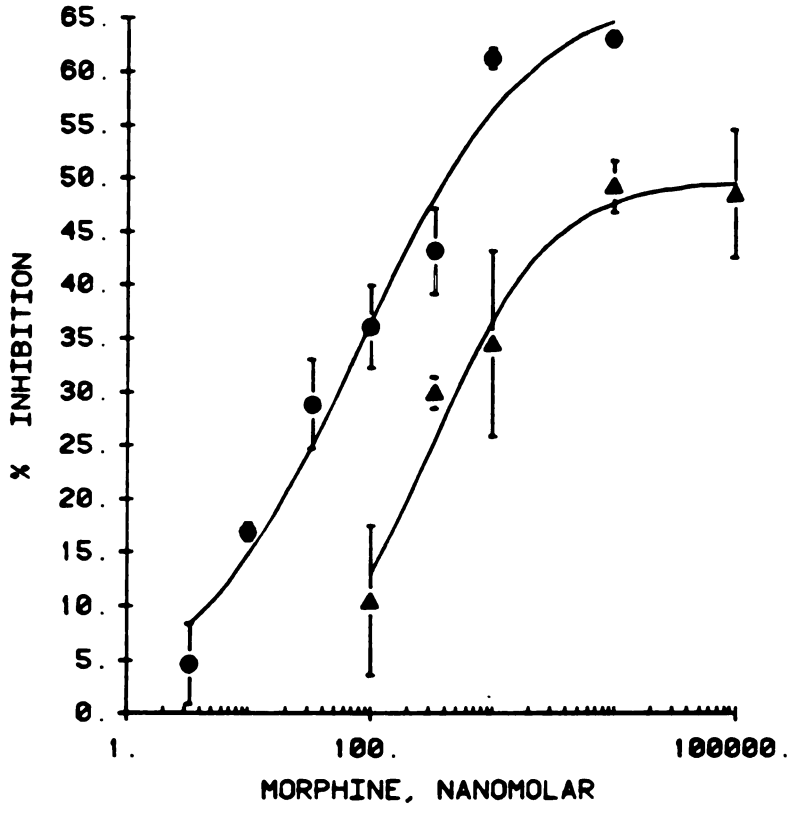

Figure V-5. Dose-response curves of morphine in naive and morphine pretreated, RA-differentiated cells. Dose-response of morphine in inhibiting forskolin-stimulated cAMP accumulation was determined in control (naive, ●) and morphine-pretreated (48 hrs, ▲) cells. Values represent means ± S.D. of 3 determinations from one representative experiment.

- cAMP response in the subclone than in the parent cell line (~20%). Use of forskolin which directly stimulates adenylate cyclase at high concentrations (Seaman et al., 1981) increased opioid inhibition of cAMP accumulation, but the maximal inhibition (35%) was still insufficient to readily perform quantitative studies. To further increase opioid regulatory effects, the relatively immature SH-SY5Y cells were differentiated with several agents, including RA, NGF and dBcAMP. While RA increased the number of opioid receptors per cell by twofold or less, dBcAMP had the opposite effect. More importantly, RA increased the maximum inhibition of the PGE_1 - cAMP response to ~50% and the forskolin - cAMP response to ~70%. NGF was less effective in this regard (unpublished data). Concomitant with the RA induced enhancement of negative control by opiates was a dramatic enhancement of the stimulatory PGE_1 - cAMP response, which was only observed with RA treatment. This enhancement appears to be related to an effect of RA on PGE_1 receptor (Chapter IV). The RA differentiated SH-SY5Y subclone shows the requisite sensitivity towards opioid drugs for quantitative studies. The inhibition of cAMP accumulation by opioid agonists was reversed by naloxone and suppressed by pertussis toxin which suggests that the inhibitory effects resulted from a direct regulation of adenylate cyclase via a G_i coupling protein (Rodbell, 1982).

Because the SH-SY5Y cells contain both μ and δ sites in a ratio of 5 to 1, it was important to establish which receptor mediates the inhibition of cAMP accumulation. Extensive previous studies on the molecular mechanisms of opioid receptors in established transformed cell lines were largely performed in neuroblastoma cells that express only δ sites (West and Miller, 1983), with the exception of a rat pituitary

tumor tissue line that expresses a moderate number of μ receptors (Puttfarcken et al., 1986). Strong evidence suggests that the opioid effects on cAMP are predominantly mediated by the μ and not the δ receptor in SH-SY5Y cells stems from the observation that the μ and δ agonists DADLE and etorphine were no more effective than the highly μ specific agonist morphiceptin. Further, morphine was considerably more potent in the SH-SY5Y cells (EC_{50} ~70 nM) than in the δ receptor-carrying NG108-15 cells (EC_{50} ~3.5 μM (Law et al., 1983)), but it showed similar potency as reported for inhibiting adenylate cyclase and prolactin release in the rat pituitary tumor 7315c (Frey and Kebabian, 1981) which apparently expresses μ receptors (Puttfarcken et al., 1986). The EC_{50} value of ~70 nM is also well within the range expected after therapeutic application of morphine in patients (Grevel et al., 1984). Further, while the μ selective agonist DAGO was fully effective and quite potent (IC_{50} 9.6 nM), the equally potent δ selective agonist DPDPE gave only 23% of maximal response at 30 nM. In contrast, the IC_{50} value for DPDPE in a δ preparation, the mouse vas deferens, was 4 nM (Corbett et al., 1984), while at higher concentrations DPDPE also acts as a μ agonist. Therefore, we concluded that the opioid effects and particularly those of morphine are predominantly mediated by the μ receptor in the SH-SY5Y cells.

Because analgesia is frequently an all-or-none phenomenon, it is difficult to establish relative efficacies among the narcotic analgesic drugs. A survey of several partial agonists of the morphinan and benzomorphan type revealed that none of these agents gave full response of cAMP inhibiton at high concentrations. The clinically effective partial agonist pentazocine gave only ~60% of the maximum response of

morphine, which supports the notion that the SH-SY5Y cells may provide a measure of efficacy among the narcotic analgesics. Further, the EC_{50} of pentazocine (Fig. V-4) in inhibiting the forskolin - cAMP response (700 nM) is similar to that established in the guinea pig ileum (250 nM) (Magnan et al., 1982), which is considered a μ receptor preparation.

We next addressed the question whether the RA-treated SH-SY5Y cells become tolerant to the effects of morphine. Indeed, within 12 hours of morphine exposure, the sensitivity of the forskolin - cAMP response to morphine inhibition was significantly reduced, but it did not change further with longer morphine exposure. The shift of the morphine dose-response curve was approximately fourfold, with only a modest decline in the maximum response. If this degree of tolerance reflects similar events in vivo, it cannot fully account for the remarkable tolerance to morphine that occurs in narcotic addiction, and additional physiological mechanisms involving the interactions of multiple cell types must be considered. Nevertheless, the results presented here should facilitate the study of the molecular mechanisms responsible for the effects of narcotic analgesics.

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