Title
Molecular mechanism underlying narcotic dependence and tolerance regulation of constitutive mu opioid receptor activity

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Publication Date
1996

Peer reviewed|Thesis/dissertation

MOLECULAR MECHANISM UNDERLYING NARCOTIC DEPENDENCE AND TOLERANCE REGULATION OF CONSTITUTIVE μ OPIOID RECEPTOR ACTIVITY

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

PHARMACEUTICAL CHEMISTRY

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

Date **University Librarian**

Degree Conferred: . . . . . .

To my parents, Mrs. Ge Yafen and Mr. Wang Yizhong,

for their love, support, and encouragement

ACKNOWLEDGMENTS

I wish to express my gratitude to:

My research advisor, Dr. Wolfgang Sadée, for his generous gifts of knowledge, time and encouragement. Without his guidance, this study would not have been possible.

Dr. Svein Øie and Dr. Kathy Giacomini, for their skillful guidance throughout this study, and their valuable suggestions and comments on this dissertation.

Dr. Frank Porreca, and Ed Bilsky, who collaborated on acute tolerance and dependence studies presented here.

My friends in San Francisco and Stockton for their support and encouragement.

Molecular Mechanism underlying Narcotic Dependence and Tolerance
---Regulation of Constitutive μ Opioid Receptor Activity
Zaijie Wang

ABSTRACT

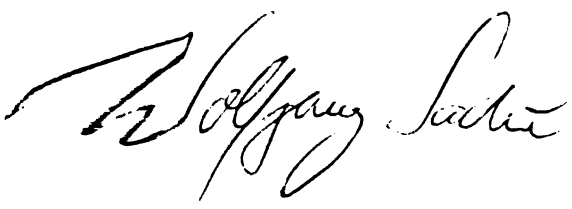
In this study, narcotic tolerance and dependence are proposed to be regulated by constitutive μ opioid receptor (μOR) activation. Morphine lowers cAMP levels by interacting with μOR in neuroblastoma SH-SY5Y cells. In morphine-pretreated SH-SY5Y cells, naloxone caused an unexpected cAMP overshoot (NICO), after morphine was removed. This inverse agonistic effect of naloxone implied the presence of constitutively active μOR state (μ*). NICO can be prevented by including in the pretreatment a protein kinase inhibitor H7, but not its congener H8, suggesting a role of phosphorylation in μ* formation.

Other opioid antagonists such as diprenorphine, naltrexone also produced cAMP overshoot similar to NICO. In contrast, the peptide antagonists CTOP and CTAP were ineffective. These results suggested two distinct classes of antagonists: antagonists with negative intrinsic activity (inverse agonists, e.g. naloxone) and neutral antagonists (e.g. CTOP).

μOR transfected in HEK293 (HEK-μ) cells (~4 x 10^6 sites/cell) was functionally coupled to adenylyl cyclase. Pretreatment of HEK-μ cells with morphine gave rise to a large NICO. Unlike in SH-SY5Y cells, morphine treatment did not lead to μOR tolerance. Activation of cAMP-dependent kinase (PKA) by forskolin or 8-bromo-cAMP caused receptor desensitization in both SH-SY5Y and HEK-μ cells, indicating that morphine-induced μOR tolerance is not mediated by PKA.

μOR phosphorylation was directly examined in HEK293 cells expressing epitope-tagged μORs. The tagged-μOR was regulated in a similar fashion to μOR, and exhibited high basal receptor phosphorylation, which was enhanced by morphine and inhibited by H7, and 3-isobutyl-1-methylxanthine (IBMX). Studies using various inhibitors and ionic conditions revealed the presence of a Ca^{++}-sensitive kinase responsible for μOR phosphorylation.

Both IBMX and H7, but not H8, disrupted morphine-induced antinociceptive tolerance and dependence in mice. Unlike naloxone, CTAP showed little effect in eliciting withdrawal in dependent mice. Therefore, μOR phosphorylation and constitutive activity may be involved in narcotic dependence and tolerance. In addition, this study yielded cellular targets and lead-compounds to design therapy for narcotic addiction.



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INTRODUCTION

OPIOIDS AND THEIR PHARMACOLOGICAL PROPERTIES

The knowledge of opium predates the written word. Opium is obtained from the dried juice of unripe poppy, *Papaver somniferum*. Isolation of morphine from opium was achieved by the German pharmacist Sertürner in 1806, and was quickly followed by purification of more than 20 other alkaloids, including codeine, papaverine, noscapine, thebaine, narceine, and narcotine.

Morphine and morphine-like drugs are collectively termed opioids, and are primarily used as pain-killers (analgesics). In addition, they cause respiratory depression, emesis, antitussive effect, decreased gastrointestinal motility, and produce effect on biliary, pancreatic and intestinal secretions, body temperature, immune system, etc. Although opioids are potent and effective analgesics, fear of the development of dependence and tolerance after repeated administrations has greatly discouraged their applications. Tolerance is a phenomenon of diminished pharmacological effect after successive uses of drugs, even where the dose and drug level remain the same. For opioids, the pharmacological effect usually refers to analgesia; however tolerance can occur to other pharmacological functions at a greater or lesser degree. Dependence is defined as the need of continuous presence of drug in the body to avoid physical discomfort (withdrawal syndrome). Addiction refers to the behavior of compulsive use of drugs despite possible

adverse consequences. Opioid addiction is almost always associated with physical dependence.

The causes of opioid dependence and tolerance remain to be elucidated. None of the current treatments for drug dependence has yielded much success. Opioid drug addiction continues to impose heavy costs on our society. On the other hand, development of tolerance requires increasingly higher dose of opioid analgesics, which can produce many unwanted adverse effects. Treatment of addiction and development of effective opioid analgesics deprived of dependence and tolerance liability is unlikely until there is a better understanding of the mechanism underlying dependence and tolerance.

OPIOID RECEPTORS AND THEIR SIGNAL TRANSDUCTION PATHWAYS

In an effort to understand structure-activity relationships (SAR) of opioids and search for better (more effective and less addictive) analgesics, a number of new opioids were synthesized based on morphine (1, 2). Although this failed to find any non-addictive opioid agonist, a number of potent analgesics, such as fentanyl, etorphine, hydromorphin were discovered. In addition, antagonists like naloxone and naltrexone, and partial agonist/antagonist, such as nalorphine, pentazocine, were made available. This kind of approach was greatly aided by the discoveries of opioid receptors in early 1970's using radioligand binding (3, 4, 5). These were followed by isolation of endogenous peptides that act through opioid receptors (6).

Three major classes of opioid receptors, μ, δ, and κ, have been extensively characterized. All of the clinically used analgesics act through the μ opioid receptor, hence "narcotic" is used for morphine-like and μ receptor-related events. This term should not be confused with the increasing use of *narcotics* in legal context as a name for any abusive substance. This study, therefore, focuses on the regulation of the μ opioid receptor. This does not preclude agonists of other subtypes to be used as analgesics. In fact, there is concrete evidence for antinociception mediated by δ and κ receptor. Because they seem to cause less dependence, non-peptide agonists of these receptors may provide analgesics with little dependence liability. However the efficacy and availability of such agents are to be demonstrated.

Despite the differences in anatomical locations and affinities to various agents, all three receptor subtypes share very similar activation and coupling mechanisms. Opioid receptors have been long regarded to interact with pertussis toxin-sensitive heterotrimeric G proteins (7, 8, 9, 10). Activation of opioid receptors inhibits adenylate cyclase [EC 4.6.1.1; ATP pyrophosphate-lyase (cyclizing)] and cAMP production via Gi (9, 10, 11, 12), and increases a potassium conductance (13) and decreases the N-type calcium-channel current (14, 15). These receptors may also couple to the inositol trisphosphate pathway (16) and directly affect cAMP phosphodiesterase activity (17). Recently, δ (18, 19), μ (20, 21) and κ (22, 23) receptors were cloned and all three sequences share characteristics of G-protein coupled receptors, such as seven putative transmembrane domains. The cloned receptors, when expressed in host cells, did couple to G-protein and adenylate cyclase (24, 25, 26, 27).

ANIMAL, TISSUE, AND CELL MODELS

The opioid functions, both acute and chronic, have been studied in a number of systems, each of which provides either general or specific information based on specific opioid and related receptors expressed. Human subjects and animals have been largely used for studies of analgesia, tolerance and dependence; however, such studies are limited in defining molecular mechanisms or receptor types. One well-established system is the mouse model, where antinociception effect can be easily tested by methods such as the tail-flick experiment. Moreover, mice can be quickly induced to dependence on and tolerance to morphine by a single large dose of morphine sulfate (e.g. subcutaneous injection of 100-150 mg/kg) (28, 29). Tolerance is demonstrated by right-shift of antinociception dose response. Physical dependence can be illustrated by administration of naloxone, which precipitates the withdrawal syndrome including animal vertical jump, body shaking, paw tremor, irritable, teeth chattering, etc. (30).

Brain slices of the locus coeruleus and hippocampus, and isolated peripheral tissues have also been used for identifying pharmacological consequences of activating receptors, such as perfused guinea pig ileum (largely μ) and mouse vas deferens (largely δ). Alternatively, cultured cells that express opioid receptors were used. Studies in neuroblastoma x glioma hybrid NG108-15 cells, which express δ receptor, have provided us with extensive information on δ opioid receptor activation and its interaction with second messenger system (12, 31). Cell lines expressing μ receptor were made possible when neuroblastoma SH-N-SH (10, 32), its subclone SH-SY5Y cells (10, 33), and primary culture of rat pituitary 7315c tumor cells (34) were developed.

SH-SY5Y cell line has been particularly useful because it is a permanent culture and expression of the μ receptor can be readily induced by applying suitable agents (10, 33, 35). Activation of opioids in cultured cells are characterized by stimulation of GTPase activity (9), and GTPγS binding (36), inhibition of adenylate cyclase and cAMP production (10, 37). When SH-SY5Y cells or NG108-15 cells are exposed to morphine for a prolonged period, the cAMP production tends to return to the uninhibited level (development of tolerance), and the adenylate cyclase and cAMP production are found to be upregulated when the agonist is removed. The latter has been considered as a biochemical correlate of withdrawal (10, 37, 38). Most recently, since the cloned receptor genes are available, it is possible to transiently or stably express a receptor in host cells (24, 26, 27). This is especially useful for such studies as receptor phosphorylation, where high receptor expression level and receptor tagging may be required to achieve intense signals (Chapter II--V).

MECHANISMS OF NARCOTIC TOLERANCE AND DEPENDENCE

The findings of opioid receptors, endogenous opioid peptide, and cloning of opioid receptors have greatly improved our understanding of opioid actions; however, none has given rise to an explanation of the development of dependence. Although the addictive and euphoric effects of cocaine may be due to the inhibition of dopamine reuptake and stimulation of monoamines release (39), studies have failed to demonstrate consistent long-term changes of particular neurotransmitter and anatomical brain location for opioid dependence (40, 41).

In terms of narcotic tolerance, no consistent changes were detectable in the numbers of opioid receptors and receptor affinity (31, 42, 43, 44, 45, 46), or

G protein levels (47) (48) after long-term treatment with opioid agonist. The matter is further complicated by inconsistent/variable results depending on agonist and antagonist used. In Chapter I and III, I demonstrated the effect of various agonists and antagonists on receptor phosphorylation and coupling to cAMP second messenger system. There have been reports of uncoupling of opioid receptor by activation of the protein kinase A (49), In Chapter IV, I present data that activation of protein kinase A desensitized the opioid receptor, but was not involved in agonist-induced receptor tolerance to morphine.

In conclusion, the mechanisms underlying narcotic tolerance and dependence remain a challenge in developing opioid analgesics and treating opioid addiction. In this study, we propose a novel hypothesis on regulation of the μ opioid receptor by invoking constitutive activity (agonist-independent receptor activity) (32, 50, 51, 52, 53). Constitutive μ receptor activation may serve as a driving force regulating the development of tolerance and dependence. Such a mechanism may also be shared by other biological systems, especially those involved in learning and memory.

OBJECTIVE OF THIS STUDY

The overall objective of this study was to probe the molecular mechanisms of μ opioid receptor tolerance and dependence. We hypothesized that prolonged exposure to opioids leads to constitutive activity of the μ opioid receptor, which, in turn, mediates opioid tolerance and dependence. Two cell lines, SH-SY5Y cells expressing endogenous opioid receptors, and HEK 293 cells stably transfected only with a cloned μ receptor,

were used as model systems to test the regulation of the μ opioid receptor by receptor constitutive activation. Results from these two cell lines were further tested in mice. The specific aims were to 1) test whether constitutive activity develops after treatment with a μ receptor agonist; 2) test if the μ receptor mediates this constitutive activity; 3) explore the importance of constitutive μ receptor activity in narcotic tolerance and dependence; 4) determine the mechanism of μ receptor constitutive activation; 5) characterize the μ receptor kinase involved in receptor basal phosphorylation and constitutive activation; 6) search for compounds that can inhibit μ receptor basal phosphorylation and disrupt animal tolerance and dependence; 7) study the μ receptor tolerance caused by agonist treatment and activation of the cAMP-dependent protein kinase.

CHAPTER I

CONSTITUTIVE μ OPIOID RECEPTOR ACTIVATION AS A REGULATORY MECHANISM UNDERLYING NARCOTIC TOLERANCE AND DEPENDENCE

SUMMARY

Chronic administration of narcotic μ opioid agonists results in tolerance and dependence. We propose that agonist stimulation causes a gradual conversion of μ receptors to a constitutively active state (μ*, a state in which the presence of an agonist is no longer required for signal transduction) as a key step in tolerance and physical dependence. We provide evidence in support of the existence of μ* in human neuroblastoma cells, SH-SY5Y, and μ* upregulation during morphine treatment. Naloxone blocked μ* activity, acting as an antagonist with negative intrinsic activity which accounts for its high potency in eliciting withdrawal. In contrast, the μ selective antagonist CTAP did not affect μ* activity but inhibited naloxone's effect. The protein kinase inhibitor H7 was found to suppress μ* formation, suggesting that μ* is phosphorylated. In a model of acute morphine

tolerance/dependence in mice, H7 prevented naloxone induced withdrawal jumping and reversed morphine (antinociceptive) tolerance. CTAP caused only mild withdrawal and attenuated naloxone induced withdrawal, as predicted for an antagonist without negative activity. These results support a role for constitutive μ receptor activation in narcotic tolerance and dependence, affording potential separation of acute and chronic narcotic effects.

INTRODUCTION

The mechanisms underlying narcotic tolerance and dependence remain largely unknown. Strong evidence invokes the cAMP second messenger system as playing a key role in narcotic dependence (37, 38, 54). In response to chronic adenylyl cyclase (AC) inhibition by opioids, the cAMP messenger system is upregulated, including enhanced activity of AC and protein kinase A (PKA). Narcotic drug removal thus leads to a *spontaneous cAMP overshoot* as the previously proposed biochemical correlate of withdrawal (37, 38, 54). Indeed, cAMP is a primary regulator of the basal neuronal firing rate in the locus coeruleus, one of the main anatomical sites proposed to mediate narcotic withdrawal (55). Numerous studies have focused on possible changes of opioid receptors in signal transduction during chronic narcotic drug exposure, but thus far, a clear picture has failed to emerge (40, 55, 56, 57). Yet, the dependent state appears to be critically regulated by opioid receptor activity in target neurons. With increasing degree of tolerance and dependence, the potency of the antagonist naloxone to elicit withdrawal at the μ opioid receptor increases by more than an order of

magnitude in animal studies (28). The increased potency of naloxone is inconsistent with a desensitized opioid receptor in the tolerant/dependent state.

To resolve these paradoxical results, we propose that narcotic agonist stimulation causes a gradual constitutive μ receptor activation , *i.e.*, no longer requiring an agonist for signal transduction (Scheme 1). Thus, the dependent state consists of an upregulated cAMP system, counterbalanced by constitutively active μ receptors (μ*). Increasing dependence is linked to increasing activation to μ*, and in parallel, increasing activity of the cAMP system. Tolerance to narcotic drugs can occur because fewer μ receptors remain activatable by agonists and because of the enhanced activity of the cAMP system. Constitutive receptor activity can be detected with the use of antagonists having negative intrinsic activity, as described for several G protein coupled receptors displaying a basal level of constitutive activity (8-10). Assuming naloxone were to block μ* activity, one would then expect its increasing potency to cause withdrawal during the development of dependence (28) because more μ* receptors are present.

This hypothesis is tested here with the use of the human neuroblastoma cell line SK-N-SH, and its subclone SH-SY5Y, which express abundant μ opioid receptors, show partial tolerance to the ability of μ agonists in inhibiting cAMP accumulation after chronic morphine exposure, and display a *spontaneous cAMP overshoot* after complete morphine removal as an indicator of upregulation of the cAMP system (10, 33, 35). In this study, the cells were pretreated with morphine to induce a tolerant/dependent state, followed by complete morphine removal. If μ* receptors were present, one would expect naloxone to increase further the cAMP levels in

tolerant/dependent cells (*naloxone cAMP overshoot).* The cell culture experiments indeed supported the proposed existence of μ*, and we identified a protein kinase inhibitor that blocked and reversed μ* formation. Moreover, we identified neutral antagonists which block the effects of both morphine and naloxone without any effect on the μ receptor when added alone. These agents were then tested in morphine pretreated mice for their ability to reverse the tolerant dependent state (kinase inhibitor), and to block the μ receptor without causing severe withdrawal (neutral antagonist). The combined results strongly support the hypothesis that μ* formation is crucial to narcotic tolerance and dependence.

EXPERIMENTAL PROCEDURES

Materials

[D-Ala^2-D-Leu^5]-enkephalin (DADLE), D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP), and D-Tic-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2 (D-Tic-CTAP) were obtained from Peninsula Labs. (Belmont, CA). [D-Ala^2, *N* -Me-Phe^4,Gly^3-ol]-enkephalin (DAMGO), [D-Pen^2,D-Pen^3]-enkephalin (DPDPE), ICI-174864, and D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2 (CTAP) were obtained from Multiple Peptide Systems (San Diego, CA). (+)-Naloxone HCl was obtained from the National Institute on Drug Abuse, Rockville, MD. The protein kinase inhibitors H7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine) and chelerythrine were purchased from Sigma Chemicals (St. Louis, MO). H8 (N-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide) was obtained from SeikagakuAmercan Inc. (Rockville, MD). The [^{3}H]-cAMP assay kit was supplied by Amersham (Arlington Heights, IL).

Cell Culture

The SK-N-SH and SH-SY5Y cell lines were provide by Dr. June Biedler of the Sloan Kettering Institute (Rye, N.Y.). The cells were grown at 37 ^{o}C in DME H-21 medium supplemented with 10% fetal calf serum containing 100 mg/ml streptomycin and 100 IU/ml penicillin (10, 33, 35). Initial results with SK-N-SH and SH-SY5Y cells were similar, and subsequent results were obtained only with SH-SY5Y cells. Optimal sensitivity to μ opioid agonist inhibition of cAMP accumulation was observed in retinoic acid pretreated cells (5 mM for 6 days), and upon AC stimulation with prostaglandin E_1 (PGE_1), in the absence of any phosphodiesterase inhibitor. These conditions were used throughout. Addition of IBMX as a phosphodiesterase inhibitor abolished the *naloxone cAMP overshoot*, which could have resulted from multiple mechanisms inherent to IBMX (14). To induce a tolerant-dependent state, the cells were pretreated with 1 μM morphine for periods ranging from 2 min to 48 h. The relatively low pretreatment concentration of 1 μM morphine was selected to achieve near maximum effects in tolerant cells (10, 33, 35) while facilitating complete washout of the drug. Immediately before the cAMP accumulation assay, cells were washed twice with medium containing 5% serum and twice more with serum free medium. Supernatants from the washed cells failed to elicit any opioid agonist like cAMP response when transferred to fresh untreated cells, suggesting effective morphine removal during washing. cAMP levels were determined by radioimmunoassay after stimulation with 1 mM PGE_1 over 15 min in the presence or absence of naloxone.

In several experiments, SH-SY5Y cell membranes, either with or without morphine pretreatment of the cells, were prepared as desribed (10, 33, 35), and cAMP accumulation over 15 min was assayed by RIA, without adding PGE_1, since it provided only a small additional stimulation.

Animal Experiments : Withdrawal

Male ICR mice (20-30 g, Harlan Industries, Cleveland, Ohio), in groups of 10 each, were made acutely dependent on morphine by subcutaneous (s.c.) injection of 100 mg/kg morphine sulfate. After 4 h, naloxone was given either intraperitoneally (i.p.) or simultaneously by intracerebroventricular (i.c.v.) and intrathecal (i.t.) injections to induce withdrawal. Alternatively, CTAP was injected i.c.v. and/or i.t. To test the ability of kinase inhibitors to reverse the dependent state, animals (under light ether anesthesia) were injected 3.5 h after the first 100 mg/kg morphine dose with saline or with H7 or H8 i.c.v. and/or i.t. Animals recovered within several minutes from anesthesia. At 4 h, withdrawal was precipitated by injection of naloxone (3 mg/kg intraperitoneally). Mice were then placed in Plexiglas cylinders and observed for a 15 min period with the number of vertical jumps recorded.

Animal Experiments : Tolerance

Mice in groups of 10 were again injected s.c. with 100 mg/kg morphine sulfate, and analgesia was measured with the tail immersion assay at 55° C by determining the time elapsed before the tail is flicked (15 sec cutoff = 100% analgesia). At 4.5 h after the first dose, either saline alone or saline containing 50 nmole H7 was injected i.c.v. At 5 h, the analgesic effect of the first

morphine dose had essentially ceased (4 ± 3 %), and a second dose of 10 mg/kg morphine sulfate (the ED90 dose in naive animals) was injected to detemine the degree of tolerance. All animal experiments were performed under an approved protocol in accordance with institutional guidelines.

RESULTS AND DISCUSSION

Regulation of cAMP in Cell Culture

After pretreatment of SH-SY5Y cells for 48 hr with morphine, and complete drug removal by thorough washing of the cells, a *spontaneous cAMP overshoot* was observed when compared to untreated cells (Fig.1.1, Panel A, $p < 0.05$), as previously reported (33, 35, 37). Moreover, addition of naloxone to the pretreated and thoroughly washed, drug-free cells caused a significant additional increase of cAMP accumulation (range: 20 to 80 % in over 40 experiments, $p < 0.001$) (Fig. 1.1). This *naloxone cAMP overshoot* is the expected result if naloxone indeed blocked μ* receptor activity (see Scheme 1). The *naloxone cAMP overshoot* was also observed in extensively washed membrane homogenates obtained from morphine pretreated cells, arguing against residual morphine as a cause (Fig. 1.1). Moreover, our results with the neutral antagonists CTAP and CTOP which do not affect cAMP levels (described below) rule out residual opioids as the cause for the *naloxone cAMP overshoot*. A dose response curve for the *naloxone cAMP overshoot* gave an Emax of 25% and an ED50 value of ~1 nM (Fig. 1.2), which is consistent with naloxone's action at a μ receptor population. A second dose-response curve gave Emax of 80% and ED50 of ~3 nM, illustrating the variability encountered between experiments. Further, the pharmacologically

inactive (+)-naloxone failed to elicit this overshoot (Table 1.1), as expected from the stereoselective interaction of naloxone with the μ receptor. Pertussis toxin was previously shown to prevent the acute and chronic effects of morphine *in vivo* (58). Similarly, pertussis toxin (200 ng/ml) added along with 1 μM morphine for 12 h pretreatment, prevented the *spontaneous* (106 ± 13% of control, n.s.) and *naloxone cAMP overshoot* (110 ± 7% of control, n.s.), and the acute inhibition by morphine (10 mM, 90 ± 8% of control, n.s.) added after the pretreatment period. Therefore, the cAMP overshoot phenomena depend on a pertussis toxin sensitive G protein. These combined results strongly support the hypothesis that naloxone exerts negative intrinsic activity at the constitutively active μ* receptor, prevalent in the dependent state (Scheme 1.1).

When naloxone was applied to thoroughly washed, untreated SH-SY5Y control cells, we observed a paradoxical decrease of cAMP accumulation (Fig. 1.1). Whereas naloxone represents the prototypal opioid receptor antagonist, under certain conditions it was shown to cause agonist-like effects (40, 59). Furthermore, opioid agonists at low doses can evoke excitatory effects on sensory neurons (60, 61) and the myenteric plexus (62) which are reversed by naloxone and mediated by G_s. Hence, μ receptors are capable of activating AC *via* G_s, while the common inhibitory effect *via* G_i becomes more prominent with increasing receptor activation. These dual effects may account for paradoxical hyperalgesic and aversive opioid effect (59), and they could play a role in narcotic dependence (60, 61). Therefore, the decrease of cAMP accumulation caused by naloxone in control cells (Fig. 1.1) suggests the presence of a low level of μ* which stimulates AC via G_s (Scheme 1.1). Significant naloxone inhibition of AC activity was also observed in

thoroughly washed untreated SH-SY5Y cell membranes (Fig. 1.1) arguing against the presence of residual endogenous opioids. Half-maximum inhibition of this stimulatory μ effect by naloxone was observed at ~0.3 nM (Fig. 1.2), which is again consistent with naloxone interacting at a μ receptor population. Pretreatment with pertussis toxin (200 ng/ml for 12 hours) to block G_i, but not G_s, suppressed the acute reduction of cAMP by 10 mM morphine (90 ± 8% of control, n.s.) without affecting the reduction of cAMP by naloxone (72 ± 15% of control, $p < 0.01$), as expected if the effect of μ* receptors were predominantly mediated *via* G_s in control cells. Such paradoxical naloxone effects must be considered in all experiments where naloxone is used as a test compound for determining the involvement of endogenous opioid systems (63). Further, the upregulation of μ opioid receptors by naloxone observed in SH-SY5Y cells (64) can be accounted for by its negative intrinsic activity at μ*. In summary, the net effect of μ* activity on cAMP accumulation can be either positive at low levels of activation, or negative at higher levels of activation. However, on the basis of these results alone we cannot exclude the possibility that two distinct μ receptor gene products mediate the stimulatory and the inhibitory effects, and that these two receptor types are differentially regulated during morphine pretreatment. Even though, the results cannot be readily interpreted without invoking constitutive activation.

Time Course of cAMP Overshoot

To account for tolerance and dependence, the *spontaneous* and the *naloxone cAMP overshoot* should occur gradually (65). Pretreatment of cells with 1 μM morphine for 20 min or less caused a small decrease of PGE_1

stimulated cAMP accumulation, which was not reversed or even enhanced by the addition of naloxone (Fig. 1.3). Therefore, this decrease could not have resulted from residual morphine after washout but it is consistent with enhance formation of μ*. A similar naloxone resistant reduction of AC activity after acute doses of morphine given to rats was previously observed to occur in the locus coeruleus when AC activity was assayed *in vitro* (58). Morphine pretreatment longer than 20 min caused gradually increasing levels of both the *spontaneous* and the *naloxone cAMP overshoot*, indicative of increasing μ*, until a maximum is reached at 12 hours (Fig. 1.3). A treatment period of 12 hours for attainment of a maximum cAMP overshoot is compatible with the slow development of tolerance and dependence (65).

The reversal of the cAMP overshoot after morphine removal was determined by incubating the pretreated cells in drug-free medium for varying times. Surprisingly, the known *spontaneous cAMP overshoot* disappeared within 30 min after morphine removal (Fig. 1.3). This result does not support the hypothesis that the *spontaneous cAMP overshoot* alone can maintain the dependent state for a prolonged time period. In contrast, the *naloxone cAMP overshoot* , indicative of μ*, lasted for at least two hours (Fig. 1.3). From 4-12 h after morphine removal, naloxone had no effect on cAMP levels, in contrast to the decrease of cAMP observed in untreated cells. These results suggest a slow decline of constitutively active μ* receptors after drug removal. Therefore, its time course could account for the delayed peak of narcotic withdrawal seen *in vivo* (65), occurring after morphine is largely eliminated from the body.

Possible Role of μ Receptor Phosphorylation

We then investigated the mechanisms contributing to the *spontaneous* and *naloxone cAMP overshoot.* Increased cAMP levels as a result of prolonged AC inhibition by α2 receptors were previously shown to depend on intact protein kinase A (PKA) activity (66). Indeed, pretreatment of the cells with the PKA inhibitor H8 (67), at 100 mM for 12 hours, resulted in a significant cAMP increase (Fig. 1.4; control *versus* H8), consistent with the hypothesis that decreased PKA activity results in enhanced cAMP accumulation (66). However, coincubation of H8 with 1 μM morphine caused an even greater *spontaneous cAMP overshoot* after drug removal (Fig. 1.4), suggesting that an additional mechanism is operative for morphine, such as the continued release of bg subunits from G_i by active μ* receptors. bg subunits were previously shown to sensitize AC subtypes II and IV and can lead to paradoxical stimulation of cAMP levels by receptors coupled to G_i (68). Furthermore, pretreatment with H8 plus morphine did not block the *naloxone cAMP overshoot* (Fig. 1.4). The latter result indicates that constitutive μ receptor activation does not result from phosphorylation *via* PKA which is fully inhibited by 100 mM H8 (67). Protein kinase C (PKC) is unlikely to play a role, because H8 at 100 mM also inhibits PKC (67), and the PKC selective antagonist, chelerythrine (1 mM) (69), failed to produce any measurable effects on cAMP accumulation (data not shown).

The general protein kinase inhibitor, H7 (1-(5-isoquinoline sulfonyl)-2-methylpiperazine) is structurally similar to H8 but has a different inhibitory profile against various protein kinases (67). Treatment with H7 alone for 12 h also caused a marked cAMP increase at concentrations known to inhibit PKA (100 mM). However, in constrast to H8, pretreatment with morphine

plus H7 abolished the *spontaneous* and the *naloxone cAMP overshoot* (Fig. 1.4). Similar results were observed with 10mM H7, whereas 1 mM had little effect. This result suggests that H7 blocks a protein kinase that recognizes agonist activated μ receptors and causes their constitutive activation to μ* by phosphorylation. Such a kinase may be related to the known b-adrenergic receptor kinases (βARK) since agonist dependent b_2 desensitization, resulting from receptor phosphorylation by βARK, was also partially prevented by H7 in human lymphocytes (70). However, H7 had no effect on β2 receptor regulation in 1321 N1 human astrocytoma cells (71), and therefore, the identity of this putative μ receptor kinase remains to be established.

If μ receptor phosphorylation were involved in its constitutive activation, one would expect a relatively rapid and continuous turnover of the phosphate group on the receptor through the action of kinases and phosphatases. In this case, the kinase inhibitor H7 should rapidly reverse activation to μ* by blocking receptor phosphorylation in the dependent state (Scheme 1.1). Indeed, after 12 h pretreatment with morphine to induce the dependent state, and complete drug removal, a 30 min incubation with 100 mM H7 alone reverted the naloxone effect to that observed in naive cells (Fig. 1.4), i.e., naloxone again lowered cAMP levels.

After 2 h of H7 treatment, the naloxone effect was completely abolished, indicating the absence of any detectable μ* activity. Similarly, 100 mM H7 added for 2 h to untreated SH-SY5Y cells abolished the naloxone induced reduction of cAMP accumulation seen in control cells (106 ± 4 % of control, n=8, n.s.). These results strongly suggest that H7 prevents and rapidly reverses constitutive μ receptor activation. In contrast, 100 mM H7 added

directly to the cAMP assay incubation had no significant effect on acute morphine (10 mM) inhibition of cAMP accumulation in naive cells (45 ± 9% of control in the absence *versus* 50 ± 9% of control in the presence of H7, n=6).

Inhibitor H7 should have also prevented and reversed morphine tolerance. Pretreatment with morphine + 100 mM H7 for 12 h indeed enhanced the subsequent inhibition by 10 mM morphine (37 ± 8 % of control cAMP accumulation, compared to 51 ± 15 % of control (n=12) after pretreatment with morphine alone ($p < 0.01$)). Therefore, H7 was capable of reversing morphine tolerance.

Antagonists with Negative Intrinsic Activity and Neutral Antagonists

The ability to detect constitutive μ* receptor activity in tolerant-dependent SH-SY5Y cells allows one to reclassify the pharmacological profiles of narcotic drugs previously termed partial agonists, mixed agonists-antagonists and antagonists. One now must also consider their potential to interact with active μ* receptors, either with negative intrinsic activity at μ*, or as neutral antagonists which bind to μ* without affecting its activity. We therefore tested a panel of opioid drugs for their ability to either decrease or increase cAMP levels in morphine pretreated SH-SY5Y cells (Table 1.1). As expected from their high potency in causing withdrawal, naloxone, naltrexone, and diprenorphine all have negative intrinsic activity, increasing cAMP levels in drug-free dependent cells. Known agonists and partial agonists decreased levels to varying degrees as expected from their agonist efficacy. Buprenorphine was found equally effective to morphine, whereas

pentazocine is less effective. DAMGO and DADLE are full μ agonists (10, 33, 35), lowering cAMP levels even below those obtained with morphine.

Nalorphine is of interest because it is considered an antagonist with little agonist activity, and in the tolerant-dependent SH-SY5Y cells, it had no significant effect on cAMP levels. Hence, nalorphine may represent a *neutral antagonist*. This result is consistent with the finding that nalorphine while having similar μ receptor affinity, is nevertheless 20 times less potent than naloxone in eliciting withdrawal in morphine dependent mice (72).

The μ selective peptide antagonist CTAP and its analogs, CTOP and d-Tic-CTAP (73), also had no significant effect on cAMP levels in dependent cells when given alone (Table 1.1). Moreover, in contrast to naloxone, CTOP (1mM) did not lower cAMP levels in untreated SH-SY5Y cells (99 ± 18 % of control, n=7). However, CTOP and CTAP reversed the effects of both morphine and naloxone in dependent cells (Table 1.1), showing them to be antagonists at μ and *neutral antagonists* at μ* opioid receptors. Reversal of the effects of naloxone by the μ selective CTOP and CTAP provides strong supportive evidence for the hypothesis that naloxone has negative intrinsic activity at the μ* receptor. Moreover, this dual action of CTAP and CTOP rules out residual morphine or endorphin activity in the cells as a factor in the *naloxone cAMP overshoot.*

The δ selective agonist DPDPE showed little effect at 1 mM in naive (10, 33, 35) and dependent cells, nor did the δ antagonist ICI 174864 have any effect on cAMP levels in dependent cells (Table 1.1). This result rules out any interference by δ opioid receptors which are also expressed in smaller number in SH-SY5Y cells (10)

Effects of H7 and CTAP in Morphine Tolerant-Dependent Mice

If μ* activity were indeed crucial in narcotic tolerance and dependence, one would predict the following drug effects in morphine pretreated animals. First, H7 should reverse tolerance and suppress naloxone induced jumping without affecting morphine analgesia, whereas H8 should be without any effect. Second, the neutral antagonist CTAP should cause less withdrawal than naloxone because it does not reverse the activity of μ*, and third, CTAP should reverse naloxone induced jumping.

Mice were made acutely dependent on morphine by a single s.c. injection of 100 mg/kg morphine sulfate, and drug induced withdrawal jumping was tested after 4 hours (74). Intraperitoneal (i. p.) administration of naloxone caused a dose-dependent increase in the number of jumps (Fig. 1.5). Administration of H7 (50 nmol, intra-cerebroventricularly (i. c. v.) 30 min before naloxone strongly suppressed the number of naloxone precipitated jumps (Fig. 1.5), without affecting gross behavior of the animals or i.c.v. morphine antinociception as measured in the 55 °C hot water tail-flick test (data not shown). In contrast, H8 was without effect on naloxone induced jumping (Fig. 1.5), as predicted from the *in vitro* results. Therefore, the protein kinase inhibitor H7 reverted the drug dependent state towards a naive state within 30 min.

Inhibitor H7 also fully reversed morphine tolerance observed 5 hours after a 100 mg/kg morphine sulfate s.c. dose in mice. The test dose of 10 mg/kg morphine sulfate (the ED90 in naive animals) produced only 22 ± 14 % analgesia in a 55 °C tail flick assay when given 5 h after the first dose, and 30

min after a control i.c.v. injection of saline. However, i.c.v. injection of 50 nmole H7 30 min before the second morphine test dose completely reversed tolerance, yielding 90 ± 10 % analgesia. Therefore, H7 rapidly reversed both morphine tolerance and dependence in an acute mouse model.

To precipitate strong withdrawal by direct injection into the CNS (for testing the polar CTAP peptide as a neutral antagonist), simultaneous injections i.c.v. and intrathecally (i.t.) were required for naloxone (47 ± 12 jumps at 10 nmol into each site). Apparently, both spinal and supraspinal sites are cooperatively involved in producing withdrawal jumping. In contrast, jumping was suppressed by H7 given either i.c.v. or i.t. to the same extent (data not shown). CTAP, in doses previously shown to antagonize PLO17 antinociception (73) (0.3 and 1 nmol i.c.v. plus i.t.) indeed caused significantly less withdrawal jumping (10 ± 9 and 12 ± 5 jumps, respectively), as expected for a neutral antagonist. Finally, CTAP given either i.c.v. or i.t. only (0.3 nmol) significantly reduced naloxone induced jumping (3 mg/kg i.p.) from 77 ± 20 jumps to 32 ± 12 jumps (i.c.v.) and 35 ± 9 (i.t.) ($p<0.05$). These *in vivo* results satisfy the three specific predictions from the *in vitro* results with SH-SY5Y cells and strongly support a role for constitutively active μ^* receptors in narcotic dependence.

Physiological and Pharmacological Implications of Constitutive μ Receptor Activation.

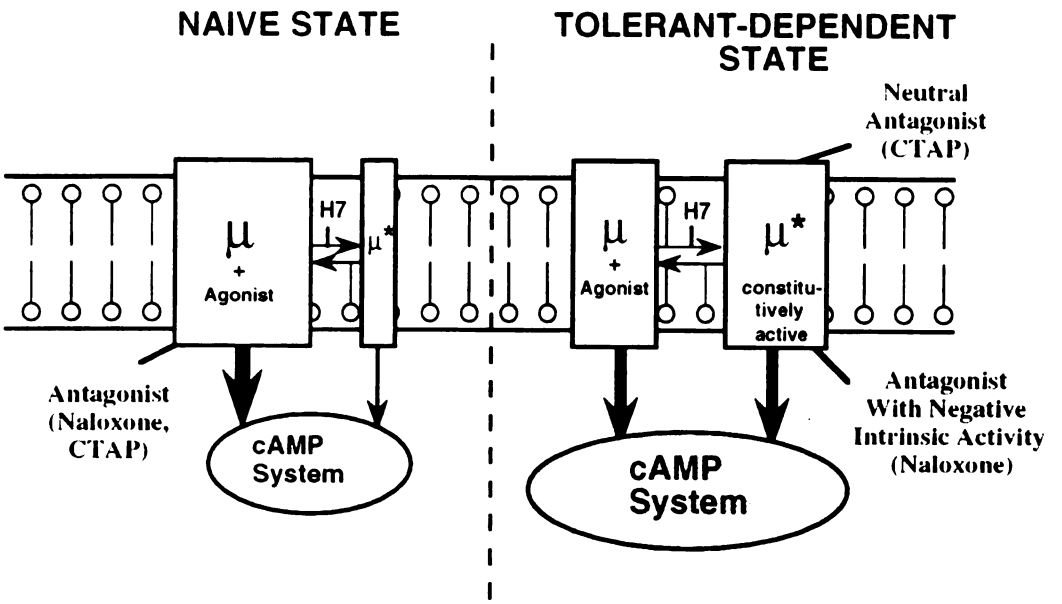
Constitutive activation as an agonist induced regulatory mechanism had not been considered previously for any G protein coupled receptor, or for any other receptor classes. However, basal constitutive activity has been

documented for several G protein coupled receptors (GPCRs), including the δ opioid receptor (75, 76, 77). Moreover, single point mutations in different receptor domains were shown to cause permanent activation of adrenoceptors, MSH, LH, and thyrotropin receptors, and rhodopsin (78, 79). Further, agonist independent phosphorylation by PKC leads to the sustained activation of heteromeric NMDA receptor channels (80), providing a precedent that phosphorylation can result in permanent activation of membrane receptors. The reversal of μ* formation by the protein kinase inhibitor H7 indeed suggests phosphorylation by a receptor kinase as a possible mechanism for mediating constitutive μ receptor activation. Since receptor kinases such as βARK selectively recognize activated receptors, receptor phosphorylation could thus serve as a feed-forward mechanism: the μ* receptor is continuously phosphorylated and trapped in a highly phosphorylated state for prolonged times.

Since constitutive μ activation occurs rapidly, it is likely to affect the time course of pharmacological effects. Indeed, the duration of analgesia is identical for many opioid drugs regardless of their elimination half-life (81). These paradoxical results may be accounted for by postulating that the duration of action depends on the slow reversal of the active μ* state, induced by a single narcotic agonist dose or release of endogenous opioids, and not on the ligand's half-life in the brain.

We further propose that μ* plays a key role in the development and maintenance of narcotic tolerance and dependence (Scheme 1.1). The regulation of μ* activity affords novel therapeutic approaches to narcotic analgesia, tolerance, dependence, and overdose. Neutral antagonists may find clinical use in treating narcotic overdose, particularly in addicts where

naloxone causes immediate withdrawal. Moreover, suitable kinase inhibitors such as H7 could permit the use of narcotic drugs as analgesics without dependence liability, or they could serve in the treatment of narcotic addiction (34). Constitutive activation could represent a common regulatory mechanism of receptor systems with long term effects, including addiction to other substances of abuse (cocaine, alcohol), memory, and mental illness, such as schizophrenia.



Scheme 1.1 Proposed constitutive μ receptor activation and upregulation of the cAMP second messenger system during narcotic tolerance and dependence. The cAMP second messenger system includes the G coupling proteins, adenylyl cyclase (AC), and protein kinase A (PKA), all of which are reported upregulated in narcotic tolerance/dependence. The model does not rule out other regulatory processes that may occur in parallel, such as μ receptor desensitization which may predominate in some cell types (34) or adaptive changes downstream of the cAMP cascade or in other cells. μ : inactive, drug-sensitive μ opioid receptor; μ* : constitutively active μ opioid receptor. The size of the arrows and boxes or ovals indicate relative activity or abundance of each of the components. Conversion of μ to μ* is proposed to be mediated by a receptor kinase which can be inhibited by H7. Antagonists can act at μ* either as antagonists with negative intrinsic activity or as neutral antagonists which bind to μ* but do not affect its activity.

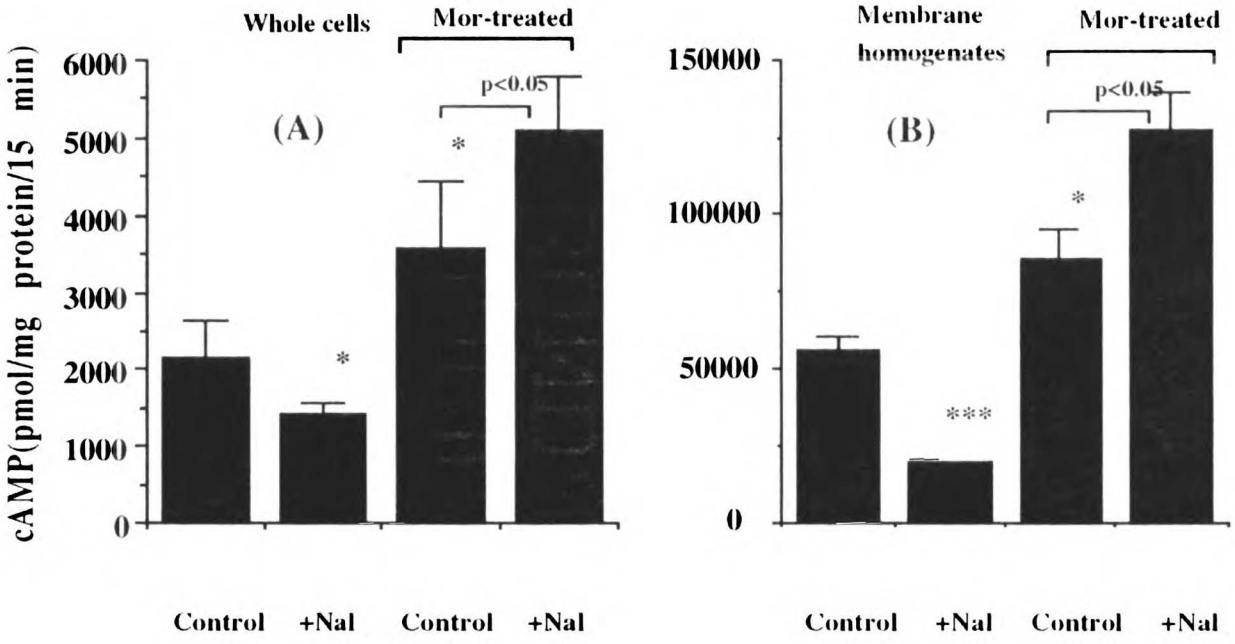


Fig. 1.1 Effects of naloxone on PGE_1-stimulated cAMP accumulation in untreated and morphine-pretreated SH-SY5Y cells.

Fig. 1.1 (legend) Cells were differentiated with retinoic acid and pretreated with morphine (1 mM) or no drug for 48 h. The difference between "Control" and "Mor-pretreated Control" is the *spontaneous cAMP overshoot* as reported previously (10), whereas the difference between "Mor-pretreated Control" and "Mor-pretreated + Nal" is the *naloxone cAMP overshoot.*, representing μ* activity. The magnitude of the cAMP overshoot values differed considerably among different experiments, but the naloxone cAMP overshoot was significant ($p < 0.05$) in at least 4 out of 5 experiments. SH-SY5Y cells are phenotypically unstable and need to be replaced after more than 6-8 weeks in culture. **Panel A.** Intact cell monolayers. **Panel B.** Cell membrane homogenates. cAMP accumulation was assayed without the addition of PGE_1. The data represent mean ± S.D. ($n = 6$-16). *$p < 0.05$, *** $p < 0.001$ *versus* control (no pretreatment) (Scheffe t test). The significance level for the difference between morphine pretreated control with and without the addition of naloxone is inserted above the brackets over these two columns (= *naloxone cAMP overshoot*).

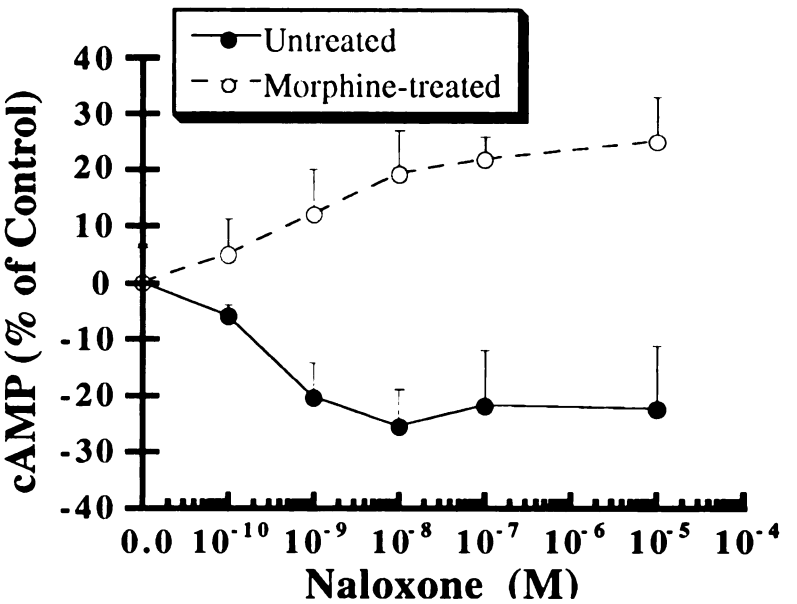


Fig. 1.2. Dose response curves for naloxone effects on PGE_1-stimulated cAMP accumulation in untreated and morphine pretreated, thoroughly washed SH-SY5Y cells. Whereas naloxone caused a decrease in untreated cells, it caused an increase in morphine pretreated cells (*naloxone cAMP overshoot*), above the *spontaneous cAMP overshoot* in treated cells. The *spontaneous cAMP overshoot* (~50% above the control level, $p < 0.05$) was subtracted from the total cAMP level achieved with naloxone to give the *naloxone cAMP overshoot*. Cell culture and cAMP assay conditions were identical to those in Fig. 1.1. The data are mean ± S.D. (n = 4).

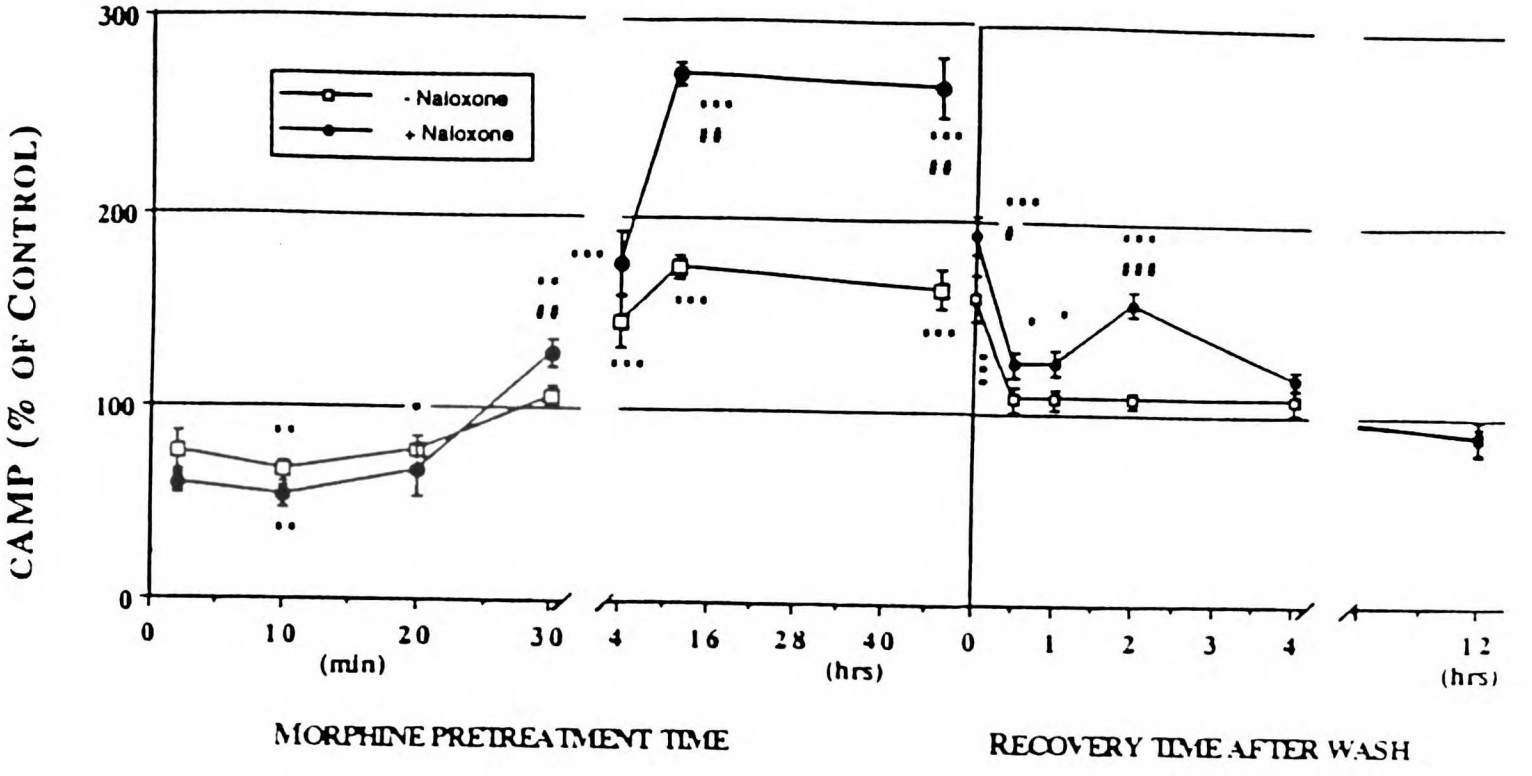

CAMP (% OF CONTROL)
300
200
100
0
- Naloxone
+ Naloxone
0
10
20
30
(min)
4
16
28
40
(hrs)
0
1
2
3
4
12
(hrs)
MORPHINE PRETREATMENT TIME
RECOVERY TIME AFTER WASH

Fig. 1.3. Time course of the *spontaneous* and *naloxone cAMP overshoot*.. Cells were pretreated for up to 48 h with 1 μM morphine, and the drug was then removed at the indicated times by thorough washing. cAMP accumulation, stimulated by PGE_1, was then determined either in the absence or presence of 10 mM naloxone (see legend of Fig. 1.1 for details). For recovery, morphine was thoroughly washed out after a 12 hour pretreatment period, and the cells were incubated in drug-free medium for an additional time period (up to 12 h). After additional washing (twice), cAMP accumulation was measured as above in the absence or presence of naloxone. Each point represents the mean ± S.D. (n = 8-12). *p < 0.05, **p < 0.01, ***p < 0.001 compared with control (no morphine pretreatment). #p < 0.05, ##p < 0.01, ###p < 0.001 for the difference between morphine pretreated control with and without addition of naloxone (=*naloxone cAMP overshoot*) (Scheffe t test). The figure is constructed with composite data from a series of multiple independent experiments with varying magnitudes of the cAMP overshoot phenomena because of phenotypic variability of the cell cultures.

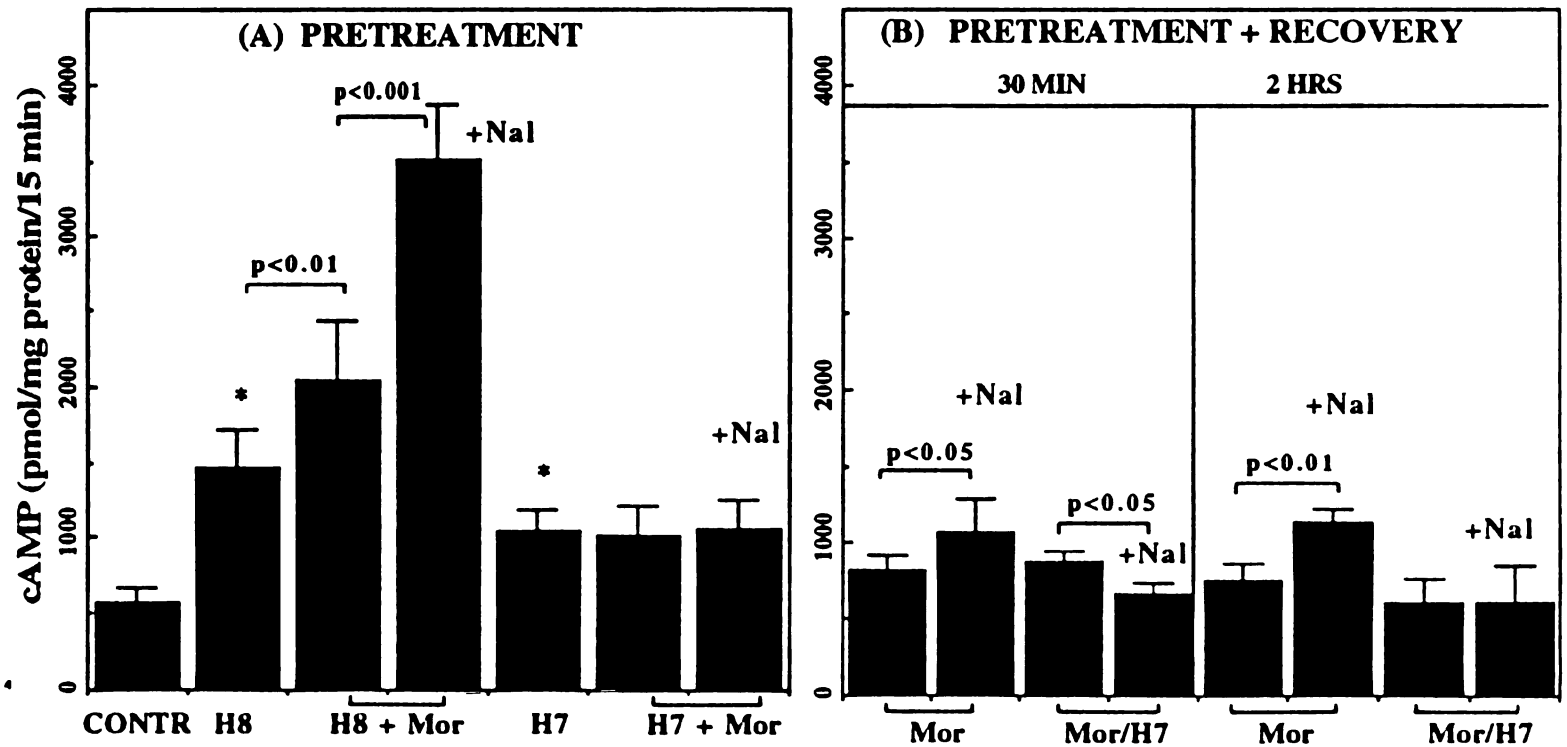

(A) PRETREATMENT
cAMP (pmol/mg protein/15 min)
0
1000
2000
3000
4000
p<0.001
+Nal
p<0.01
*
*
+Nal
CONTR
H8
H8 + Mor
H7
H7 + Mor
(B) PRETREATMENT + RECOVERY
30 MIN
2 HRS
+Nal
p<0.05
p<0.05
+Nal
+Nal
p<0.01
+Nal
Mor
Mor/H7
Mor
Mor/H7

Fig. 1.4. Effect of the protein kinase inhibitor H7 and H8 on cAMP accumulation. Panel A: H7 or H8 (100 mM) was added to SH-SY5Y cell incubates for 12 h, either without or together with 1 μM morphine. After complete removal of the drugs by washing, PGE_1 stimulated cAMP accumulation was measured in the presence or absence of 10 mM naloxone. Panel B: SK-N-SH cells were treated for 12 h with 1 μM morphine, and after drug removal by thorough washing, the incubations were continued for 30 min or 2 h in morphine-free medium in the presence or absence of 100 mM H7. After again washing out the protein kinase inhibitor, PGE_1-stimulated cAMP accumulation was measured in the presence or absence of 10 mM naloxone. Data are mean ± S.D. (n = 6-12). *$p<0.05$ versus control; significance levels for other comparisons are given above the brackets over the respective columns (Scheffe t test).

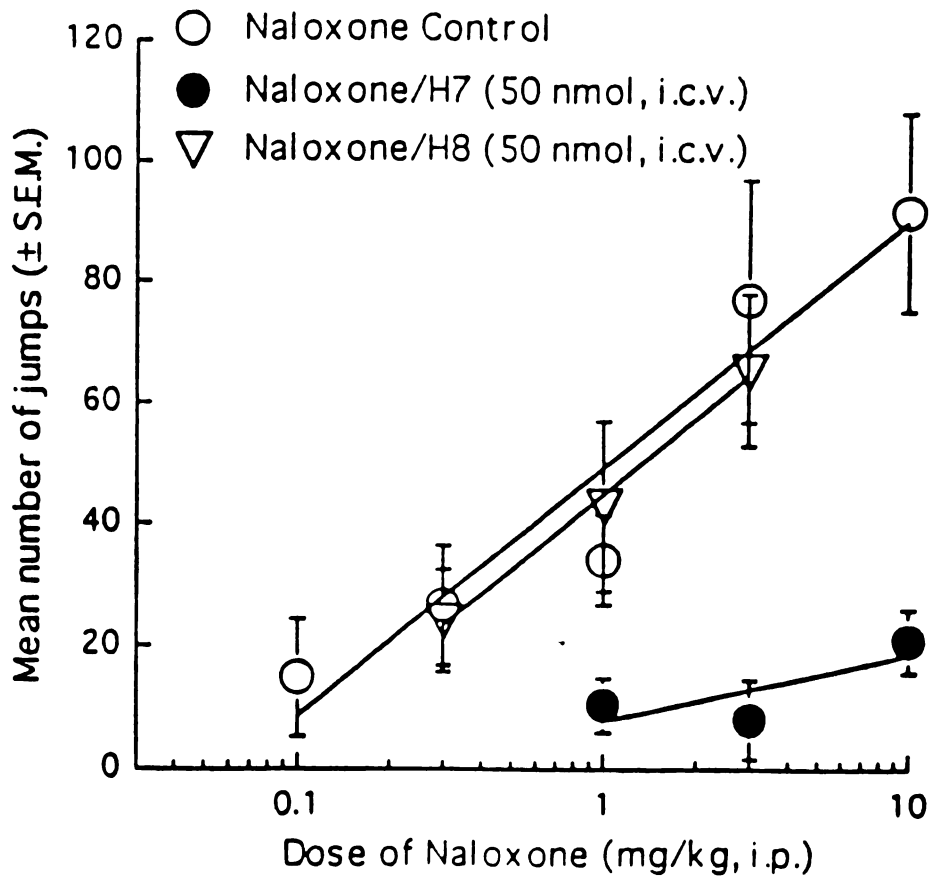


Fig. 1.5. Effect of the protein kinase inhibitors H7 and H8 on naloxone induced withdrawal jumping in mice.

The animals were made dependent with an s.c. dose of 100 mg/kg morphine sulfate. Only H7, but not H8, suppressed withdrawal jumping when injected i.c.v. 30 min before naloxone (3mg/kg i.p. 4 h after morphine). The H7 dose of 50 nmole was found to be maximally effective without producing deficits in motor activity at the time of withdrawal testing.

TABLE 1.1

Effect of selected opioids on cAMP accumulation in morphine pretreated SH-SY5Y cells.

Compound (concentration)	% Control (Mean ± S.D.)	n	Statistical levels (p)[1] vs Control	vs Naloxone
Diprenorphine 10 mM	149 ± 21	8	< 0.001	n.s.
Naltrexone 10 mM	146 ± 39	8	< 0.001	n.s.
Naloxone 10 mM	144 ± 8	24	< 0.001	_ _
Naloxone 1 mM	142 ± 35	8	< 0.001	n.s.
Naloxone 1 mM +CTAP10mM	111 ± 7	8	n.s.	n.s.
Naloxone 0.1 mM	140 ± 20	8	< 0.001	n.s.
Naloxone 0.1 mM +CTOP 10 mM	110 ± 6	8	n.s.	< 0.001
(+)-Naloxone 10 mM	94 ± 19	8	n.s.	< 0.05
Nalorphine 1 mM	112 ± 15	8	n.s.	n.s.
CTAP 1 mM	107 ± 5	16	n.s.	<0.01
D-Tic-CTAP 1 mM	91 ± 10	8	n.s.	<0.01
ICI-174,846 1 mM	91 ± 23	8	n.s.	< 0.001
DPDPE 1 mM	77 ± 18	8	n.s.	< 0.001
CTOP 1 mM	91 ± 3	8	n.s.	< 0.001
CTOP 1 mM +Morphine 10 mM	115 ± 23	8	n.s.	< 0.05[2]
Pentazocine 10 mM	70 ± 6	8	< 0.05	< 0.001
Morphine 10 mM	51 ± 15	8	< 0.001	<0.001
EKC 1mM	51± 15	8	<0.001	<0.001
Buprenorphine 1 mM	43 ± 14	8	< 0.001	< 0.001
DADLE 1 mM	29 ± 2	8	< 0.001	< 0.001
DAMGO 1 mM	20 ± 4	8	< 0.001	< 0.001

TABLE 1.1. NOTES:

The cells were pretreated with 1 μM morphine for 12 h and thoroughly washed. Then, cAMP accumulation was measured for 15 min in the presence of 1 mM PGE_1, plus the indicated opioid drug. The 100% control is the cAMP accumulation in the absence of any opioid drug (*spontaneous cAMP overshoot*). Cell culture and cAMP assay conditions were identical to those in Fig. 1.1.

1. vs Control : cAMP accumulation after morphine pretreatment and complete drug removal (=100%); vs Naloxone : cAMP accumulation under the same conditions but in the presence of naloxone. Scheffe t test; n.s.: not significant.

2. $p < 0.001$ compared with 10 μM morphine alone.

CHAPTER II

EXPRESSION, COUPLING, CONSTITUTIVE ACTIVITY, AND PHOSPHORYLATION OF A CLONED μ OPIOID RECEPTOR STABLY EXPRESSED IN HEK 293 CELLS

SUMMARY

The μ opioid receptor expressed in SH-SY5Y neuroblastoma cells appeared to display constitutive activity in the absence of any agonist (Wang, Z., Bilsky., E. J., Porreca, F., and Sadée, W. (1994) *Life Sci.* 54, PL339-350) On the basis of results obtained in SH-SY5Y neuroblastoma cells, we have proposed the hypothesis that prolonged agonist stimulation enhances the conversion of the μ receptor to a constitutively active state, termed μ*, by stimulating μ receptor phosphorylation via an H7-sensitive protein kinase. To test this hypothesis further, we functionally expressed a cloned rat μ opioid receptor gene (Chen, Y., Mestek, A., Liu, J., Hurley, J.A., and Yu, L. (1994) *Mol. Pharmacol.*. 44, 8-12) in human embryonic kidney (HEK) 293 cells. One selected clonal HEK-μ cell line expressed 4 x 10^6 sites per cell. The μ receptor in HEK-μ cells inhibited forskolin (10 μM)-stimulated cAMP accumulation with an EC50 of ~30 nM, and a maximum inhibition of 76 ± 6 %.

Constitutive μ receptor activity (μ*) was assessed by measuring the effects of the proposed negative antagonist naloxone in morphine-pretreated HEK-μ cells. [^{3}H]Morphine labeling of HEK-μ and other control tests demonstrated that morphine was functionally removed before cAMP accumulation assay. The addition of naloxone to the drug-free cAMP accumulation medium elicited a large cAMP overshoot at 554 ± 60 % over the control (cAMP level in the absence of naloxone) ($p<0.001$), indicative of the presence of substantial μ* activity, as seen in SH-SY5Y cells.

Using an epitope-tagged μ receptor with similar pharmacological properties, receptor phosphorylation was measured in permeabilized cells. There was high basal receptor phosphorylation in the absence of agonist, which was differentially affected by agonists and antagonists.

INTRODUCTION

It has been previously proposed that agonist stimulation of the μ receptor in SH-SY5Y neuroblastoma cells results in its gradual conversion to a constitutively active state, termed μ*, by a process sensitive to the protein kinase inhibitor H7 (50, 53). Naloxone was identified as a μ* antagonist with negative intrinsic activity (i.e. inverse agonist), which could account for its increasing potency in eliciting withdrawal with increasing narcotic dependence (50). The proposed neutral antagonist CTAP not only caused significantly less withdrawal jumping than naloxone in morphine-tolerant mice, but it also reversed the intense withdrawal jumping elicited by the negative antagonist naloxone (52). A central role for μ* in narcotic tolerance and dependence was further supported by the finding that H7 rapidly

reversed acute tolerance and dependence in morphine treated mice (50). However, we cannot exclude the possibility that additional, yet unknown opioid receptor subtypes could have contributed to the unique results obtained in SH-SY5Y cells which natively express μ and δ opioid receptor subtypes (10, 33).

In order to test these hypotheses, we stably transfected a cloned rat μ opioid receptor gene (20) into HEK 293 cells. One resulting HEK-μ cell line expressed high levels of receptors (4 x 10^6 sites per cell), and functionally coupled to the adenylate cyclase. This cell line was used for this study to examine the acute and chronic effects of morphine and other opioids.

Regulation of μ receptor phosphorylation by opioid ligands was also important to understand the vastly different acute and chronic effects of opioids. Phosphorylation of neurotransmitter and hormone receptors is recognized as a common regulatory process leading to desensitization during agonist stimulation (82). Yet, phosphorylation activates many cellular proteins including some ion channels (80). Moreover, tyrosine phosphorylation plays an integral role in the signaling of growth factor receptors (83). Recent evidence suggests that the activation of G-protein coupled receptors (GPCRs) is facile, because of a low energy barrier between inactive and active states. Several GPCRs including receptors coupled to the inhibitory G_i (adenosine A1, muscarinic m4, dopamine D2, and opioid δ and μ receptors) display basal activity in the absence of agonists (50, 76, 84, 85). Numerous point mutations in different GPCR domains have been shown to result in constitutive activation (78, 84), and several such mutations were linked to genetic disorders (78, 84). These findings suggest that GPCR phosphorylation might be able to enhance basal (or constitutive) receptor

activity, and if agonist dependent, would constitute a feed-forward loop with profound physiological implications.

Although more detailed accounts of regulation of the constitutive μ receptor activity will be discussed in later Chapters (III, IV), here we directly measured μ receptor phosphorylation in HEK 293 cells stably expressing a tagged μ receptor (HEK-EE-μ cells) (25). Epitope (EE) tagging of the N terminus of the μ receptor (EE-μ) greatly facilitates immunoprecipitation, western blotting of the μ receptor (25). The pharmacological and biochemical properties of HEK-μ and HEK-EE-μ were indistinguishable. Immunohistochemistry demonstrated that the tagged μ receptors were localized at the plasma membrane when stably expressed in HEK 293 cells (25), or transiently or stably expressed in human neuroblastoma SH-SY5Y cells (Wang, Z., unpublished data). We reported here the regulation of basal μ receptor phosphorylation by opioid agonists and antagonists.

EXPERIMENTAL PROCEDURES

Materials. N-methyl-[^{3}H]-morphine (79 Ci/mmol) was purchased from NEN (Boston, MA). Morphine sulfate, [15,16-$^3H_2$]-diprenorphine (specific activity 47 Ci/mmol), naloxone sulfate, D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2 (CTAP) (73), [N-MePhe3,D-Pro4]morphiceptin (PLO17), etorphine, and [D-Ala2, MePhe4,Glyol5]enkephalin (DAMGO) were provided by the National Institute on Drug Abuse, Rockville, MD. The protein kinase inhibitor H7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine) and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) were purchased from Sigma Chemicals (St. Louis, MO). H8 (N-[2-

(methylamino)ethyl]-5-isoquinolinesulfonamide) was obtained from Seikagaku American Inc. (Rockville, MD). Calyculin A was a product of RBI (Natick, MA). Monoclonal antibody to the epitope EYMPME was from Onyx Inc. (Richmond, CA). The [^{3}H]-cAMP assay kit was supplied by Amersham (Arlington Heights, IL). GRK2 cDNA was provided by Dr. Tatsuya Haga, Tokyo University.

Cell culture and transfection

Human embryonic kidney cells, HEK 293, were obtained from the American Type Culture Collection (ATCC CRL-1573), and were used as receipt cells for transfection of a cloned rat μ opioid receptor gene (20) (provided by Dr. Lei Yu, Indiana University) by calcium phosphate precipitation method (86). Clonal cells stably expressing the transfected μ receptor (HEK-μ cells) were selected with 400 μg/ml G418. To facilitate the immunoprecipitation of the μ receptor, this receptor was tagged with EYMPME, immediately downstream of the Met initiation codon. An HEK cell line stably expressing the tagged μ receptor (HEK-EE-μ cells) was also established with the above method (25).

HEK 293 cells were also transiently transfected with 2-18 μg of μ receptor cDNA in pRC/CMV vector using calcium phosphate precipitation method. Cells were allow to grow for 60 h after transfection in the absence of G418, before they were harvested for receptor binding study.

Cells were maintained in DMEM/F-12 (50%/50%) medium supplemented with 10 % fetal calf serum containing 100 μg/ml streptomycin and 100 units/ml penicillin. G418 (200 μg/ml) was added in HEK-μ and HEK-EE-μ cultures to maintain stable selection. Passage numbers 3-12 (~ 2 months

in culture) were used, and we have observed declining binding sites at high passages.

Receptor binding assay

Receptor expression was examined by specific binding of [^{3}H]-diprenorphine to intact cell monolayers. Cells were harvested with phosphate buffered saline (PBS) containing no Ca^{++} and Mg^{++} (PBS/CMF) and resuspended into 50 mM Tris·HCl (pH 7.4) containing 0.5% bovine serum albumin. For [^{3}H]diprenorphine saturation binding, triplicate samples (~ 1x 10^6 cells/sample) were incubated with increasing concentrations of [^{3}H]diprenorphine. Non-specific binding was determined by addition of 20 μM cold diprenorphine, except for transient transfection experiment, where non-specific binding was also calculated from the cells transfected with pRC/CMV vector alone. Cells were incubated for 1 hours at room temperature. Binding reactions were terminated by harvesting (vacuum filtration) cells onto Whatman GF/C glass fiber filters, which were washed three time with cold PBS, and ^{3}H retained on the filters were quantified by liquid scintillation counting. Protein was measured by the Bradford method (87), using bovine serum albumin as standard.

Saturation binding curve was fitted by the logistic function:

$$B = B^*L^n/(Kd^n+L^n) + NSB$$

where B is the tracer bound, L is the free ligand concentration, NSB is non-specific binding. n is the Hill coefficient. Kd is the equilibrium dissociation constant.

Displacement of [^{3}H]-diprenorphine binding by morphine and naloxone

HEK-μ cells were treated with morphine (1μM) for 12 h, and rinsed twice with culture medium and 3 times more with PBS. Cells were harvested into PBS/CMF and spun down at 1,000rpm for 10min. These cells were resuspended in 50 mM Tris·HCL (pH 7.4) containing 0.5% bovine serum albumin and divided into ~1 x 106 cells/tube. They were incubated at room temperature for 1 h with 1 nM [^{3}H]-diprenorphine and varying concentrations of morphine or naloxone at 0.1, 1, 10, 100, 1,000, 10,000, and 100,000 nM. Cell harvesting and filtration were performed as the above method. Displacement data were fitted to the logistic function:

$$B = Bmax - [Bmax * L^n]/[IC50^n + L^n] + NSB$$

where B is the tracer bound, Bmax is total numbers of binding sites, L is free ligand concentration, n is the Hill coefficient, and NSB is the nonspecific binding.

Cell treatment and cAMP accumulation assay

Cells were pretreated with 1 μM morphine for 0-12 h. The relatively low concentration of 1 μM morphine was selected to achieve maximum effects while facilitating removal of the drug. Immediately before the cAMP accumulation assay, cells were washed twice with medium containing 5% serum and twice more with serum-free medium.

For the assay of cAMP accumulation, washed cell monolayers were incubated for 7 min at 37 °C in the presence of 10 μM forskolin, and cAMP was determined by RIA as described previously (27, 88)(see also Chapter IV).

Removal of morphine from the pretreatment medium

In order to quantify morphine removal by the above washing protocol. untransfected HEK-293 cells and HEK-μ cells were treated with [^{3}H]-morphine (2 μCi/well), in the presence or absence of unlabeled morphine (1 mM), and washed with media in exactly the same fashion to estimate residual morphine. Washed cells were incubated with 300μl cAMP assay medium (27, 32) in the presence of 10 μM forskolin for 7 min and centrifuged, and tritium content in the supernatants and pellets was determined by liquid scintillation counting.

Immunoblot of EE-μ receptor

HEK 293 and HEK-EE-μ cells were grown to confluence in T75 flasks, and washed briefly with PBS. Cells were harvested with PBS/CMF, and resuspended in lysis buffer (5 mM Tris, protease inhibitors cocktail: 1 mM benzamidine, 1μg/ml leupeptin, 1 mg/ml aprotinin, and 100 μg/ml phenylmethylsulfonyl fluoride (PMSF), and 100 nM diprenorphine to stabilize the receptor during purification) for 15 min on ice. Cells were homogenized, and unbroken cells and nuclei were removed by centrifugation at 1000 x g for 10 min. The supernatant was centrifuged at 30,000 g for 45 min and the membrane pellets were used for immunoblotting.

The membrane pellets were quantified for protein content (87), and aliquots of membranes were resuspended in sodium dodecyl sulfate (SDS)-loading buffer. The proteins were separated by 8% polyacrylamide gel electrophoresis (PAGE), and transferred onto a PVDF membrane (Millipore, Bedford, MA) for 2-5 h at 12 voltage in Tris-glycine buffer. PVDF membrane was blocked with 3% gelatin, and incubated with 1: 500 dilution of monoclonal antibody against the EYMPME peptide (anti-EE mAb) at room temperature for 2 h. The anti-EE mAB was recognized by anti-mouse antibody (1: 2,000 dilution) coupled to alkaline phosphatase, which catalyzes a reaction with chromagenic substance (Bio-RAD) to generate colors.

Phosphorylation and immunoprecipitation of the epitope-tagged μ receptor

Phosphorylation of EE-μ was determined in permeabilized cells by a procedure described earlier with some modifications (25, 89). Confluent EE-μ cells (2×10^7) were pretreated with opioid agonists and antagonists, washed 4-6 times, and incubated for 1-2 h at 37°C in phosphate-free medium. Cells were harvested, resuspended and permeabilized with digitonin (150-200 μM to achieve 90% efficiency measured by trypan blue dye uptake) . Permeabilized cells were labeled with 250 μCi/ml [γ-^{32}P]-ATP (7000 Ci/mmol, Dupont/NEN, Boston, MA) in a final volume of 0.5 ml at 25°C for 15 minutes. Labeling medium was removed to stop the reaction and cell pellets were washed twice with ice-cold Tris-NaCl buffer containing phosphatase and protease inhibitors (50 mM Tris, 100 mM NaCl, the above protease cocktail, pH 7.4). Cell pellets were resuspended in lysis buffer for 15 min, and homogenized. Unbroken cells and nuclei were removed by centrifugation at 1000 x g for 10 min, and the 30,000 x g membrane pellet was solubilized in 10 mM CHAPS, containing

100 nM diprenorphine, and centrifuged at 105,000 x g for 40 minutes. Solubilized EE-μ was immunoprecipitated with anti-EE mAb , subjected to 8% SDS-PAGE, and autoradiographed at -80 °C. The ^{32}P labeled band corresponding to EE-μ was quantitated by scanning densitometry.

Isolation of total RNA and mRNA, and northern blot analysis

Total RNA was prepared from cells by the guanidium isothiocyanate method (90). Poly(A)-selected RNA was isolated by two sequential purification steps of the total RNA over oligo(dT)-cellulose (Pharmacia, Piscataway, NJ). Total RNA or mRNA (~10 μg) was denatured in formamide, fractionated on a 1% agarose-formaldehyde gel, and transferred onto a nitrocellulose membrane (Schleicher & Schuell, Keene, NH) with 20 x saline sodium citrate (SSC). The RNA blot was prehybridized in 5 x SSC solution containing 5 x Denhardt's at 42°C for 5 h. [α-^{32}P]-dCTP labeled cDNA probe was generated by using random hexamer priming (Pharmacia, Piscataway, NJ) with βARK1 (GRK2) cDNA as a template, and used for hybridization at 42 °C in 5 x SSC for 16 h. The blot was washed in 2x SSC and 0.1 % SDS at 42 °C for 1 h, and subjected to autoradiography at -80°C for 12 to 72 h.

RESULTS

Stable expression of μ receptor in HEK-μ cells

HEK 293 cells, which do not possess high-specific binding for opioid ligands, were transfected with a cloned μ receptor cDNA in pRC/CMV vector

containing a geneticin resistance sequence. Of the 12 stable clones resistant to 400 μg/ml G418, one clone expresses 4 x 10^6 sites per cell (Bmax= 34.6 ± 4 pmol/mg protein) measured in intact cell monolayer binding with [^{3}H]diprenorphine. An HEK-μ cell line was established from this clone and used for further studies. The Kd of [^{3}H]diprenorphine was 2.4 ± 0.5 nM from a saturation binding curve (Fig. 2.1). The IC50 for displacement of [^{3}H]diprenorphine was 687 ± 159 nM by morphine, and 59 ± 8 nM by naloxone. When HEK 293 were treated with 1 μM morphine for 12 h, the IC50 for displacement of [^{3}H]diprenorphine was decreased to 444 ± 99 nM by morphine (n.s.), and 35 ± 5 nM by naloxone ($p<0.01$) (Fig 2.2). Therefore there was no evidence of decreased affinity to morphine (agonist) or naloxone (antagonist) after treatment with morphine. The total specific [^{3}H] diprenorphine binding was slightly reduced from 42100 ± 2103 dpm to 38449 ± 1847 dpm (9.5%, n. s.). So there was no significant downregulation of receptor (loss of receptor number), either.

Transient expression of μ receptor in HEK 293 cells

In an attempt to correlate the Kd values with receptor expression levels, HEK 293 cells were transiently transfected 2-18 μg μ receptor cDNA (Fig 2.3). The expression of μ receptor increased from 60 ± 6 fmol/mg protein ($p<0.001$ vs 4 μg), to 322 ± 36 fmol/mg protein ($p<0.001$ vs 18 μg), to 1595 ± 129 fmol/mg protein after transfection with 2, 4, 18 μg cDNA, respectively (Table 2.1). Meanwhile, the Kd of [^{3}H]diprenorphine increased from 0.25 ± 0.1 nM ($p<0.05$ vs 4 μg), to 1.0 ± 0.4 nM ($p<0.05$ vs 18 μg), to 1.8 ± 0.4 nM, respectively. There were no significant difference of Kd, or Bmax after transfections with 8, 12, or 18 μg cDNA, suggesting the saturation of DNA uptake and expression by the cells.

Inhibition of cAMP accumulation by morphine in HEK-μ and HEK-EE-μ cells

In untreated HEK-μ cells, morphine caused a 76 ± 6 % (n=20) maximum inhibition of forskolin (10 μM)-stimulated cAMP accumulation with an EC50 of ~30 nM (Fig. 2.4 & 2.6). Following pretreatment with 1 μM morphine for 12 hours, the EC50 was unchanged, and the Emax was 85 ± 3 % (n=15) (Fig. 2.5 & 2.7). A moderate degree of tolerance to morphine occurs in SH-SY5Y neuroblastoma cells expressing μ receptors natively (35). These differences may have been caused by differences in the host cells or by the higher receptor expression in the transfected cell lines (see also Chapter IV). The maximum cAMP inhibition by 10 μM morphine in HEK-EE-μ cells, before and after treatment with morphine (1 μM), are 74 ± 42 % and 73 ± 45 %, respectively (Fig 2.7).

It was noted that low morphine concentrations (~10 nM) paradoxically stimulated cAMP production in morphine pretreated HEK-μ cells (at 10 nM : 50 ± 22 % above control, n=14, $P<0.01$). At the morphine concentrations used little stimulation was detectable in untreated cells; however, morphine did not show inhibitory effect until the concentration is 1 nM or higher.

Effectiveness of morphine washout after pretreatment.

To test the efficiency of removing morphine before the cAMP accumulation assay, HEK 293 and HEK-μ cells were incubated with N-methyl-[^{3}H]-morphine (2 μCi/well) for 9 hours. The [^{3}H] labeled cells were washed twice with medium containing 5% serum and twice more with serum-free medium, 0.32% of the added tracer was retained in the HEK-μ cell pellets.

Additional washing steps did not further reduce residual [^{3}H] activity in the cell pellet.

Maximum [^{3}H]-morphine labeling of the cells was reached within 0.5-2 hours, which was substantially faster than the development of the naloxone-induced cAMP overshoot. Moreover, addition of 1 mM unlabeled morphine did not affect the washing efficiency, nor did HEK-μ cells retain more tracer than nontransfected HEK 293 cells. Therefore, the residual tracer (0.32%) did not appear to be uniquely bound to the receptors nor any saturable binding sites.

In order to interact with μ receptor at the surface, residual tracer must redistribute into the medium. When the washed HEK-μ cells were incubated in 300 μl fresh cAMP assay medium at 37 °C for 7 min, only 0.12% of the total added tracer redistributed into the medium during that time. Therefore, maximum morphine levels after preincubation with 1 mM and washout would be 1.2 nM in the cAMP assay at the end 7 min incubation.

The spontaneous and naloxone-induced cyclic AMP overshoots after morphine pretreatment.

Agonist stimulation of μ receptors in SH-SY5Y cells results in a compensatory upregulation of the cAMP second messenger system (27, 35) which has been proposed as a biochemical correlate of the dependent state (27, 29, 35, 37, 91). Similarly, pretreatment of HEK-μ and HEK-EE-μ cells with 1 μM morphine, followed by extensive washing to remove the agonist, elicited a cAMP rebound phenomenon (spontaneous cAMP overshoot) (Fig. 2.6 & 2.7) with a doubling of the forskolin stimulated cAMP levels (201 ± 28 % of control, n=24, P <0.001) in for HEK-μ cells, and 44 ± 13 % of control (n=4, n.s.

probably due to large errors) in HEK-EE-μ cells. These results suggest that a dependent state had been attained, similar to that observed in SH-SY5Y cells (27, 35).

To test for the presence of constitutive μ* activity in the dependent state, HEK-μ and HEK-EE-μ cells were pretreated with morphine, and after removing the agonist by washing, the proposed negative antagonist naloxone (27) was added. If substantial μ* activity accumulated during morphine pretreatment, one would expect naloxone to increase cAMP levels further even in the absence of any agonist, as observed previously in SH-SY5Y cells (27) . Indeed, naloxone caused a profound additional cAMP overshoot above the spontaneous cAMP overshoot in HEK-μ (554 ± 60 %, n=26, $P < 0.001$) and HEK-EE-μ cells (467±14, n=4, $P<0.001$) (Fig. 2.6 & 2.7). A naloxone dose response-curve in morphine pretreated HEK-μ cells gave an EC50 value of 0.5 nM (Fig. 2.5, the naloxone-induced overshoot was 250% above the spontaneous overshoot in this experiment), consistent with its high affinity at the μ opioid receptor. In contrast, naloxone lowered cAMP accumulation in untreated and thoroughly washed HEK-EE-μ cell by, (Fig. 2.7) as well as HEK-μ cells by 40 ± 10% (n=20, $P <0.01$) with an EC50 value of 0.5 nM (HEK-μ, Fig. 2.4, & 2.6), which may suggest the presence of basal μ receptor constitutive activity.

Other non-selective opioid antagonists, such as naltrexone, and diprenorphine were also added to the morphine-pretreated HEK-μ cells to test if they, too, cause the naloxone-like cAMP overshoot. Naltrexone (10 μM) was as effective as naloxone (10 μM) in increasing cAMP levels, whereas diprenorphine (10 μM) was slightly less effective (Fig. 2.8). Nalorphine, like in SH-SY5Y cells, seemed to act like a neutral antagonist that did not affect

cAMP levels. However, the previously identified neutral antagonist, CTAP and dic-CTAP, also increased cAMP in those cells. In other experiments, when both CTAP (10 μM) and naloxone (1 μM) were added in the cAMP accumulation assay, CTAP reduced naloxone (1 μM)-induced overshoot ($p<0.05$ naloxone vs naloxone plus CTAP) (Fig. 2.9), suggesting that they may act through different mechanisms.

We also tested if the naloxone-induced cAMP develops after treatment with other narcotic agonists. Whereas naloxone again showed inhibitory effect in the control (untreated) cells, the naloxone-induced cAMP overshoot was seen after treatments with the μ selective peptide agonists DAMGO (1 μM) (300 ± % $p<0.001$) and PLO17 (1 μM) (200 ± %, $p<0.001$). Both also led to significant spontaneous cAMP overshoots. We have not performed any tests to show if DAMGO or PLO17 was removed from the system, although it has been reported that PLO17 (92) can be readily washed out from the cells. Etorphine (100 nM), however, was not capable of inducing either the spontaneous or the naloxone-induced cAMP overshoot (Fig. 2.10).

Labeling of constitutively active μ receptor by opioid ligands

In an effort to specifically label the consitutively active portion of μ receptors (μ*), HEK-μ cell were bound with 1 nM [^{3}H] diprenorphine before and after treatment with morphine (1 μM 12 h). If the constitutively active μ* receptor has differential affinity for opioid agonist or antagonist, one should be able to label the μ* receptor. When the [^{3}H] diprenorphine binding was displaced by morphine or naloxone, both ligands were able to efficiently displace up to 98% of the receptor-bound [^{3}H] diprenorphine (Fig. 2.2). Naloxone showed about 10 fold lower IC50 than morphine in displacing [^{3}H]

diprenorphine either before or after treatment with morphine. Treatment with morphine seemed to reduce the displacement of [^{3}H] diprenorphine by low concentrations of morphine or naloxone.

Immunoblot of the EE-μ receptor

In order to identify the EE-μ receptor expressed in HEK-EE-μ cells, HEK-EE-μ membrane was solubilized in SDS-loading buffer and separated on SDS-PAGE. The EE-μ receptor band was identified by western blot using mAB agonist the EE-epitope-tag. The mAB labeled specific signal was located at 69 Kda, which was absent in untransfected HEK 293 cells even when 123 μg membrane protein was loaded onto the gel (Fig. 2.11). On closer examination, there were two separated, but adjacent bands, which may both relate to the EE-μ receptor and represent to different receptor phosphorylation states. It was reported that the cAMP receptor of Dictyostelium gave rise to distinct protein bands due to receptor phosphorylation(93).

Direct measurement of μ receptor phosphorylation.

Studies in SH-SY5Y cells suggested that the constitutive μ receptor activity was regulated by a phosphorylation mechanism. To address the difference in chronic effects of opioid compounds, we examined the receptor phosphorylation in HEK-EE-μ cells after pretreatment with several representative agonists. We also compared the phosphorylation affected by two antagonists, naloxone and CTAP. All these compounds were removed after treatment.

Treatment with morphine (1 μ) for 12 h, enhanced μ receptor for over 3 fold (Fig. 2.12) as previously reported (25, 88). Treatment with DAMGO (1

μM); however, largely abolished receptor phosphorylation. In another experiment, treatment with 100 nM etorphine also led to diminished μ receptor basal phosphorylation.

Treatment with naloxone (10 μM) enhanced the μ receptor phosphorylation for over 4 fold, whereas incubation with CTAP (1 μM) reduce μ receptor phosphorylation.

Northern blot analysis

G-protein coupled receptor kinases (GRKs) are a newly identified family of kinases that specifically phosphorylate the activated GPCRs. They are capable of introducing multiple phosphates into GPCRs at Ser and Thr residues (23,40), richly represented in the C-terminal tail of the μ receptor (21). Since the GRKs, especially GRK2 (βARK1), have been shown to play important roles in receptor activation and tolerance, we examined the mRNA signals of GRK2 in cell lines. Northern blot analysis revealed the presence of a GRK2 mRNA signal at 4 kb in both HEK 293 cells as well as SH-SY5Y cells (Fig. 2.13). The mRNA preparation of HEK 293 showed a stronger signal due to the enriched mRNA. The RNA preparation from a HEK 293 cell line stably transfected with a GRK2 cDNA was used as a positive control here, and there is a greatly enhanced band at 4 kb. The lower hybridized band (~2.5 kb) may indicate alternative gene splicing or the presence of a similar kinase.

Discussion

The cloned μ receptor, when stably transfected in HEK 293 cells, showed a high receptor expression level. Moreover, the receptor was functionally coupled to the adenlate cyclase. [^{3}H] Diprenorphine binding

yielded a high Kd value. The published Kd of diprenorphine falls below nanomolar (20). One reason for this discrepancy may be due to the difference in binding procedures. The subnanomolar Kd was obtained in cell membrane preparation using unphysiological buffer, which has been shown to yield higher receptor affinity (94). This may also be due to the high expression level of the μ receptors. When transiently introducing the μ receptor into HEK 293 cells, which allowed us to titrate the receptor expression levels, we found that the affinity of diprenorphine increased (Kd decreased) as the receptor expression increased. However, higher expression may not necessarily result in lower coupling efficiency. When beta 2-adrenoceptor expressed at two different levels in NCB20 cells, it was found that the receptor expressed at higher level had lower EC50 value in stimulating cAMP production (95).

HEK 293 cells appeared to be a suitable host cells to study the receptor activation and phosphorylation, since this cell line does not express endogenous opioid receptor as judged from lack of both high specific binding for [^{3}H] diprenorphine and inhibition of cAMP accumulation even by high concentrations of morphine (data not shown). Moreover, it has been known to express different types of G proteins and adenylate cyclases, and protein kinases. Using northern blot analysis, we have shown here that both HEK 293 and SH-SY5Y cells have mRNA for endogenous GRK2 or a related GRK. Therefore, HEK-EE-μ cells serves as a good cell system to study the involvement of GRK2 in μ receptor phosphorylation and activation, which will be addressed in Chapter III.

When HEK-μ and HEK-EE-μ cells were treated with morphine, a significant cAMP overshoot was revealed when naloxone was added into cell

incubation, previously rinsed with medium to remove morphine. This supported our previous conclusion from the studies in SH-SY5Y cells that this naloxone-induced cAMP overshoot is an indication for the presence of constitutive μ receptor activity.

However, this conclusion was based on the fact that the naloxone-induced overshoot is an agonist-independent receptor event. To address possible residual morphine from the pretreatment, we incubated cells with [^{3}H]morphine, and found that 1.2 nM morphine may be present in the cAMP assay incubation. This is an over-estimation, since it did not account for impurities or metabolites of [^{3}H]-morphine. Addition of 1.2 nM morphine did not change cAMP levels measurably in forskolin (10 μM)-stimulated HEK-μ and HEK-EE-μ cells, since the inhibitory EC50 of morphine was 30 nM.

Other evidence also supported the absence of morphine or endogenous opioid activity. First, when the morphine-pretreated cells were stimulated by 100 μM forskolin, the naloxone-induced cAMP overshoot was much smaller than that in the presence of 10 μM forskolin. In many cases, the naloxone-induced cAMP overshoot was undetectable in the presence of 100 μM forskolin (data not shown). Secondly, the naloxone-induced cAMP overshoot was invisible in the presence of the cAMP phosphodiesterase inhibitor, IBMX (Chapter V). In both the above cases, any residual opioid activity would have been more sensitively observed in the presence of a phosphodiesterase inhibitor and higher forskolin stimulation. There is no reason that residual morphine activity would not be detected under these condition if the naloxone-overshoot were caused just by residual morphine. Yet, the lower forskolin concentration, in combination with the absence of any cAMP phosphodiesterase inhibitors, optimized evaluation of μ receptor regulation

and maximized the naloxone-induced cAMP overshoot observed after morphine pretreatment.

Thirdly, removal of morphine was supported by bioassay. When morphine-pretreated cells were washed 4 times and incubated with forskolin at 37 °C for 7 min, supernatant from the above incubation did not contain any opioid activity that can be reversed by 10 μM naloxone (50, 96) (data not shown for HEK-μ cells).

In addition, by varying the morphine pretreatment time, we found that the naloxone-induced cAMP overshoot has a slow onset, reaching maximum at approximately 6-12 hours (data not shown), similar to the results in SH-SY5Y cells (27). Such a slow onset is consistent with a gradual development of the dependent state, but is inconsistent with the maximum uptake of [^{3}H]morphine achieved within 0.5-2 h.

Development of the naloxone-induced cAMP in HEK-μ cells allowed us to attribute the effect to the μ opioid receptor, since only the μ receptor was transfected in this cell line and μ receptor-untransfected cells did not exhibit naloxone-regulated cAMP alteration. It argues against the possibility that the equivalent naloxone-induced cAMP overshoot observed in morphine pretreated SH-SY5Y cells (50) resulted from the presence of any unknown μ receptor subtypes with unusual properties. Previously, it was concluded only from the results that subnanomolar EC50 of naloxone and failure to cause the naloxone-induced cAMP overshoot by a selective antagonist of the δ receptor, which was also present in SH-SY5Y cells. The rather dramatic magnitude of the naloxone-induced cAMP overshoot is consistent with the high μ receptor expression level (4 x 10^6 sites/cell), which is about 80 times higher than that in ratinoic acid-differentiated SH-SY5Y cells.

However, ligand binding failed to identify the constitutively active receptor by ligand binding, suggest that the μ* has similar binding profile as the resting μ receptor.

Like in SH-SY5Y cells, the naloxone-induced cAMP overshoot was shared by other opioid antagonists such as naltrexone and diprenorphine, indicating that it is receptor related, instead of naloxone-specific event. Moreover, the naloxone-induced cAMP overshoot can be induced by the μ receptor peptide agonist PLO17. However, treatment with etorphine did not lead to the same effect. This is very interesting, since one of its derivatives dihydroetorphine has been reported to cause only a very mild physical dependence in animals and human subjects (97). We also found that treatment with etorphine (100 nM) abolished μ receptor phosphorylation. This could well be another example that μ receptor phosphorylation and constitutive activation are molecular mechanisms leading to dependence. Although DAMGO treatment also resulted in the naloxone-induced cAMP overshoot, it is not known if DAMGO was functionally removed. DAMGO may diffuse or internalize with μ receptor(25) into cells. However, judging from the rather low diffusion rate, redistribution of DAMGO from the intracellular compartment may be to slow to affect the cAMP levels.

The mechanism of constitutive μ receptor activation seems to be receptor phosphorylation related, which will be discussed in more length in the Chapters III and V. We studied chronic effects of several opioid compounds on μ receptor phosphorylation. Etorphine and DAMGO, but not morphine internalize transfected μ receptor (25). Dephosphorylation, observed in this study after treatments with etorphine and DAMGO, may be important, among other mechanisms, for their recycling to the cell surface. Such a mechanism has been suggested for other GPCRs (98, 99).

Treatments with CTAP and naloxone produced opposite effects on μ receptor phosphorylation, suggesting that they are different types of antagonists. Naloxone enhanced the phosphorylation signal, suggesting that it might serve as an agent to maintain the dependence state as defined by high receptor phosphorylation. Naloxone and naltrexone have been suggested and used at low doses to aid and stabilize withdrawal from narcotic addiction (100). There has been some success by using this regime, however it was never scientifically justified. The effect of naloxone on μ receptor phosphorylation appeared to support this type of approach. The inhibitory effect of CTAP was of interest, because if this effect has a fast on-set, it may decrease the receptor phosphorylation, during the cAMP incubation. This will lead to fewer constitutively active μ receptors, and hence a increase in cAMP level.

In several tissues containing μ opioid receptors, low agonist concentrations were shown to stimulate, and high concentrations to inhibit downstream effectors (101, 102). Indeed, low morphine concentrations (~10 nM) stimulated cAMP production in morphine pretreated HEK-μ cells. This result supports the view that μ receptor activation can have biphasic effects on cAMP levels which affect the shape of the morphine dose-response curves under varying conditions. Using 100 μM forskolin to stimulate maximally cAMP production, an inhibitory EC50 value ~1 nM was obtained for morphine in the same HEK-μ cells (25). This substantially lower EC50 value than EC50 of 30 nM obtained in the presence of 10 μM forskolin, suggests that maximum stimulation of cAMP production masks any stimulatory effects of morphine at the μ receptor.

In conclusion, the naloxone-induced cAMP overshoot was not confined to SK-N-SH cells and its subclone SH-SY5Y cells, nor was it caused only by morphine treatment. The development of this phenomenon in the μ receptor-transfected HEK cells, suggested that it may be a general event associated with the μ opioid receptor and chronic effect of narcotic opioids. Studies of phosphorylation and constitutive activity of the μ receptor will provide us with more information on molecular mechanism of narcotic dependence and tolerance.

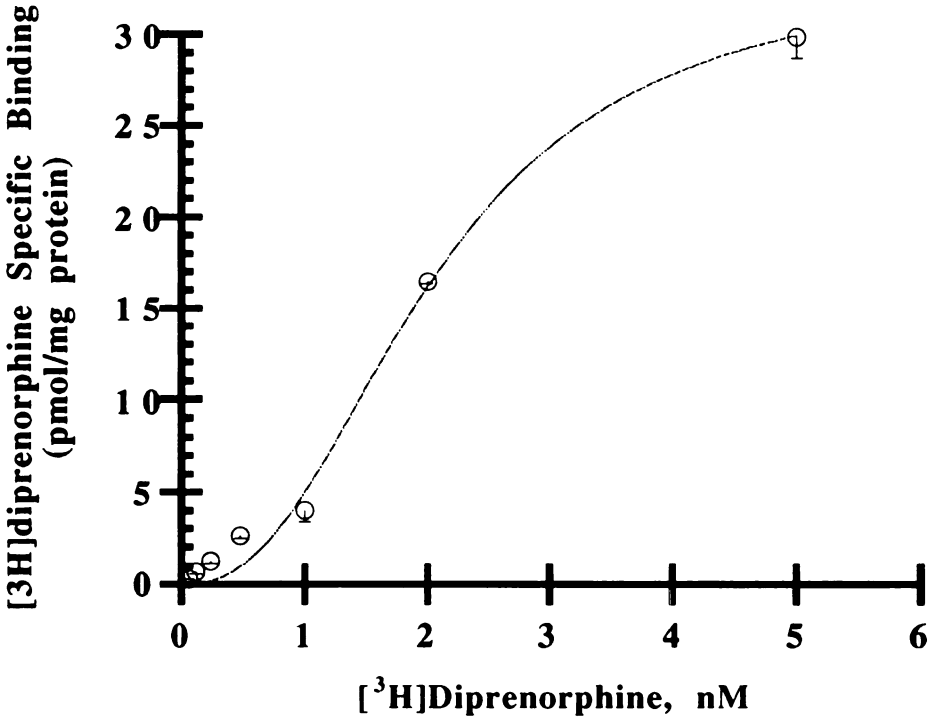


Fig. 2.1. Saturation binding of [^{3}H] diprenorphine to the HEK-μ cells in monolayer. Each point represents mean ± SD from triplicate samples.

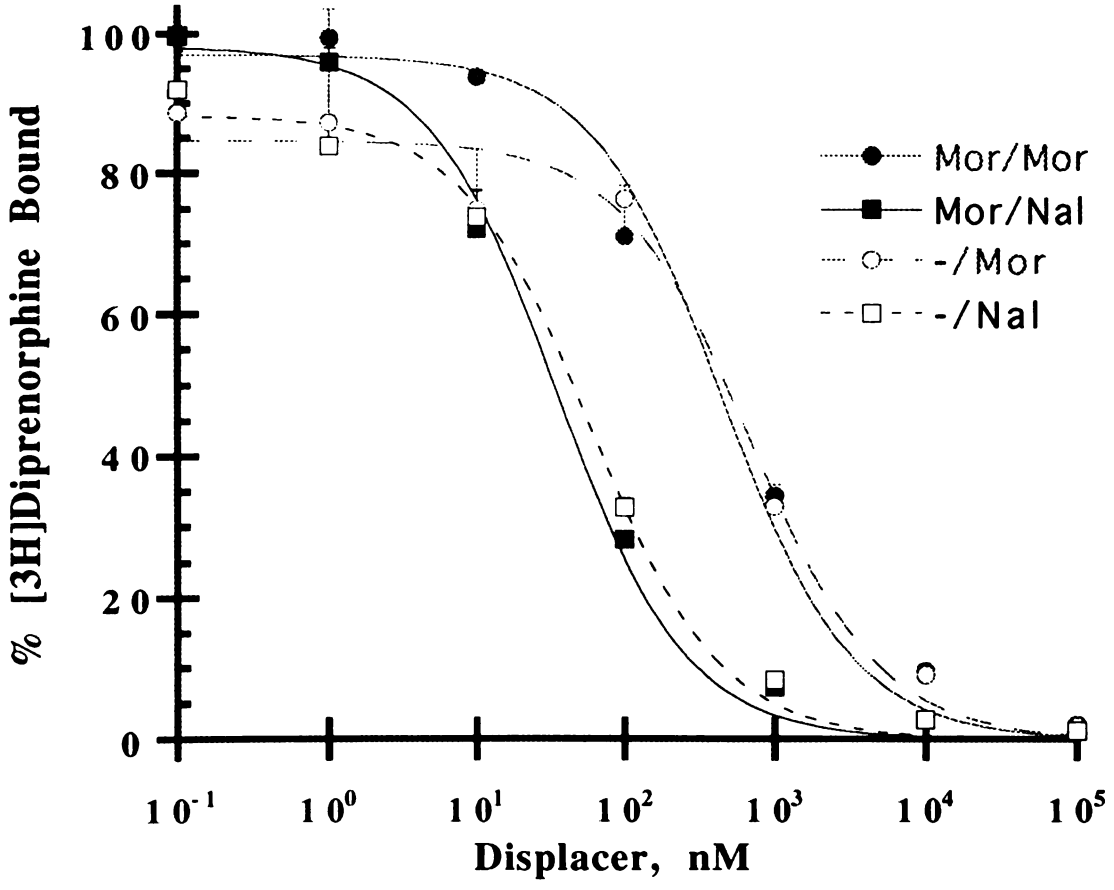


Fig. 2.2. Displacement of [^{3}H]Diprenorphine binding by morphine (Mor, solid line) or naloxone (Nal, dashed line) in HEK-μ cells before (--/Mor, --/Nal) and after (Mor/Mor, Mor/Nal) treatment with morphine. Each point represents mean ± SD from triplicate samples.

Table 2.1

[^{3}H]Diprenorphine binding of HEK 293 cell transiently transfected with a cloned μ opioid recepter

cDNA (μg)	Bmax (fmol/mg protein)	Kd (nM)
2	59 ±6	0.2 ± 0.1
4	322 ± 36	1.0 ± 0.4
8	1455 ± 180	1.3 ±0.5
12	1020 ± 88	1.6 ± 0.4
18	1595 ±129	1.8 ± 0.4

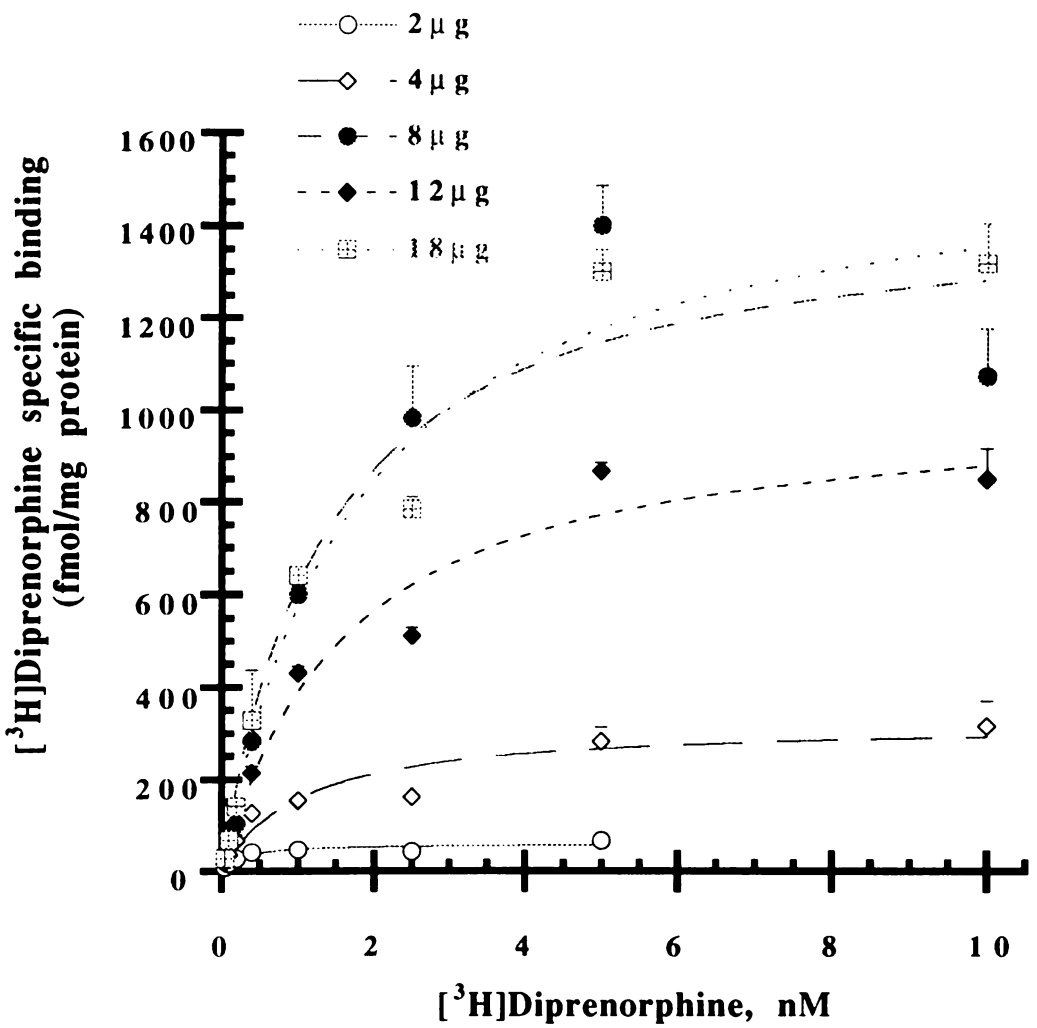


Fig 2.3. Saturation binding of [^{3}H] diprenorphine to the HEK 293 cells transiently transfected with different amount (2-18 µg) of a rat µ receptor cDNA. Each point represents mean ± SD from triplicate samples.

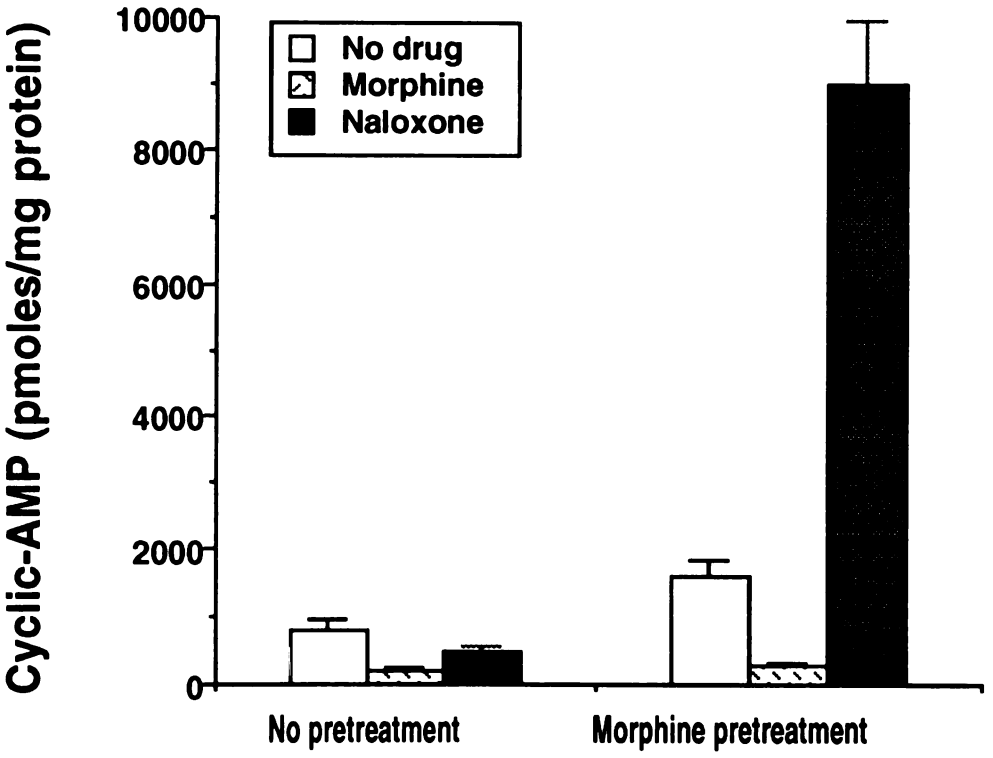


Fig. 2.4. Effects of morphine and naloxone on forskolin-stimulated cAMP accumulation in untreated and morphine-pretreated HEK-μ cells. Cells were pretreated with medium containing 1 μM morphine or no drug for 12 h. Immediately before the cAMP accumulation assay, cells were washed to remove any agonist. cAMP accumulation by 10 μM forskolin was then determined in the presence of 10 μM morphine, or 10 μM naloxone, or no drug. Data are mean ± S.D. (n=4) of a representative experiment.

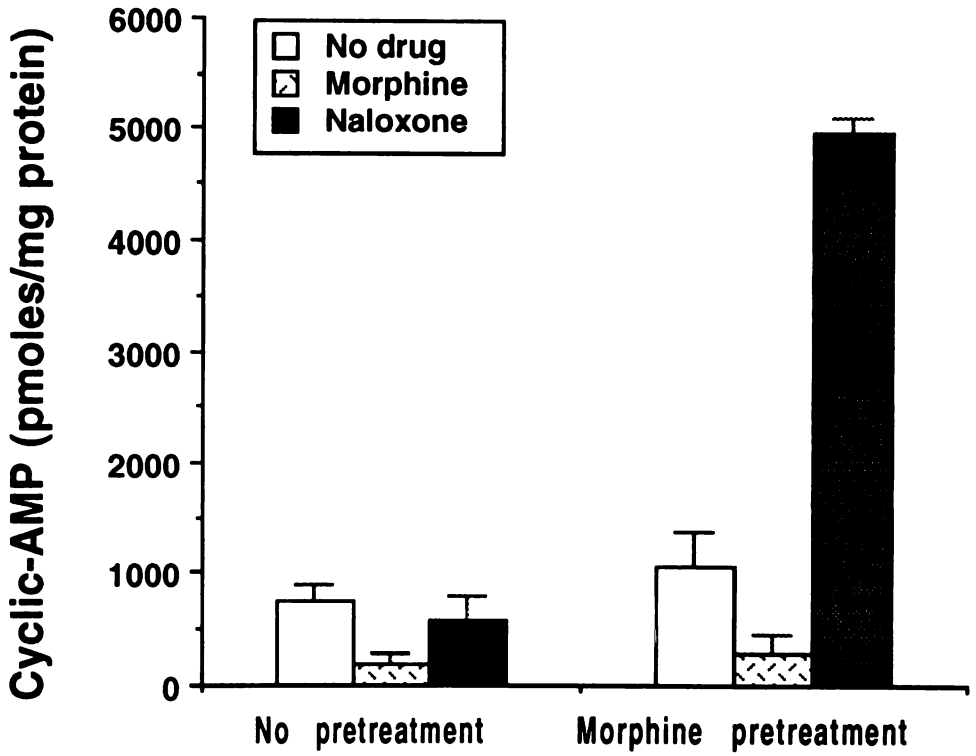


Fig. 2.5. Effects of morphine and naloxone on forskolin-stimulated cAMP accumulation in untreated and morphine-pretreated HEK-EE-μ cells. Cells were pretreated with medium containing 1 μM morphine or no drug for 12 h. Immediately before the cAMP accumulation assay, cells were washed to remove any agonist. cAMP accumulation by 10 μM forskolin was then determined in the presence of 10 μM morphine, or 10 μM naloxone, or no drug. Data are mean ± S.D. (n=4) of a representative experiment.

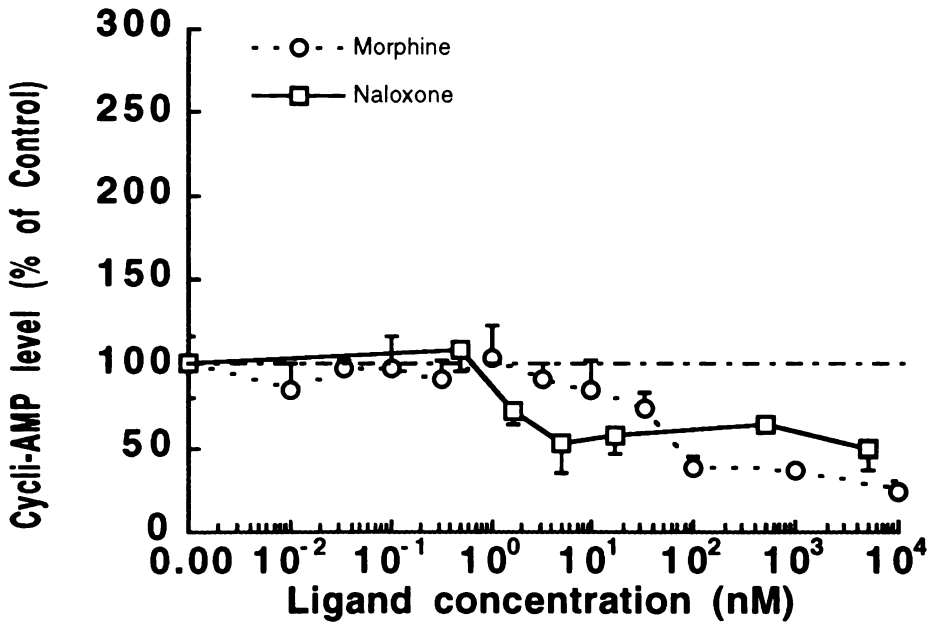


Fig. 2.6. Dose-response curve of the effects of morphine and naloxone on cAMP levels in HEK-μ cells. Data represent the mean ± SD of several experiments (n=6-20 per point for the morphine curves, and n=3-6 for the naloxone curves). cAMP levels were normalized to 100% in drug-free control cells.

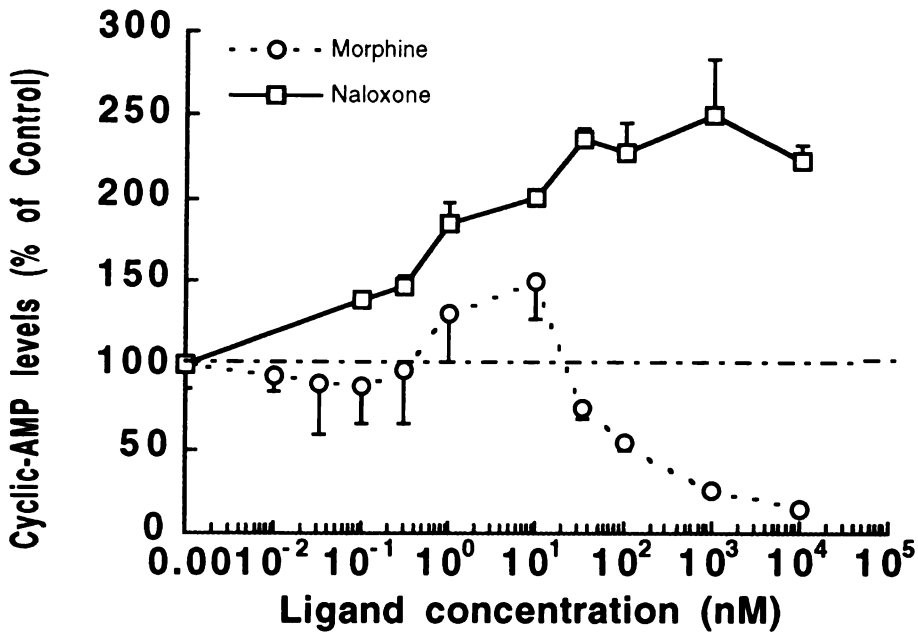


Fig. 2.7. Dose-response curve of the effects of morphine and naloxone on cAMP levels in HEK-μ cells pretreated with morphine. HEK-μ cells were treated with 1 μM morphine for 12 h, and cAMP accumulation was assayed after removing morphine by washing. Data represent the mean ± SD of several experiments (n=6-20 per point for the morphine curves, and n=3-6 for the naloxone curves). cAMP levels were normalized to 100% for the control (cAMP level in the washed morphine-pretreated cells). cAMP levels in pretreated cells were two-fold higher than in untreated controls (see Fig. 1A).

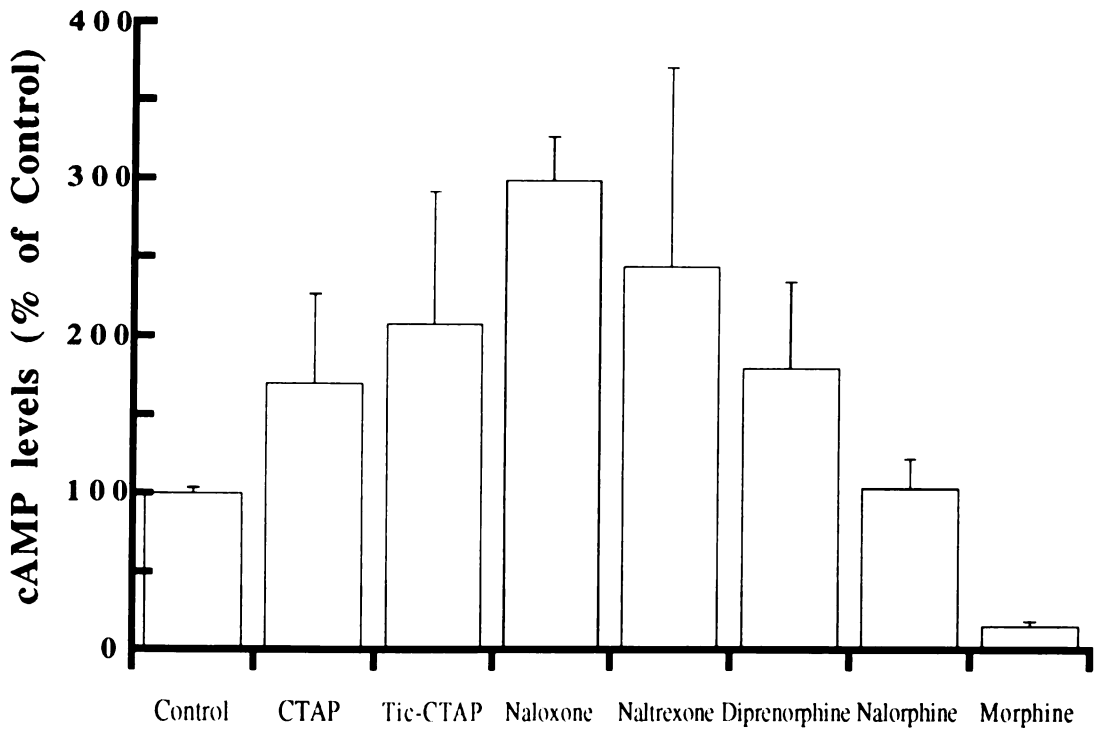


Fig 2.8. Effects of opioid antagonists and agonists on forskolin-stimulated cAMP accumulation in morphine-pretreated HEK-EE-μ cells. Cells were pretreated with medium containing 1 μM morphine or no drug for 12 h. Immediately before the cAMP accumulation assay, cells were washed to remove any agonist. cAMP accumulation by 10 μM forskolin was then determined in the presence of the following compounds: no drug (Control), 1μM CTAP, 1 μM Tic-CTAP, 10 μM naloxone, 10 μM naltrexone, 10 μM diprenorphine, 1 μM nalorphine, and 10 μM morphine. Data are mean ± S.D. (n=4) of a representative experiment.

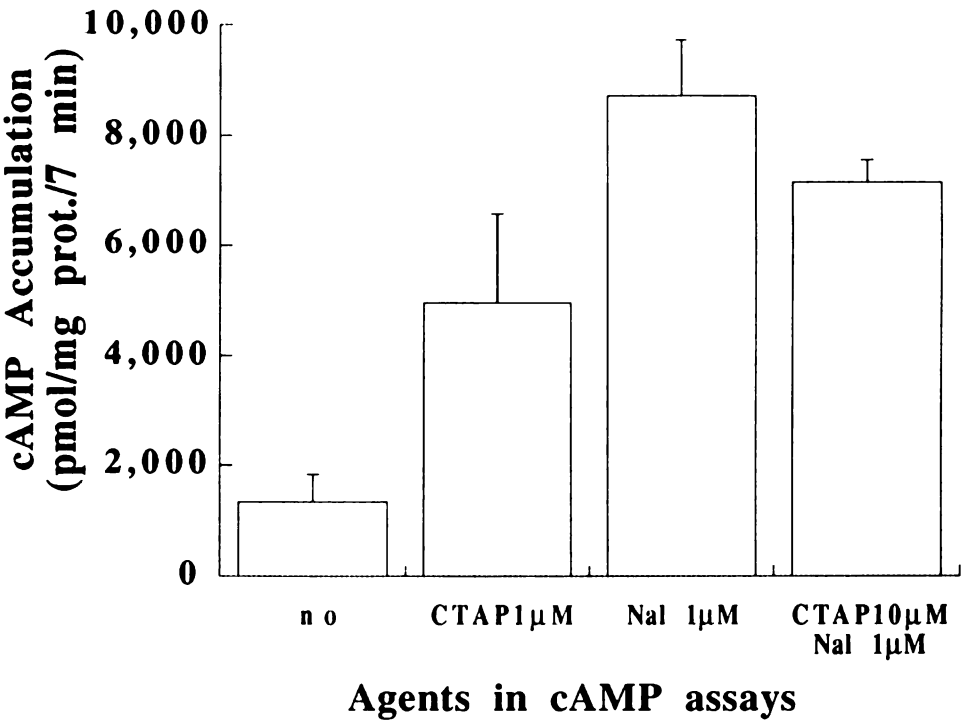


Fig. 2.9. Effects of naloxone and/or CTAP on forskolin-stimulated cAMP accumulation in morphine-pretreated HEK-μ cells. All the cells were pretreated with medium containing 1 μM morphine 12 h. Immediately before the cAMP accumulation assay, cells were washed to remove any agonist. cAMP accumulation by 10 μM forskolin was then determined in the presence of no drug (no), 1 μM CTAP, 1 μM naloxone, and 10 μM CTAP plus 1 μM naloxone, Data are mean ± S.D. (n=4) of a representative experiment.

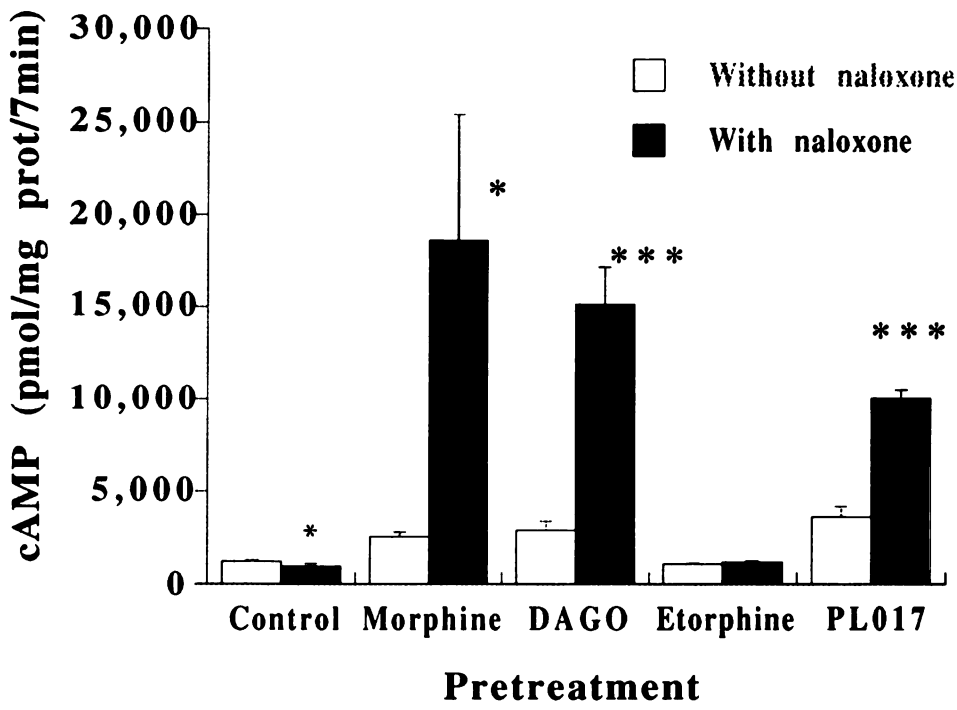


Fig. 2.10. Effects of morphine, DAMGO, etorphine and PL017 pretreatment on the naloxone-induced cAMP overshoot in HEK-μ cells. Cells were pretreated no drug (Control), 1 μM morphine, 1 μM DAMGO, 100 nM etorphine, and 1 μM PLO17 for 12 h. Immediately before the cAMP accumulation assay, cells were washed 4 times in am attempt to remove any agonist. cAMP accumulation by 10 μM forskolin was then determined in the presence and absence of 10 μM naloxone. Data are mean ± S.D. (n=4) of a representative experiment.

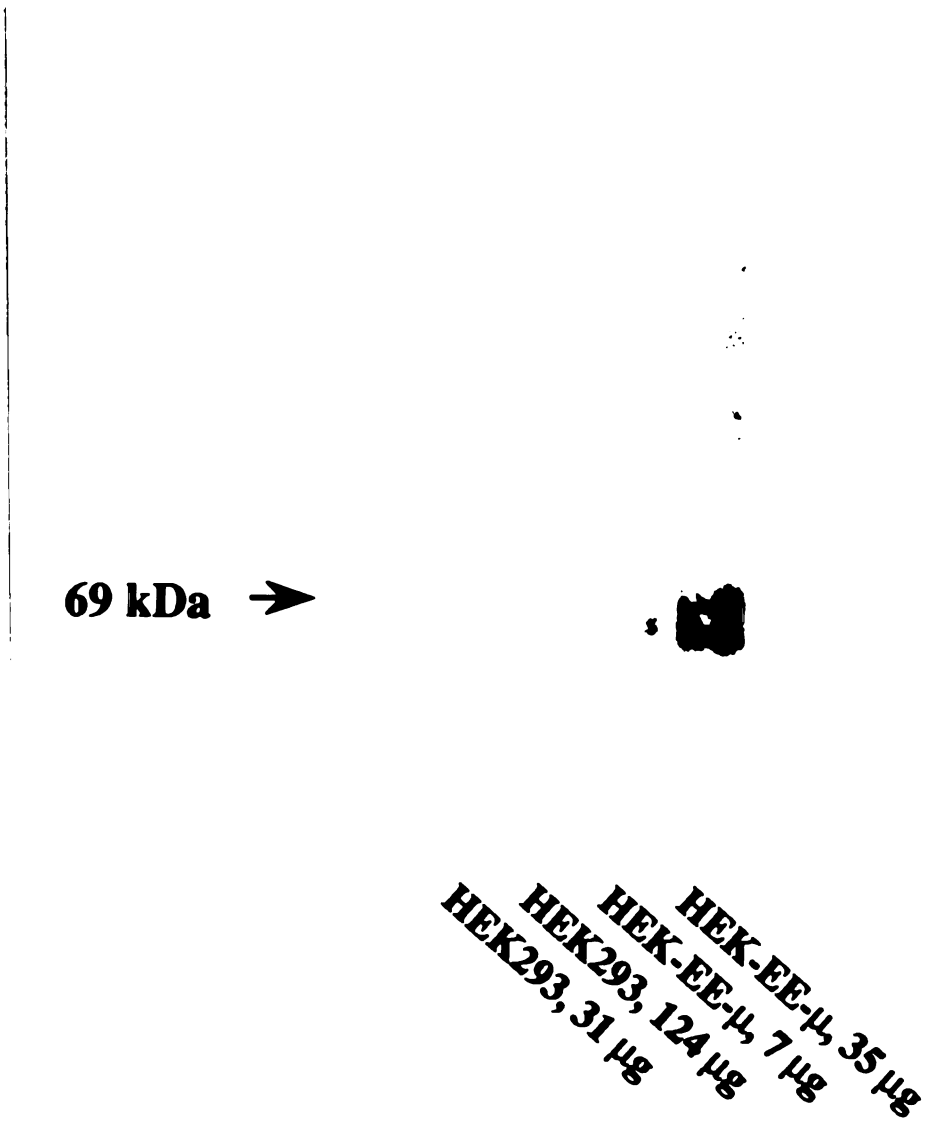


Fig. 2.11. Immunoblot of EE-μ receptor expressed in HEK-EE-μ cells. 30,000g membranes from HEK 293, or HEK-EE-μ were solubilized in SDS-loading buffer, and separated on 8% SDS-PAGE, and blotted with anti-EE mAB.

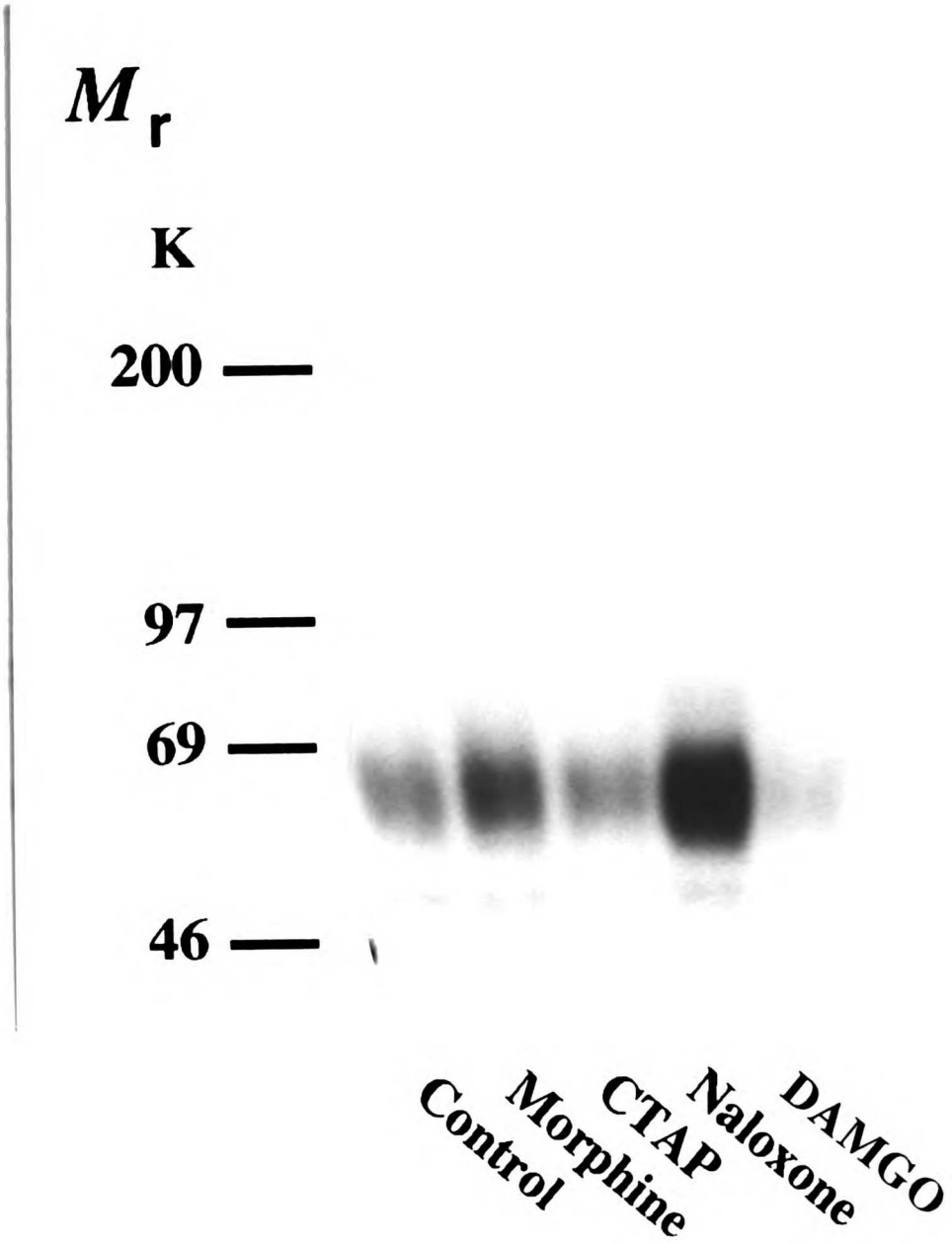


Fig. 2.12 Phosphorylation of EE-μ receptor expressed in HEK-EE-μ cells after treatments with morphine, DAMGO, CTAP, and naloxone. HEK-EE-μ cells were treated with 1 μM morphine, 1 μM DAMGO, 1 MM CTAP, and 10 μM naloxone for 12 h, followed by 6 times washes to remove these compounds. Cells were permeabilized with digitonin and labeled with 32g-ATP. 30,000g membranes were prepared from these cells, solubilized with CHAPS, and immunoprecipitated with anti-EE mAB, before separated on 8 % reducing SDS-PAGE.

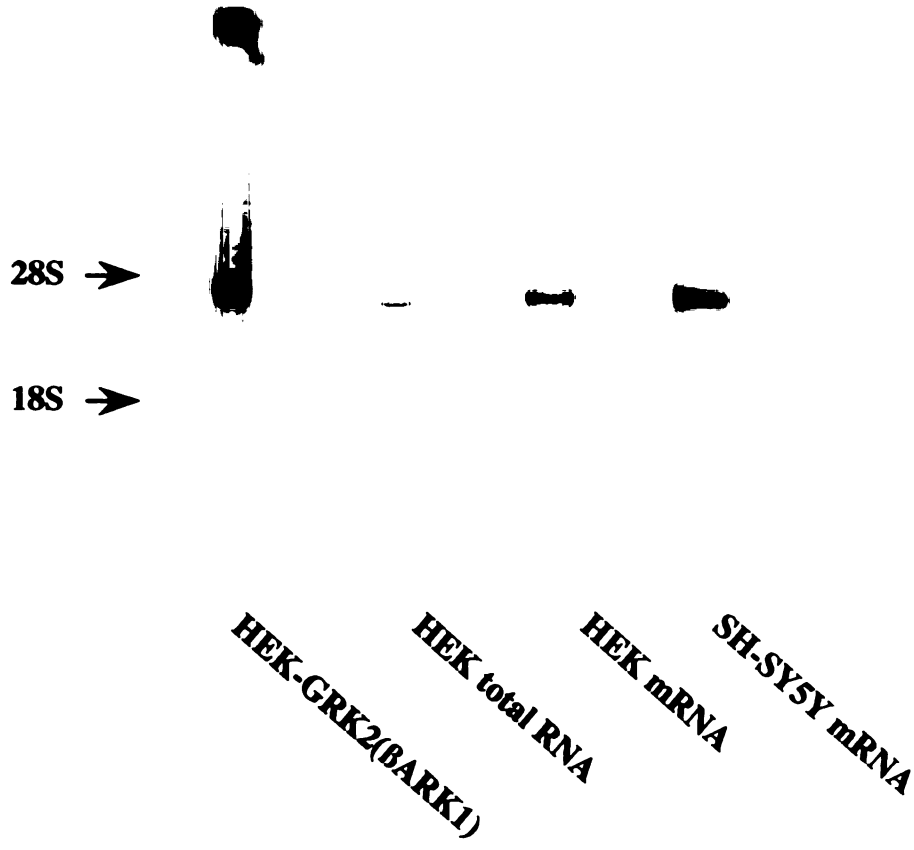


Fig. 2.13. Nothern blot analysis of RNA from SH-SY5Y cells and HEK 293 cells. RNA and mRNA were extracted as described under the method. The RNA blot was hybridized with [α-^{32}P]-dCTP labeled GRK2 cDNA probe generated by radium priming.

CHAPTER III

CHARACTERIZATION OF THE μ OPIOID RECEPTOR KINASE: BASAL PHOSPHORYLATION OF μ OPIOID RECEPTOR IS AGONIST MODULATED AND CA^{++} DEPENDENT

SUMMARY

The μ opioid receptor was shown to be phosphorylated at a basal rate in the absence of agonist, measured in permeabilized HEK 293 cells transfected with an epitope tagged μ receptor (EE-μ) (Arden, J., Segredo, V., Wang, Z., Lameh, J., and Sadée, W. (1995) *J. Neurochem.* 65, 1636-1645). In the present study , basal phosphorylation was found to be Ca^{++} dependent; however, several inhibitors of protein kinase C and Ca^{++}-calmodulin dependent kinases failed to affect basal μ receptor phosphorylation. Thus, the basal μ receptor phosphorylating activity differed from the main kinases involved in receptor regulation. The general kinase inhibitor H7 (100 μM) suppressed basal μ receptor phosphorylation. Pretreatment with the agonist morphine, followed by drug removal, resulted in a sustained increase of basal μ receptor phosphorylation. The gradual agonist dependent modulation of basal μ receptor phosphorylation suggests a novel regulatory mechanism which may play a role in narcotic tolerance and dependence.

INTRODUCTION

The μ opioid receptor was shown to be continuously phosphorylated at a basal rate in the absence of agonist, using [γ-^{32}P]-ATP labeling of permeabilized HEK 293 cells transfected with an epitope-tagged μ opioid receptor (HEK-EE-μ) (25). A basal rate of μ receptor phosphorylation has been confirmed to occur in other cells with heterologous or native expression of the μ receptor, including brain slices (103, 104), suggesting that this phenomenon is not limited to HEK 293 cells. Similarly high basal levels of receptor phosphorylation were previously observed with a constitutively active β_2 receptor mutant (85), and with the serotonin 5-HT$_{2C}$ wild-type receptor which displays basal signaling activity (105). These results are consistent with the hypothesis that the μ opioid receptor also has a basal (constitutive) signaling activity which might be modulated by concurrent phosphorylation (50). Basal μ receptor phosphorylation, i.e., the rate in the absence of any agonist, was further enhanced in the presence of morphine (25), as observed upon agonist stimulation of the constitutively active β_2 receptor mutant (85).

We have previously demonstrated that morphine pretreatment sensitized the μ opioid receptor "inverse agonist' effects of naloxone in SH-SY5Y neuroblastoma cells, as a possible element of the dependent state (50). This sensitization to naloxone was prevented by the general kinase inhibitor H7, but not its congener H8 (50). To determine the possible involvement of a phosphorylation reaction in narcotic tolerance and dependence, we tested the general kinase inhibitors H7 and H8 in mice made acutely tolerant to and dependent on morphine (50, 52). Compound H7, but not its congener H8, reversed morphine tolerance and dependence (50, 52), in parallel to *in vitro* results in SH-SY5Y cells (50, 52) These findings supported the hypothesis

that a phosphorylation event contributes to the expression of narcotic tolerance and dependence.

This report characterizes basal μ receptor phosphorylation with regard to ionic requirements, spectrum of inhibitors, and response to the agonist morphine. Basal μ receptor phosphorylation was found to be Ca^{++}-dependent, modulated by morphine pretreatment, and suppressed by H7.

EXPERIMENTAL PROCEDURES

Materials

N-methyl-[^{3}H]-morphine (79 Ci/mmol) was purchased from NEN (Boston, MA), and [15,16-$^3H_2$]-diprenorphine (specific activity 47 Ci/mmol) was provided by the National Institute on Drug Abuse, Rockville, MD. The following protein kinase inhibitors were used (source given in parentheses): H7, calmodulin dependent protein kinase II fragment 290-309 (CaM kinase inhibitor peptide) (Sigma Chemicals, St.Louis, MO), H8 (Seikagaku American Inc., Rockville, MD), H89, HA1004, staurosporin, W7, herbimycin A, heparin (Calbiochem, LaJolla, CA), PKC(19-31) inhibitor peptide, chelerythrine chloride, PKI(5-24) peptide (PKA inhibitor), K-525a, KN-62, and peptide A (tyrosine kinase inhibitor) (LC Laboratories, Woburn, MA).

The rat μ opioid receptor cDNA in vector pRC/CMV (20), provided by Dr. Lei Yu, Indiana University, was tagged with a sequence encoding the epitope EYMPME, immediately downstream of the Met initiation codon (EE-μ) (25).

Cell culture

The plasmid encoding the EE-μ receptor was transfected into HEK 293 cells, and stably transfected clonal cell lines were established as described (25). The cell line with the highest receptor expression initially contained $5x10^6$ sites per cell for HEK-EE-μ (25). As a result of gradual loss of μ receptor sites during culture, experiments were performed with cultures expressing 5-$10x10^5$ sites per cell.

Receptor binding and cAMP assays

Receptor expression was examined with 2 nM [^{3}H]-diprenorphine in intact cell monolayers, and cAMP levels by radioimmunoassy as described (25) (also Chapter II).

Drug pretreatment protocol

Cells were pretreated with 1 μM morphine and/or the indicated inhibitors for 0-12 hours. Immediately before the ^{32}P-labeling incubation, cells were washed twice with medium containing 5% serum and twice more with serum-free medium, requiring 10 min for effective agonist removal. Studies with ^{3}H-morphine indicated that this procedure removed 99.7% of the ^{3}H activity added to the preincubation.

Phosphorylation and immunoprecipitation of the epitope tagged μ receptor

Phosphorylation of EE-μ was determined in washed, permeabilized cells as described (25). Briefly, digitonin permeabilized cells were labeled with 0.2 mCi [γ-^{32}P]-ATP (200 μM, 0.5 ml) at 25°C for 15 minutes in the presence or absence of morphine or kinase inhibitors. The incubation medium consisted of either DMEM medium (containing Ca^{++} and Na^{+} ions) [1], or the buffers indicated in the text. Optimal labeling conditions for basal μ receptor phosphorylation were observed in a medium containing 150 mM NaCl, 5 μM $CaCl_2$, 10 mM HEPES (pH 7.1), 2 mM $MgCl_2$, 5 mM glucose, and 200 μM [γ-^{32}P]-ATP. Labeled cells were washed and homogenized in the presence of 100 nM diprenorphine and protease inhibitors (25). The 30,000xg membrane pellet was solubilized in 10 mM CHAPS, containing 100 nM diprenorphine. Solubilized EE-μ was immunoprecipitated with anti-EE mAb , subjected to SDS-PAGE, autoradiographed, and analyzed by scanning densitometry, as described previously (25). The identity of the labeled EE-μ band was confirmed by Western blotting (25).

RESULTS

Ionic requirements for basal μ receptor phosphorylation

We have previously shown that the μ receptor in HEK-EE-μ cells is phosphorylated in the absence of any agonist (25). ^{32}P-labeling of the EE-μ receptor was obtained in DMEM medium which contains 2 mM Ca^{++} and 150 mM Na^{+} (25). To determine the ionic requirements for this reaction, we incubated the permeabilized cells in medium containing 150 mM K^{+}, 7 mM

Mg^{++}, and 5 mM EGTA to chelate Ca^{++}, commonly used conditions to study intracellular kinases, including the GRKs (85, 106, 107). Under these conditions, no basal phosphorylation was detectable at 65 kb (not shown), and only a moderate level of phosphorylation appeared at 65 kb during acute stimulation with 1 μM morphine (Fig. 3.1, lane 1). It remains to be determined whether the labeling at higher molecular weights is related to the μ opioid receptor, but Western blot analysis did not reveal immunoreactive bands at high molecular weights (25). The morphine-enhanced phosphorylation activity at 65 kD may be mediated by one of the known G protein coupled receptor kinases (GRKs). This putative agonist dependent GRK-like activity is being investigated in a separate study.

Replacement of K^+ with 150 mM Na^+ (in the absence of Ca^{++}) generally reduced detectable phosphorylation, as expected since Na^+ inhibits many intracellular kinases, including the GRKs (Fig. 3.1, lane 3). In contrast, the addition of 2 mM Ca^{++} stimulated μ receptor phosphorylation, yielding a clear band at 65 kb, regardless of the presence or absence of Na^+ (Fig. 3.1, lanes 2 and 4). Therefore, to measure μ receptor phosphorylation, we included both Ca^{++} and Na^+ ions, the latter to suppress GRKs and extraneous kinase activities. To determine the Ca^{++} concentration required for basal μ receptor phosphorylation, the reaction was carried out at 5, 50, 500, and 2000 μM Ca^{++}. Maximum μ receptor phosphorylation was already attained at 5 μM Ca^{++}, the lowest Ca^{++} concentration tested (data not shown).

The results shown in Fig. 3.1 were obtained in the presence of 10 μM morphine to enhance EE-μ labeling (25). In the absence of morphine, basal μ receptor phosphorylation also depended upon Ca^{++} and was not suppressed by high levels of Na^+ (data not shown). Therefore, basal and morphine

stimulated μ receptor phosphorylation measured under these conditions appear to be mediated by the same or a similar kinase activity.

Effect of kinase inhibitors on basal μ receptor phosphorylation

We first tested the general kinase inhibitors H7 and H8. Addition of 100 μM H7 to permeabilized HEK-EE-μ cells strongly inhibited basal phosphorylation to undetectable levels (n=3) (Fig. 3.2, lane 2). Compound H8 (100 μM) inhibited basal μ receptor phosporylation only partially in permeabilized HEK-EE-μ cells (n=2, Fig. 3.2, lane 3).

To characterize the μ receptor kinase activity, we tested inhibitors with selectivity for kinases commonly involved in receptor regulation, such as PKA and PKG, and the main classes of Ca^{++}-dependent kinases, including protein kinase C (PKC) and Ca^{++}-calmodulin dependent (CaM) kinases (108). A representative experiment is shown in Fig. 3.3. The chosen concentrations of these kinase inhibitors were ~20 fold higher than published IC50 values for the respective kinases, except for W7 which was tested at 200 μM, or only ~5-fold the IC50 for calmodulin, to avoid nonspecific effects (see legend, Fig. 3.3). None of these inhibitors strongly suppressed basal μ receptor phosphorylation (Fig. 3.3). In a second experiment with W7, a reduction of basal μ receptor phosphorylation was detectable (~60%), and therefore, this experiment was repeated a third time with 200 and 500 μM W7. In the third experiment, a clear inhibition (>50%) was seen only with the 500 μM concentration. Therefore, W7 appeared to be a weak inhibitor of basal μ receptor phosphorylation at best, but at concentrations that exceed its expected IC50 concentration for calmodulin inhibition (~40 μM). It should be noted

that these experiments were designed for semiquantitative ahalysis suitble for detecting strong inhibition (>50%) only, since each control (basal phosphorylation without any inhibitor) was run only once per experiment, and the absolute level of ^{32}P labeling varied from one experiment to another, probably owing to variable recovery of the receptor on the gel.

To test a spectrum of potential inhibitors, we then initiated a screen of known inhibitors of various kinases, using single experiments. None of the following inhibitors affected μ receptor phosphorylation in single experiments: 5 μM H89 (PKA inhibitor), 10 μM herbimycin A (tyrosine kinase inhibitor), 1 μM heparin (GRK2 inhibitor), and selective peptide inhibitors of PKC (1 μM), PKA (0.2 μM), and tyrosine kinases (20 μM) (see Materials).

Regulation of basal μ receptor phosphorylation by pretreatment with morphine, H7, and H8

Figure 3.4 compares the effects of acute addition and of pretreatment on basal μ receptor phosphorylation. Acute treatment of permeabilized cells with 1 μM morphine stimulated, whereas 100 μM H7 abolished, μ receptor phosphorylation (Fig. 3.4A) (see also Fig. 3.2, lane 2 and ref.(25). To determine the effect of chronic μ receptor stimulation, we next determined basal μ receptor phosphorylation after morphine pretreatment, followed by complete agonist removal. Pretreatment of intact cells with 1 μM μorphine for 7 hours significantly increased subsequent μ receptor phosphorylation nearly three-fold (Fig. 3.4B, lane 5, 2.6±0.4 -fold, n= 5), even though the agonist was removed before labeling. A morphine dose-response experiment and a time course with 1 μM morphine pretreatment are shown in Fig. 3.5. Maximum

stimulation after a 12 hour pretreatment was attained with 100 nM morphine (Fig. 3.5, lane 3). Furthermore, a 2 hour pretreatment with 1 μM morphine was required to reach maximum (Fig. 3.5, lanes 5-7). After morphine removal, elevated basal μ phosphorylation persisted for at least 2 hours (Fig. 3.7, lanes 8-10).

Preincubation of intact HEK-EE-μ cells with 100 μM H7 alone for 7 hours, followed by removal of the inhibitor, suppressed subsequent basal μ receptor phosphorylation by >90% (n=2) even though the inhibitor was no longer present (Fig. 3.4B, lane 8). The same strong inhibition was seen with a 100 μM H7 preincubation for 2 hours. Basal phosphorylation of the μ receptor was also suppressed when HEK-EE-μ cells were preincubated with H7 (100 μM) together with morphine (1 μM) (Fig. 3.4B, lane 7, n=3). The ability of the μ receptor to undergo phosphorylation was not abolished by H7 pretreatment, since after removal of the inhibitor, addition of morphine to the labeling incubation now caused a significant increase of receptor phosphorylation (approximately 3-5 -fold, n=3) (Fig. 3.4B, lane 9). However, the absolute intensity of this morphine-induced phosphorylation was considerably lower than that without H7 pretreatment.

In contrast to the results with H7, pretreatment of intact HEK-EE-μ cells with 100 μM H8 alone (data not shown) or together with 1 μM morphine, followed by drug removal, failed to reduce subsequent basal μ receptor phosphorylation (Fig. 4B, lane 6; 110±20 % (n=3) relative to the level with morphine pretreatment alone, lane 5).

Lack of direct effects of H7 and H8 on μ opioid receptor

Compounds H7 and H8 were recently shown to interfere with ^{3}H-DAMGO binding to rabbit brain homogenates (109), suggesting a possible direct interaction with the μ opioid receptor. We therefore determined the effects of 100 μM H7 and H8 on ^{3}H-diprenorphine binding to intact HEK-μ cells, and cAMP levels in the presence and absence of 10 μM morphine. In the buffer solution used in the cAMP accumulation assay (PBS buffer), neither H7 nor H8 affected [^{3}H]-diprenorphine tracer binding significantly. Further, H7 did not alter cAMP levels in the absence of morphine (106 ± 9 % of control), nor did it affect morphine (1 μM) inhibition of cAMP accumulation (~95% inhibition in this series of experiments). H8 added alone enhanced cAMP levels in untreated cells (171 ± 7 % of control), but it also did not block the inhibition by 10 μM morphine (~95 % both in the presence and absence of H8). Therefore, H7 and H8 had no detectable acute effects on μ receptor activity in HEK-μ cells.

DISCUSSION

The present study describes unique characteristics of the kinase activity responsible for basal μ receptor phosphorylation that differ from those of known receptor kinases (25) First, basal μ receptor phosphorylation appears to be mediated by a kinase with an unusual spectrum of inhibitors and ionic requirements. Second, basal μ receptor kinase activity is gradually regulated by an agonist over a time period of several hours, and it can be disrupted by pre-incubation with a suitable kinase inhibitor (H7).

Basal μ receptor phosphorylation was suppressed by addition of the Ca^{++} chelator EGTA, and the addition of Ca^{++} restored the phosphorylating activity. Under these ionic conditions, including Ca^{++} and Na^{+}, any role of a

known GRK in μ receptor phosphorylation can be excluded, because GRKs are independent of Ca^{++} and inhibited by Na^{+} (20, 106). Moreover, the Ca^{++} requirement argues against a role for PKA and PKG which was further supported by insensitivity of basal μ receptor phosphorylation to inhibitors of these kinases. Further, basal μ receptor phosphorylation was not inhibited by Na^{+}, which was therefore included with the incubations to suppress other intracellular kinases. Finally, the failure of a panel of selective kinase inhibitors, including chelerythrine, KN62 and W7, to strongly inhibit basal μ receptor phosphorylation argues against participation of PKC and Ca^{++}-calmodulin dependent kinase II. Future studies will focus on identifying the kinase(s) responsible for basal μ phosphorylation.

Among the inhibitors tested in this study, H7 was shown to be the most effective inhibitor of basal μ receptor phosphorylation, whereas H8 was only partially effective at 100 μM when added to the permeabilized cells (Fig. 3.2). However, when intact cells were pretreated with H7 and H8, only H7 fully suppressed basal μ receptor phosphorylation, and H8 was inactive (Fig. 3.4B). The discrepancy between the partial inhibition with H8 in permeabilized cells (see above) and no effect in intact cells may be related to lower membrane permeation by the more polar H8, compared to H7. Nevertheless, H8 is genererally considered a more potent inhibitor than H7 of PKC and PKA, and at 100 μM, it appears to penetrate sufficiently into the cell to inhibit PKA (50).

The distinct effects of H7 and H8 on basal μ receptor phosphorylation correlate with their different effects on the naloxone induced cAMP overshoot in SH-SY5Y cells (50). Following morphine pretreatment, naloxone was shown to cause an increase in cAMP levels even though

morphine had been completely removed afte the pretreatment period. We attributed this paradoxical effect of naloxone to an inverse agonist effect at a constitutively active μ receptor state, μ*, characteristic of the dependent state (50). Similarly, inhibitor H7, but not H8 reversed morphine tolerance and naloxone induced withdrawal jumping in mice (50, 52). Both H7 and H8 failed to interact directly with the μ receptor under our experimental conditions. These results suggest a relationship between basal μ receptor phosphorylation and morphine tolerance and dependence. However, several kinases are likely to mediate μ receptor phosphorylation *in vivo* and to modulate narcotic effects. For example, in the absence of Na^+, morphine stimulated some μ receptor phosphorylation (Fig. 3.1, lane 1), which could have been mediated by a GRK activity. Further, stimulation of PKC with phorbol esters leads to μ receptor phosphorylation (103). Therefore, the *in vivo* phosphorylation pattern of the μ opioid receptor is likely to depend on several kinases.

Following inhibition of basal μ receptor phosphorylation by H7, phosphorylation remained suppressed even though the inhibitor was removed from the incubation (Fig. 3.4). Conversely, morphine enhanced the basal rate which persisted even after agonist removal (Fig. 3.4B). These results suggest a positive feed-back loop between receptor and kinase which is enhanced by a μ agonist and disrupted by a kinase inhibitor. Studies on the time course indicated that the modulation of basal μ receptor phosphorylation occurs over several hours (Fig. 3.5).

Several mechanisms can account for long-term changes in receptor phosphorylation. Agonist activation of a G protein coupled receptor not only converts the receptor into a better substrate for GRK phosphorylation, but it

also stimulates GRK activity directly (110, 111). If phosphorylation, possibly involving more than one kinase, were to maintain the receptor in an active state, a positive feed-back loop would be established (53). Another example of positive reinforcement of an initial signal is the translocation to the membrane and persistent activation of PKC as a result of receptor stimulation (112, 113). Similarly, stimulation of Ca^{++}-calmodulin dependent kinase II (CaM-kinase II) results in auto-phosphorylation and enhanced activation (114). Such activation mechanisms outlast the initial signal and have been implicated in memory mechanisms. By analogy, continuous basal phosphorylation of the μ opioid receptor observed in this study could result from a positive feed-back loop as a novel regulatory mechanism, with possible relevance to narcotic tolerance and dependence.

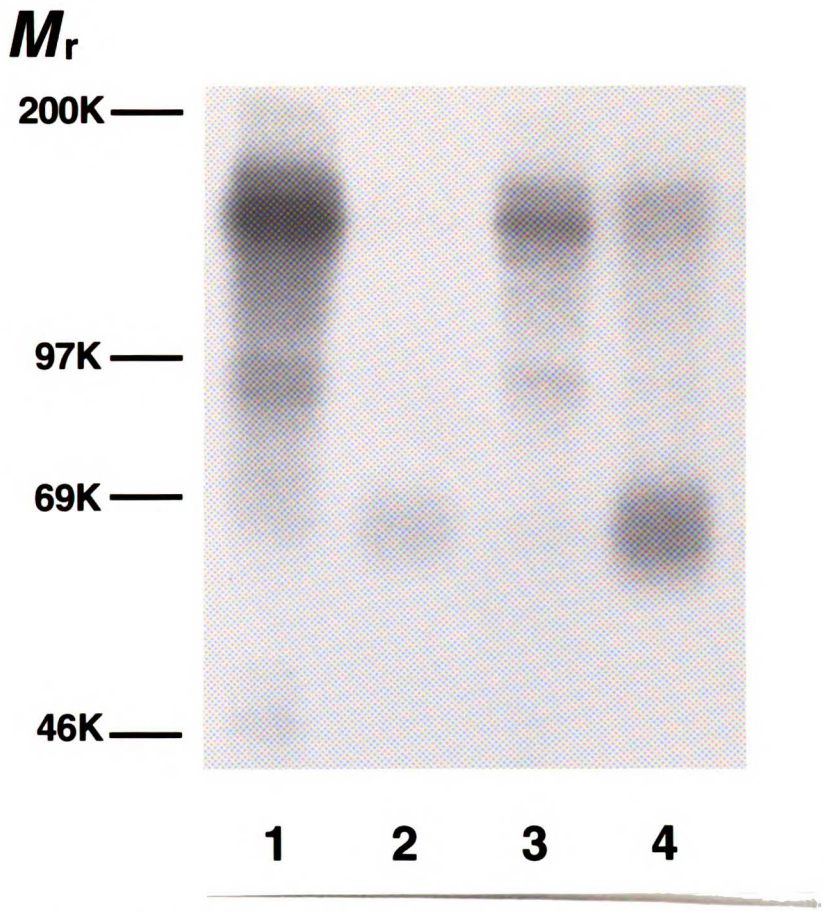


Fig. 3.1. Phosphorylation of the EE-μ receptor: ionic requirements . HEK-EE-μ cells were permeabilized, labeled with [γ-^{32}P]-ATP in the presence of 10 μM morphine, immunoprecipitated, and separated on SDS-PAGE, as described(25). The ^{32}P-labeling reaction was performed in 10 mM HEPES buffer, pH 7.1, 5 mM glucose, and the following ionic conditions: Lane 1: 150 mM KCl, 7 mM $MgCl_2$, 5 mM EGTA; lane 2: 150 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$; lane 3: 150 mM NaCl, 7 mM $MgCl_2$, 5 mM EGTA; lane 4: 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$. The requirement for Ca^{++} was confirmed in three additional experiments with varying ionic compositions, both in the presence and absence of morphine. Even though the labeling intensity of the 65 kD band in lane 2 (Ca^{++}) was lower than that in lane 4 (Ca^{++} and Na^{+}), the presence of Na^{+} did not enhance labeling in other experiments.

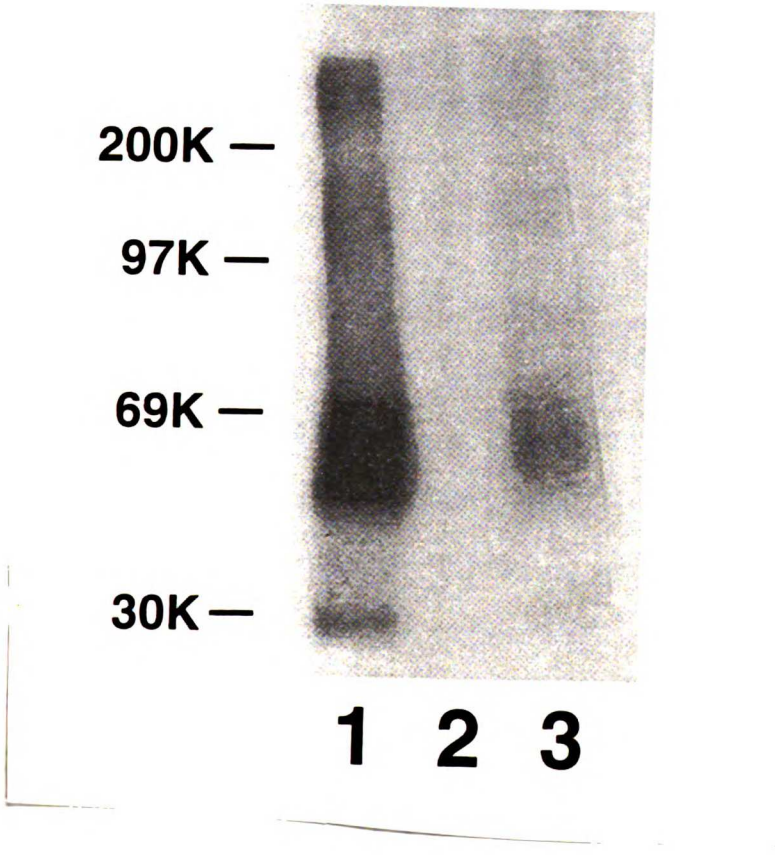


Fig. 3.2. Basal phosphorylation of the μ receptor: inhibitors H7 and H8. Permeabilized HEK-EE-μ cells were incubated for 15 min with a medium containing 150 mM Na^+, 5 μM Ca^{++}, 200 μM [γ-^{32}P]-ATP, and 100 μM H7 or H8. Lane 1: control cells; lane 2: H7; lane 3: H8.

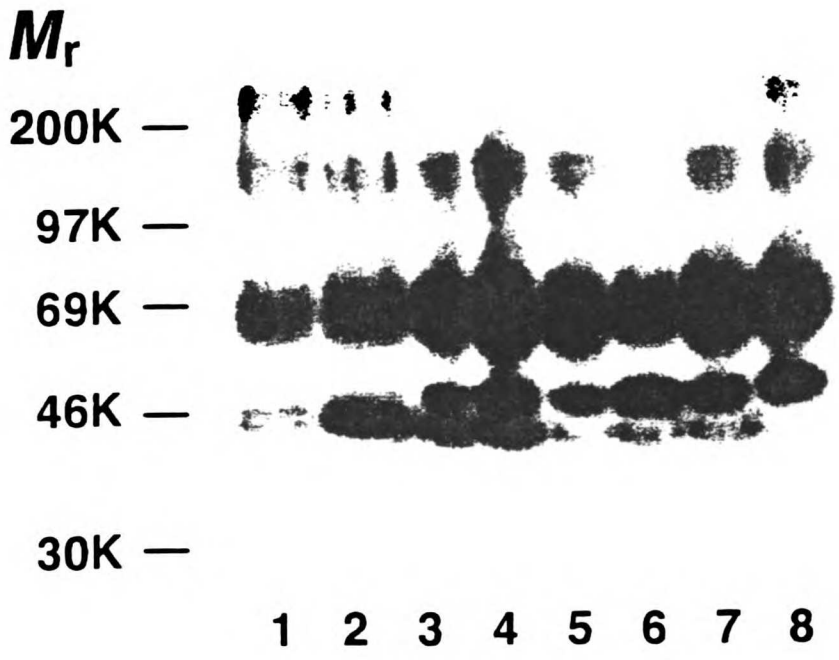


Fig. 3.3. Basal phosphorylation of the μ receptor: effect of protein kinase inhibitors. HEK-EE-μ cells were labeled as described for Fig. 3.1. The following inhibitors were added to the labeling medium (the kinases expected to be strongly inhibited are given in parentheses): lane 1: control without any inhibitor; lane 2: 50 nM K252a (CaMK); lane 3: 200 μM W7 (CaMK, MLCK); lane 4: 20 μM chelerythrine (PKC); lane 5: 20 μM KN62 (CaMK); lane 6: 30 μM HA1004 (PKA, PKG); lane 7: 1 μM calmodulin dependent protein kinase II fragment 290-309 (CaMK); lane 8: 10 nM staurosporin (MLCK, PKC). These results were repeated in independent experiments for the following inhibitors: W7, chelerythrine, KN62, and CaM kinase II fragment (for additional inhibitors see text).

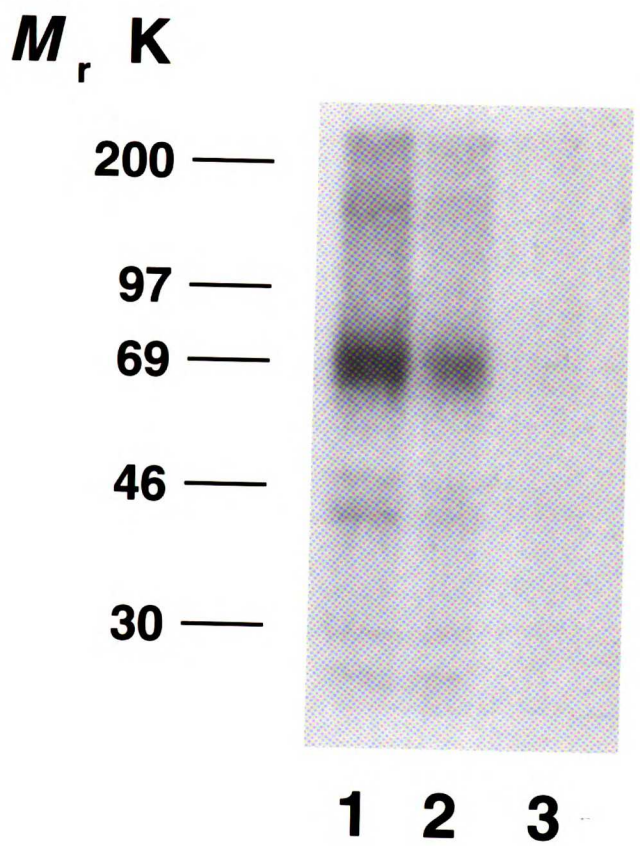


Fig. 3.4 A. Effects of morphine, H7, and H8 on μ receptor phosphorylation. HEK-EE-μ cells were labeled as described for Fig. 3.1. *Panel A. Acute treatment of permeabilized cells.* Receptor labeling was determined over 15 min in the presence of 10 μM morphine (lane 1), no drug (lane 2), or 100 μM H7 (lane 3). Each condition in panels A was repeated at least twice , with similar results.

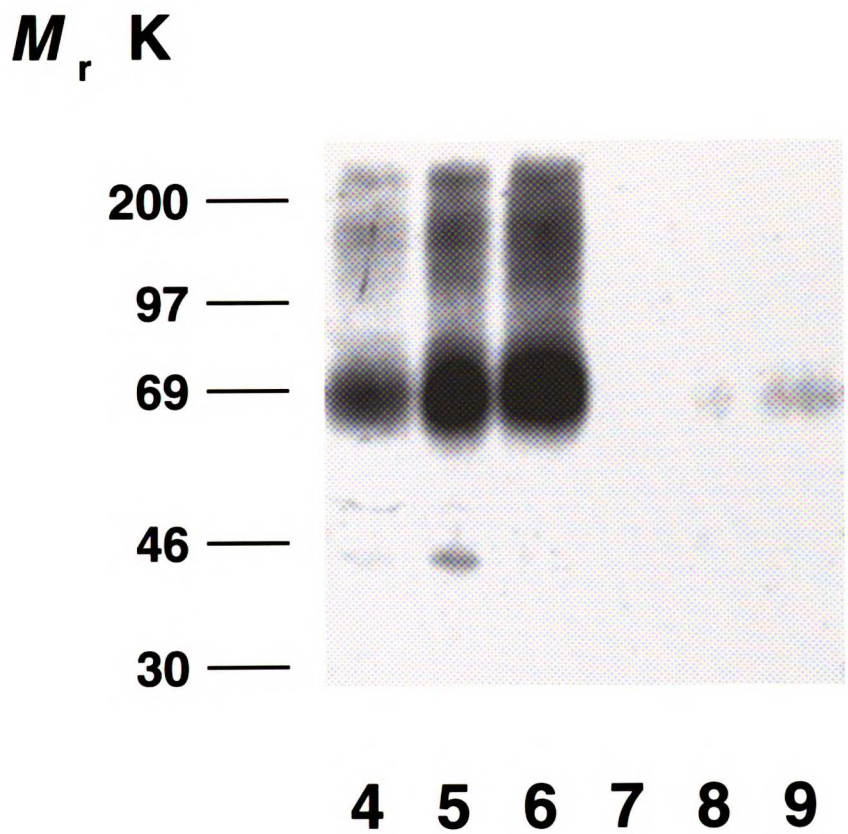


Fig. 3.4 B. Effects of morphine, H7, and H8 on μ receptor phosphorylation. HEK-EE-μ cells were labeled as described for Fig. 3.1. *Panel B. Pretreatment of intact cells with morphine, H7 and H8* , followed by removal of the drug. Pretreatment of the intact cells was performed for 7 hr with no drug (lane 4), 1 μM morphine (lane 5), 1 μM morphine plus 100 μM H8 (lane 6), 1 μM μorphine plus 100 μM H7 (lane 7), 100 μM H7 (lane 8), and 100 μM H7 with subsequent ^{32}P-labeling in the presence of 10 μM morphine (lane 9). Drugs in the pretreatment medium were removed by 4x washing before permeabilizing the cells. Thus, only the experiment shown in lane 9 contained morphine during ^{32}P-labeling. Each condition in panels B was repeated at least twice , with similar results.

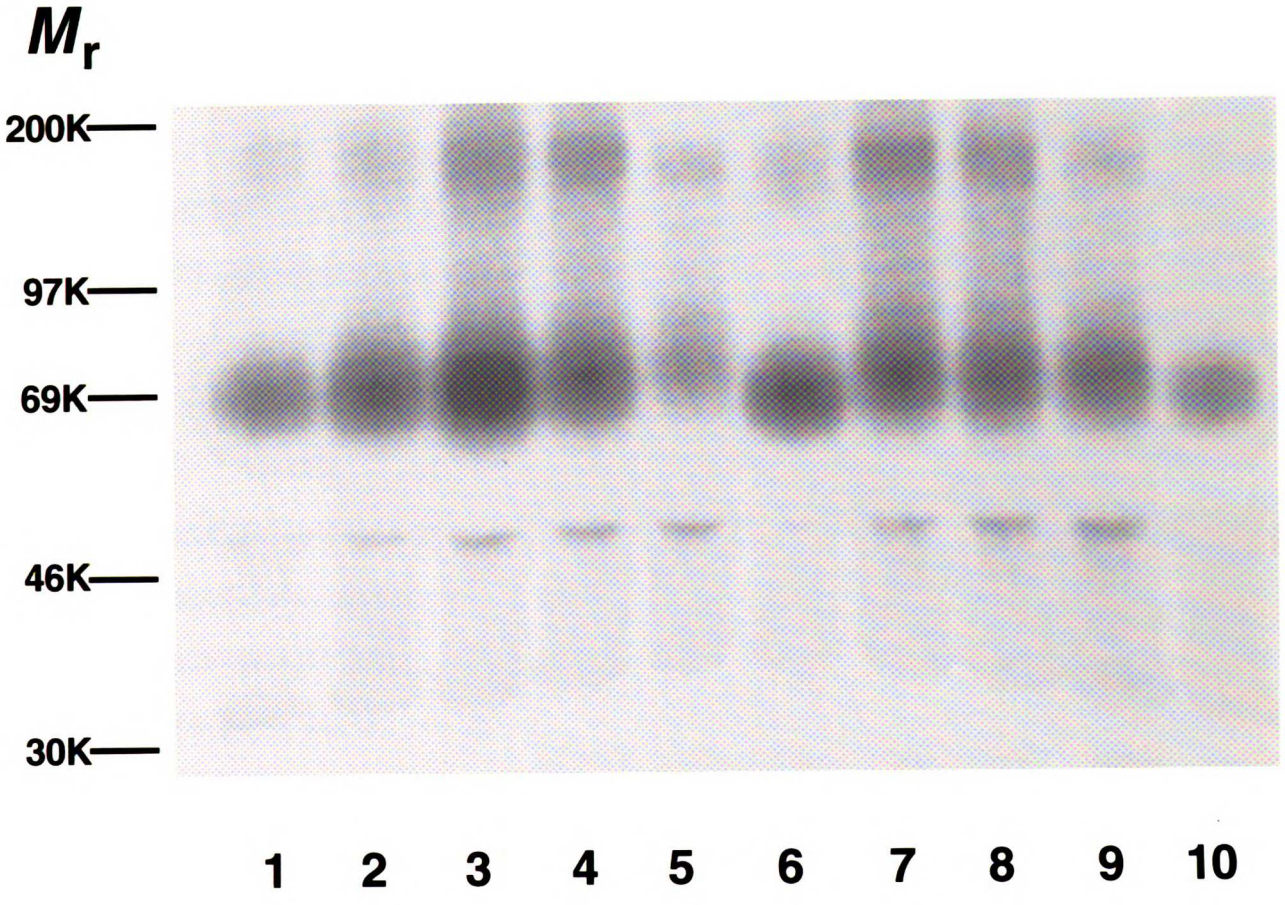


Fig. 3.5. Dose-response curve and time course of the effects of morphine pretreatment on μ receptor phosphorylation. HEK-EE-μ cells were labeled as described for Fig. 3.1. For the dose-response experiment, cells were preincubated for 12 hours with 0, 10, 100, 1000 nM morphine (lanes 1-4, respectively), followed by washout and ^{32}P labeling. For the time course (lanes 5-10), cells were pretreated for 0.5, 2, 6 hours (lanes 5-7), and allowed to recover after agonist removal for 0.5, 2, and 6 hours (lanes 8-10).

CHAPTER IV

MORPHINE TOLERANCE AND CYCLIC AMP-DEPENDENT PROTEIN KINASE-INDUCED DESENSITIZATION OF THE μ OPIOID RECEPTOR IN CELL CULTURE

ABSTRACT

Morphine tolerance and μ opioid receptor desensitization was examined in SH-SY5Y cells expressing endogenous μ opioid receptors and HEK 293 cells stably transfected with a cloned μ receptor gene (HEK-μ). Morphine tolerance was determined as a diminished effect of morphine in inhibiting cAMP accumulation upon prolonged exposure. In SH-SY5Y cells, treatment with morphine (1 μM) over 4 h reduced both the efficacy and potency of morphine. Pretreatment with forskolin (25 μM), an adenylyl cyclase (AC) activator, reduced the maximum response to morphine by 38 %, suggesting the involvement of cAMP-dependent protein kinase (PKA) in μ receptor desensitization. Forskolin-induced receptor desensitization was reversed by including H8 (100 μM), a PKA inhibitor, in the pretreatment. Incubation with both morphine and forskolin induced stronger morphine tolerance than that resulted from treatment with either agent alone. In HEK-μ cells, no morphine tolerance occurred after morphine pretreatment. In contrast, pretreatment with forskolin (25 μM) reduced the maximum

inhibition on cAMP accumulation by morphine to 50 % of control, while 8-bromo-cAMP (1 mM), a cell permeable cAMP analog and PKA activator, abolished the effect of morphine on cAMP inhibition. Desensitization induced by forskolin or 8-bromo-cAMP was reversed by H8. These results demonstrate that activation of PKA causes desensitization of the μ receptor to morphine in both SH-SY5Y and HEK-μ cells, whereas morphine tolerance resulting from morphine pretreatment cells occurs only in SH-SY5Y by a mechanism different from PKA activation.

INTRODUCTION

The opioid receptors belong to the family of G-protein coupled receptors (GPCRs). Activation of opioid receptors leads to the inhibition of adenylyl cyclase (AC) via Gi, and thus reduction of cAMP levels (9, 10, 12). Tolerance after repeated administration of narcotic opioids is well documented in clinical practice; however, the mechanisms underlying narcotic tolerance remain to be elucidated. Downregulation, internalization, and uncoupling from the second messenger system of the μ opioid receptor have been proposed to contribute to narcotic tolerance. However, no consistent changes were detectable in the numbers of μ receptors and receptor affinity (40, 46), or G protein levels (47, 48) after prolonged treatment with morphine. Recently, it has been shown that the μ receptor is not internalized after treatment with morphine; however, internalization does occur with DAMGO (25). Therefore, receptor activation by different agonists may lead to different mechanisms of tolerance.

Agonist-induced receptor desensitization is a common phenomenon in neurotransmitter signal transduction leading to reduced biological responses during continuous agonist stimulation. Among the GPCRs,

desensitization has been extensively characterized for the β-adrenergic receptor (bAR) (82). Phosphorylation of bAR by both the cAMP-dependent protein kinase (PKA) and GPCR kinase 2 (GRK 2) are, among other mechanisms, involved in rapid desensitization of bAR (82).

Using a reconstituted *in vitro* system, phosphorylation of the μ opioid receptor by PKA was shown to disrupt coupling of the receptor to Gi (49, 115). Since chronic exposure to morphine results in a compensatory upregulation of AC activity and cAMP production (37, 50), which in turn activates the PKA, it is possible that enhanced receptor phosphorylation by PKA during prolonged morphine treatment could contribute to μ opioid receptor tolerance. Phosphorylation of the μ opioid receptors is enhanced during chronic morphine exposure (88). Furthermore the cloned μ receptor contains a putative consensus sequence for the PKA (R-R-I-T*) at the end of the third intracellular loop (20). On the other hand, enhanced PKA activity by other mechanism may also cause μ receptor desensitization.

We examined μ receptor tolerance in human neuroblastoma SH-SY5Y cells, and HEK 293 cells stably transfected with a cloned μ receptor (HEK-μ) (25). In both cell lines, μ receptor tolerance can be assessed by the decreased effect of morphine in inhibiting cAMP accumulation. SH-SY5Y cells natively express both μ and δ opioid receptors (ratio ~4.5:1) (33, 35). Tolerance to morphine by pretreatment with this agonist has been demonstrated in SH-SY5Y cells (33, 35, 50). Whether activation of the PKA induces receptor desensitization in either cell line remained to be determined. PKA can be activated by treating cells with forskolin, an AC activator, which increases cAMP production, hence enhanced PKA activity (116, 117). More directly, cAMP analogs such as 8-bromo-cAMP can activate PKA in intact cells (113).

In this study, we report that tolerance/desensitization to morphine developed in SH-SY5Y cells either by treatment with morphine or by activation of the PKA, but through different mechanisms. In HEK-μ cells, tolerance occurred only after treatment with forskolin or 8-bromo-cAMP, but not with morphine. In this report, we use morphine tolerance for the reduced receptor response to morphine after prolonged exposure to morphine, whereas μ receptor desensitization refers to tolerance caused by mechanisms other than agonist treatment including PKA activation.

EXPERIMENTAL PROCEDURES

Materials

SH-SY5Y cells were provide by Dr. June L. Biedler of the Sloan-Kettering Institute for Cancer Research (Rye, NY). Forskolin, PGE1, all-trans-retinoic acid (RA), H7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine), 8-bromo-cAMP (8-bromoadenosine 3′:5′-cyclic monophosphate), and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma Chemical Co. (St. Louis, MO). H8 (N-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide) was obtained from Seikagaku America, Inc (Rockville, MD). The cAMP assay kit was supplied by Amersham, Inc. (Arlington Height, IL). Morphine sulfate was furnished by the National Institute on Drug Abuse (Bethesda, MD). The rat μ opioid receptor cDNA in vector pRC/CMV was provided by Dr. Lei Yu, Indiana University, and stably transfected into HEK 293 cells with calcium phosphate precipitation method (25, 27).

Cell culture

SH-SY5Y and HEK-μ cells were grown at 37°C in DMEM (50) and DMEM/F12 (50 %/50 %) (25) medium, respectively. Both media were supplemented with 10 % fetal calf serum containing 100 μg/ml streptomycin and 100 units/ml penicillin. G418 (200 μg/ml) was added in the HEK-μ culture to maintain stable selection. SH-SY5Y cells were pretreated with retinoic acid (5 μM) for 6 days to induce partial neuronal differentiation (35).

Cyclic AMP accumulation assay

Cells were treated with morphine (1 μM) and/or a PKA activator (25 μM forskolin or 1 mM 8-bromo-cAMP) for 12 h, unless otherwise specified. At the end of treatment, cells were rinsed twice with medium containing 5 % serum and twice with serum free medium to remove these compounds. Accumulation of intracelluar cAMP was then assayed by incubating cells in serum free medium with 1 μM PGE1 for 15 min (SH-SY5Y) (10) or forskolin at concentration and time indicated in the text (HEK-μ). Reactions were stopped by adding hydrochloric acid to a final concentration of 0.1 N. The cAMP content was determined by RIA (10, 27). Student's t-test (118) was used for statistical comparisons.

RESULTS

Tolerance to morphine after treatment with morphine in SH-SY5Y cells

Upon prolonged exposure to morphine, SH-SY5Y cells develop a moderate degree of tolerance to morphine while the cAMP second messenger system is upregulated (35, 37, 50). To determine the time course of the development of tolerance and cAMP upregulation, SH-SY5Y cells were pre-incubated for 0-24 h with 1 μM morphine. At the end of treatment, cells were thoroughly washed to ensure removal of morphine. As indicated in Fig. 4.1,

pretreatment of SH-SY5Y cells with morphine for 20 min or less caused a small decrease of PGE1-stimulated cAMP accumulation, which was not reversed by addition of naloxone (data not plotted), ruling out the presence of residual morphine as a cause for this decrease. The reason for this initial decrease is not very clear (50), and similar phenomena has been reported by others in perfused brain tissue (58). The cAMP levels recovered after incubation with morphine for 30 min or longer, and after 4 h, a rebound cAMP overshoot was detected, which peaked at 12 h with a 50-75 % increase over the control ($P<0.001$).

Unlike the rapid desensitization seen with other GPCRs including δ opioid receptor (82), tolerance to morphine developed slowly in SH-SY5Y cells (Fig. 4.1). The maximum inhibition of cAMP accumulation by morphine (10 μM) was significantly attenuated from 62 ± 3 % to 47 ± 5 % ($p<0.001$) after 4 h incubation with morphine (1 μM), and the tolerant state lasted for as long as morphine was present (fig. 4.1, and (33, 50). Treatment with morphine increased the EC50, resulting in a shift of the cAMP inhibition dose-response curve (35).

Desensitization to morphine induced by forskolin in SH-SY5Y cells

We next tested whether activation of PKA by forskolin desensitizes the opioid receptor. After pretreated with forskolin (25 μM) for 12 h, SH-SY5Y cells showed reduced sensitivity to morphine (38 ± 6 % inhibition vs 62 ± 3 % inhibition in control cells, $p<0.001$) (Fig. 4.2). A PKA inhibitor, H8 (67), (100 μM, added in the pretreatment with forskolin), largely reversed the tolerance caused by forskolin, resensitizing morphine inhibition to 52 ± 8 % ($P< 0.005$ vs treatment with forskolin alone, $P>0.05$ vs control). Therefore activation of PKA results in desensitization of the opioid receptor to morphine.

If tolerance seen after morphine pretreatment were to occur by the same mechanism as that induced by forskolin, then addition of morphine and forskolin would not produce additive effects. However, cotreatment with forskolin (25 μM) and morphine (1 μM) led to further loss of receptor sensitivity to morphine, producing only 21 ± 10 % inhibition of cAMP levels by 10 μM morphine ($P<0.001$ vs control, $P<0.01$ vs either forskolin or morphine treatment alone).

Coupling and tolerance of μ Opioid receptor expressed in HEK-μ cells

Mu receptor tolerance and desensitization were further studied in HEK-μ cells expressing a cloned rat μ receptor (20) at ~10^6 receptor sites/cells (25). Addition of 10, 50, 100 μM forskolin resulted in 30, 160, 333 folds increases in cAMP levels, respectively (Fig. 4.4 insert). The time course of forskolin-stimulated cAMP accumulation was studied by incubating HEK-μ cells with 10 μM forskolin at 37 °C for 0-10 min before the reactions were stopped by addition of HCL. Cellular cAMP levels increased within minutes after addition of forskolin and peaked at 7 min in cells either pretreated or untreated with morphine (Fig. 4.3). The expressed receptors were functionally coupled to adenylyl cyclase, as morphine (10 μM) produced 68-90 % inhibition of cAMP accumulation depending on the concentrations of forskolin used (Fig. 4.4 & 4.5). Moreover, morphine-induced cAMP inhibition increased with increasing forskolin concentration. Like in SH-SY5Y cells, pretreatment with morphine resulted in cAMP upregulation in HEK-μ cells, ranging from 75 % to 200 % increase (Fig. 4.3, and (27)). In contrast to the results in SH-SY5Y cells, pretreatment with morphine from 2 min up to 48 h did not cause any detectable loss of maximum cAMP inhibition by 10 μM morphine (data from 12 h treatment were shown in Fig.

4.4 & 4.5). On the contrary, we repeatedly found a trend of enhanced inhibition after morphine treatment ($p<0.05$ in the presence of 50 μM forskolin (Fig. 4.4).

To address further the apparent lack of morphine-induced tolerance, we examined the morphine dose response curves after treatment with 1 μM morphine for 12 h. Again, maximum inhibition by morphine was unchanged by prior morphine exposure (71 ± 5 % inhibition vs 76 ± 6 % inhibition after treatment, n.s.) (Fig. 4.5). Morphine treatment did not increase the EC50 of morphine, either (29 ± 10 nM vs 29 ± 11 nM before treatment, n.s.). There was possible stimulation of cAMP accumulation at low concentrations of morphine in control cells (1 nM) and pretreated cells (1 nM to 10 nM). The unexpected stimulation by morphine may be due to coupling of the μ receptor to Gs at low receptor activity, which has been observed in brain tissue (101, 102) and cultured cells (50), especially after exposure to a certain agonist. However, stimulation of cAMP by low concentrations of morphine was highly variable in our experiments, therefore, we did not further investigated this effect.

These results were obtained in the absence of any phosphodiesterase (PDE) inhibitors in the cAMP accumulation assay medium. In the presence of PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX), morphine (10 μM) produced 79 ± 6 % inhibition in control cells and 85 ± 2 % inhibition in morphine (1 μM)-pretreated, washed cells ($p<0.01$). Therefore, pretreatment with morphine failed to induce tolerance in HEK-μ cells regardless the addition of PDE inhibitors.

Desensitization to morphine induced by PKA activation in HEK-μ cells

To test the effect of PKA activation, HEK-μ cells were treated with forskolin (25 μM) for 12 h. The maximum inhibition by 10 μM morphine after exposure to forskolin was reduced from 68 ± 7 % to 30 ± 8 % ($p<0.001$) (Fig. 4.5), indicating the development of receptor desensitization. In parallel, the response of AC to forskolin (10 μM) stimulation was reduced after 12 h incubation with 25 μM forskolin, and forskolin-stimulated cAMP levels were 56 % lower than those in control cells ($p<0.01$), suggesting some AC tolerance to forskolin. However, the EC50 of morphine was reduced from 29 ± 10 nM to 8 ± 4 nM ($p< 0.01$) by forskolin treatment.

Forskolin-induced desensitization to morphine in HEK-μ cells was completely reversed by cotreatment with 100 μM H8 (Fig. 4.6), which resensitized the response to 10 μM morphine to 80 ± 23 % ($p<0.05$ vs forskolin treatment alone, not significantly different from the control: 79 ± 4 % inhibition in this experiment).

HEK-μ cells were also treated with the PKA activator, 8-bromo-cAMP, to induce desensitization to morphine. Pretreatment with 8-bromo-cAMP (1 mM) for 12 h led to complete desensitization to 10 μM morphine ($p<0.001$) (Fig. 4.6). The response to morphine was restored to 40 ± 36 % when H8 (100 μM) was present in the pretreatment ($p<0.05$ vs 8-bromo-cAMP alone; not significantly different from the control).

DISCUSSION

Exposure of SH-SY5Y cells to the μ receptor agonist morphine leads to partial tolerance as measured by morphine inhibition of cAMP accumulation. The delayed onset of tolerance parallels the development of antinociceptive tolerance *in vivo* (65) , supporting SH-SY5Y cells as a suitable model to study μ receptor tolerance. Desensitization of the μ receptor response to morphine

was also evident after treatment with forskolin. Forskolin activates PKA by increasing cAMP levels, therefore the effect of forskolin was most likely caused by PKA activation. This was further supported by reversal of forskolin-induced desensitization by co-pretreatment with H8, a PKA inhibitor with an IC50 of 10 μM (67). However, forskolin and morphine produced additive effect on receptor tolerance, which suggested different mechanisms contributing to morphine tolerance and PKA-induced desensitization.

In HEK-μ cells, only activation of PKA by forskolin or 8-bromo-cAMP effectively induced desensitization, whereas pretreatment with morphine did not lead to tolerance, suggesting that mechanism other than PKA activation may have caused tolerance in SH-SY5Y cells.

Failure to develop tolerance after morphine treatment may be due to the high expression level of μ receptors in HEK-μ cells, or the absence of factors responsible for tolerance. When the cloned rat μ receptor was co-expressed with a K^+ channel in Xenopus oocytes, tolerance developed after treatment with morphine as measured by K^+ conductance (119).

The physiological significance of PKA-induced receptor desensitization remains to be clarified. Since all Gi-coupled receptors, such as the α2 receptor, generally induce cAMP upregulation after prolonged treatment with agonists (27), PKA-induced desensitization may be involved in cross-tolerance. However, there is little evidence supporting cross-tolerance between μ receptor and other opioid receptors (120), or between μ and α2 receptor (121), and this mechanism did not appear to contribute to morphine tolerance in this study.

We have previously demonstrated that a novel H7-sensitive μ receptor kinase activity may play a role in narcotic tolerance (50, 88). Moreover

morphine-induced tolerance in mice can be blocked by the protein kinase inhibitor H7, but not H8 (50), in agreement with the finding from this study that PKA is not involved . On the other hand, we showed that PKA activation desensitized the μ receptor. These results and other studies (122) indicate that μ receptor phosphorylation may be regulated by multiple protein kinases with distinct effects on receptor functions.

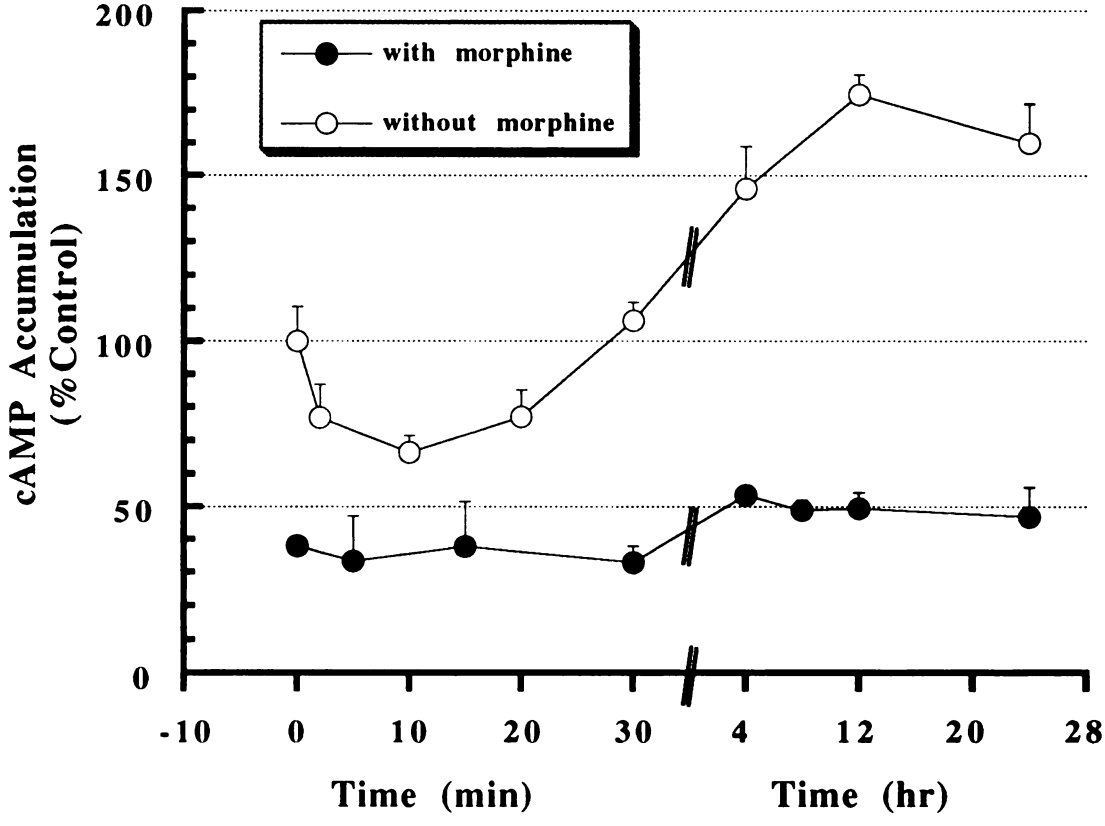


Fig. 4.1. Time course of cAMP up-regulation and morphine tolerance in SH-SY5Y cells. SH-SY5Y cells were treated with medium alone or containing 1 μM morphine for 0 - 24 h. PGE1- stimulated cAMP accumulation were assayed, after washing out morphine, in the absence (cAMP up-regulation) and presence (cAMP inhibition) of 10 μM morphine. Each point represents the mean ± S. D.

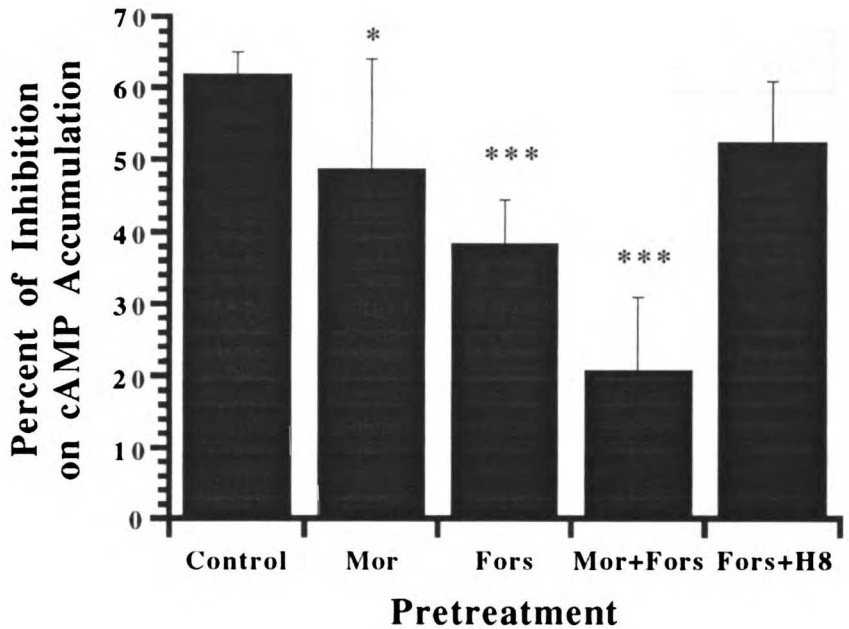


Fig. 4.2. Effect of pretreatment with morphine and/ or forskolin on cAMP inhibition caused by morphine. SH-SY5Y cells were pretreated for 12 h with DME medium (Control), or medium containing 1 μM morphine (Mor) and / or 25 μM forskolin (Fors) for 12 h, or medium containing both 25 μM forskolin and 100 μM H8 (Fors+H8). PGE1- stimulated cAMP accumulation was assayed in the absence or presence of 10 μM morphine after washing out these compounds. % inhibition was plotted for each treatment. The asterisks indicate significant differences from control: *$p<0.05$; ***$p<0.001$.

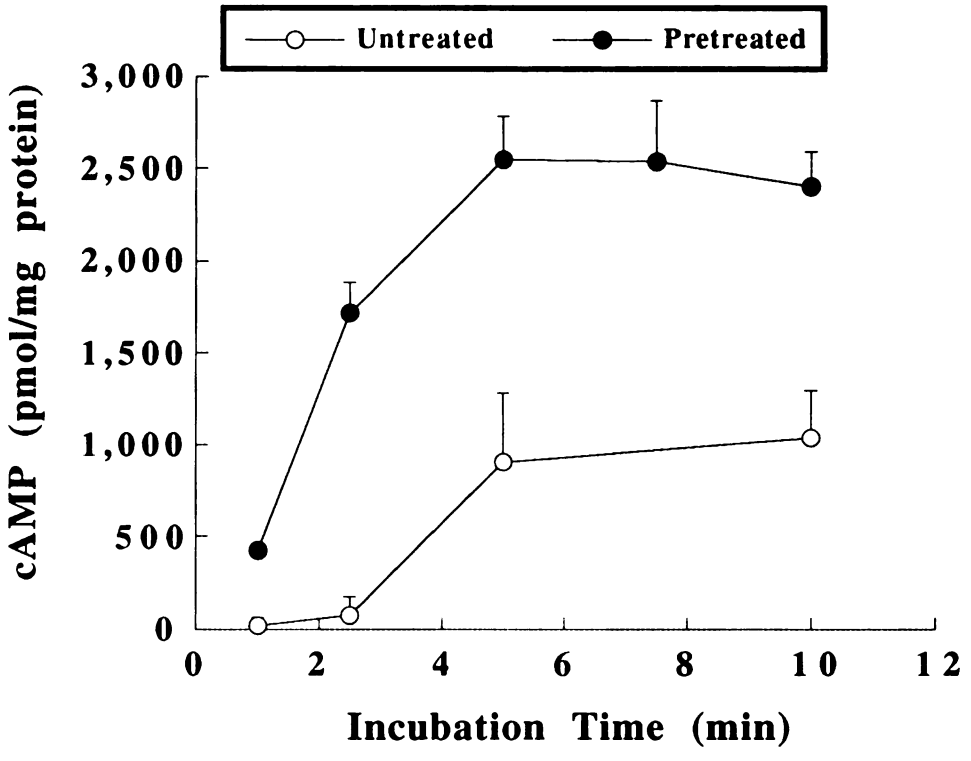


Fig. 4.3. Time course of forskolin (10 μM) stimulated-cAMP accumulation levels in HEK-μ cells. Control or morphine (1 μM) pretreated cells were washed 4 times, and incubated with 10 μM forskolin for 1-10 min in the absence of any phosphodiesterase inhibitor.

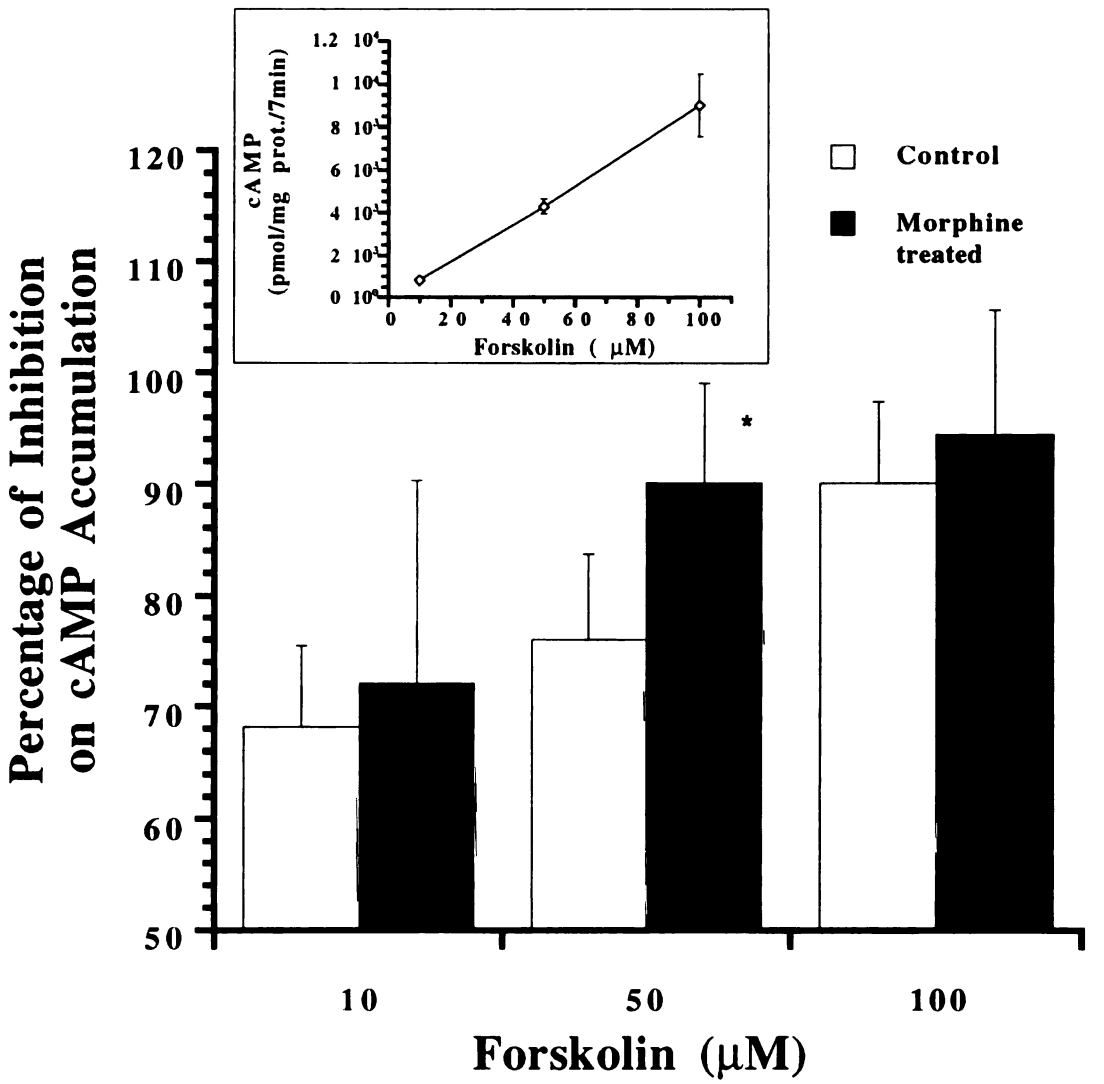


Fig. 4.4. Inhibition of cAMP by morphine in untreated and morphine-pretreated HEK-μ cells in the presence of 10 μM, 50 μM, 100 μM forskolin. the insert shows cAMP levels in relation to forskolin concentrations. Forskolin-stimulated cAMP accumulation was assayed before (open column) or after (solid column) morphine pretreatment (1 μM, 12 h). The asterisk indicates significant difference from control (before treatment): *$p<0.05$.

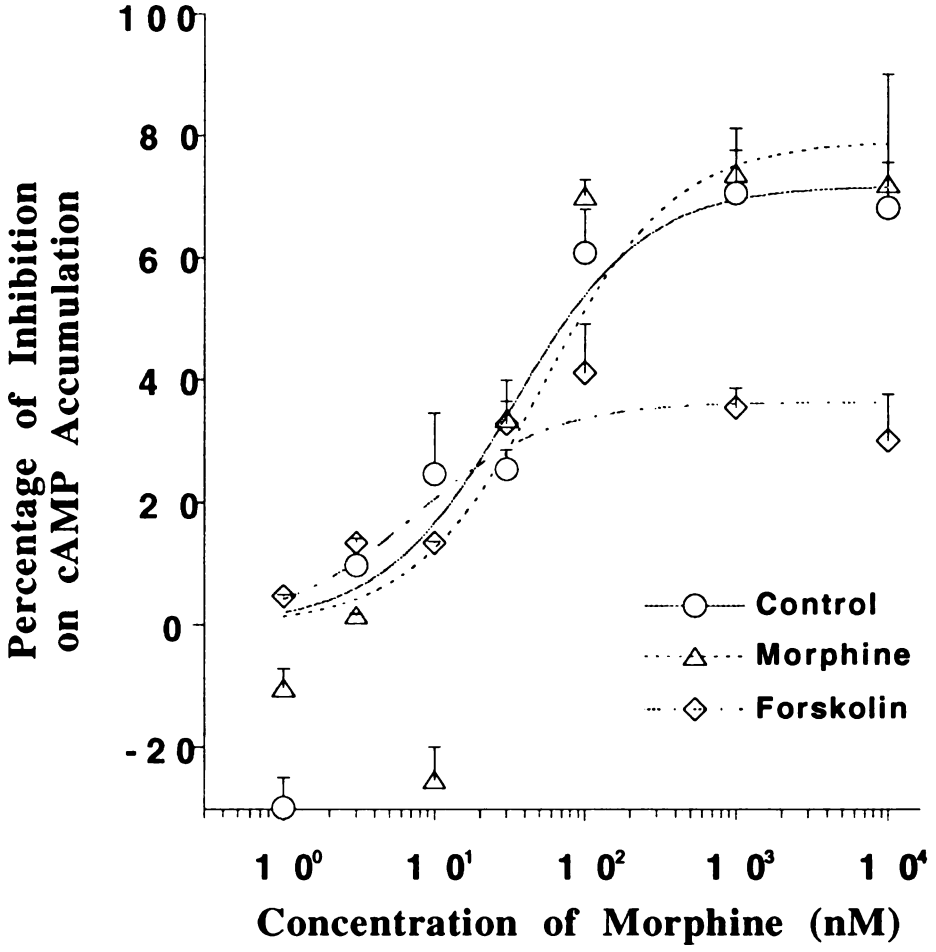


Fig. 4.5. Dose response curves of cAMP inhibition by morphine before or after treatment with morphine or forskolin. HEK-μ cells were pretreated with 1 μM morphine or 25 μM forskolin followed by washing out these compounds, before the cells were challenged with varying concentration of morphine in cAMP assay buffer containing 10 μM forskolin. The curves were fitted with equation: E=Emax*C/(C+EC50), with slope factor fixed to 1.

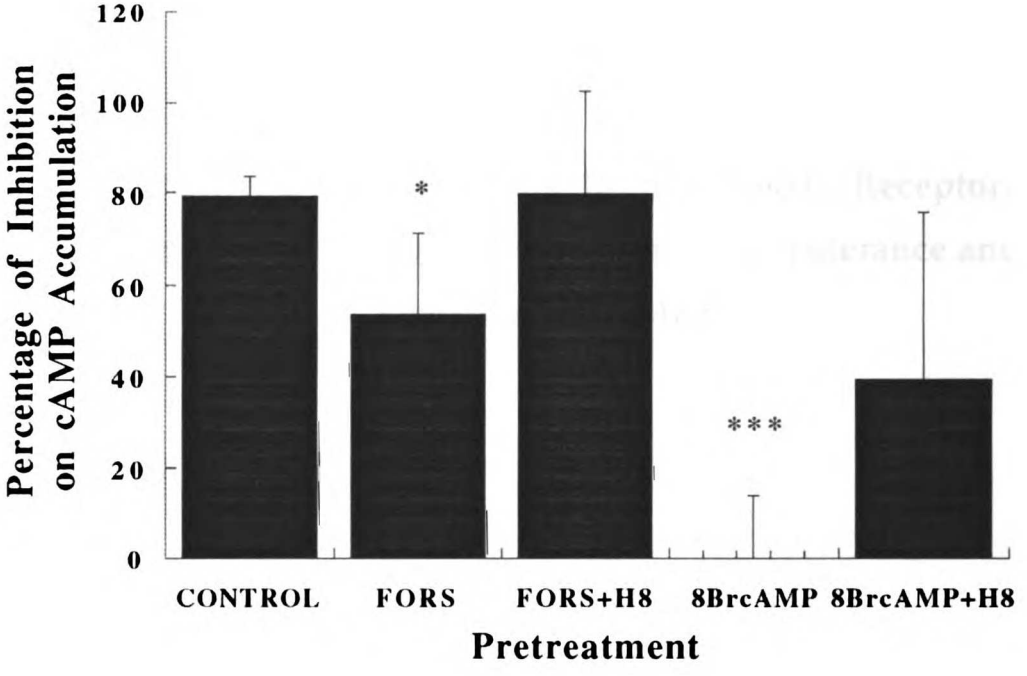


Fig. 4.6. Effect of forskolin, or 8-bromo-cAMP on morphine-mediated cAMP inhibition. HEK-μ cells were pretreated for 12 h with the following media: DME medium (CONTROL), medium containing 25 μM forskolin (FORS), medium containing both 25 μM forskolin and 100 μM H8 (FORS+H8), medium containing 1 mM 8-bromo-cAMP (8BrcAMP), or medium containing both 1 mM 8-bromo-cAMP and 100 μM H8 (8BrcAMP+H8). Forskolin (10 μM)- stimulated cAMP accumulation were assayed after washing out these compounds. The asterisks indicate significant difference from control: *$p<0.05$; ***$p<0.001$.

CHAPTER V

3-Isobutyl-1-methylxanthine Inhibits Basal μ Receptor Phosphorylation and Reverses Morphine Tolerance and Dependence in Mice

SUMMARY

Induction of a narcotic tolerant and dependent state *in vitro* and *in vivo* was previously proposed to involve μ receptor phosphorylation by an H7-sensitive kinase (Wang et al., Life.Sci. PL 54, 339-350 (1994)). The cAMP phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX), had effects similar to those of H7 on μ receptor regulated cAMP levels in morphine pretreated SH-SY5Y cells and HEK-293 cells transfected with the μ receptor. After removal of the agonist present in the pretreatment medium, IBMX was found to block a naloxone-induced rise in cAMP levels, a possible indicator of the formation of a sensitized, phosphorylated state of the μ receptor. To test whether IBMX acts as a kinase inhibitor, we measured μ receptor phosphorylation in permeabilized HEK 293 cells transfected with an epitope tagged μ receptor (Arden et al., J. Neurochem. 65,1636-1645 (1995)). Indeed, pretreatment or acute treatment with IBMX strongly inhibited basal (agonist independent) μ receptor phosphorylation (IC50 ≤10 μM). Furthermore, in

mice made acutely tolerant to and dependent on morphine, i.c.v. injection of IBMX 30 min before testing partially reversed morphine tolerance and suppressed naloxone induced withdrawal jumping, at doses that did not alter the antinociceptive effects of morphine. These results demonstrate a new mechanism of action for the xanthine derivative, IBMX, as an inhibitor of a putative protein kinase mediating basal μ receptor phosphorylation. Further, these results support the hypothesis that μ receptor phosphorylation plays a role in narcotic tolerance and dependence.

INTRODUCTION

3-Isobutyl-1-methylxanthine (IBMX) is a potent cAMP phosphodiesterase inhibitor commonly used in assays of cellular cAMP levels to prevent cAMP degradation (123). Here we demonstrate that IBMX also functions as an effective inhibitor of μ receptor phosphorylation. We have previously demonstrated that the kinase inhibitor H7 suppresses basal μ receptor phosphorylation and reverses biochemical and pharmacological indicators of narcotic tolerance and dependence (27, 50, 88, 96, 124). These results support the hypothesis that μ receptor phosphorylation plays a key role in narcotic tolerance and dependence (50). This study shows that an inhibitor of basal μ receptor phosphorylation, IBMX, with a chemical structure distinct from that of H7 similarly reverses narcotic tolerance and dependence.

Narcotic dependence is characterized at the cellular level by an upregulated cAMP second messenger system, resulting in elevated cAMP levels upon agonist removal (35, 40, 124). Thus, morphine treatment of cells

expressing μ opioid receptors, e.g., the human neuroblastoma cells SH-SY5Y (33) and human embryonic kidney HEK 293 cells transfected with the μ receptor (HEK-μ) (25), results in a subsequent cAMP overshoot after agonist removal (27, 50, 96) and a moderate degree of tolerance in SH-SY5Y cells (33, 50). Moreover, the μ receptor was shown to be converted into a new state uniquely sensitive to the antagonist naloxone (27, 33, 88): even though morphine was thoroughly removed from the pretreated cells, naloxone caused an additional cAMP increase. We use the terms *spontaneous cAMP overshoot* and *naloxone-induced cAMP overshoot* to distinguish the enhanced cAMP levels observed in the absence and the presence of naloxone, respectively (50). The direct action of naloxone at the μ receptor in dependent tissue, opposite to that of the agonist morphine, suggested an inverse agonist effect (negative antagonism) at a basally (constitutively) active μ receptor (50). Such unique action of naloxone is consistent with its high potency in eliciting withdrawal in morphine-dependent animals (28). Therefore, the naloxone-induced cAMP overshoot could represent a salient feature of narcotic dependence, in addition to the general upregulation of the cAMP second messenger system.

Results with the protein kinase inhibitor H7 suggested that morphine tolerance and dependence may involve phosphorylation of the μ opioid receptor (50). Pretreatment of SH-SY5Y cells with morphine together with H7 prevented the naloxone-induced cAMP overshoot; therefore, μ receptor phosphorylation may be required for its sensitization towards naloxone (50). Moreover, i.c.v. injection of H7 reversed morphine tolerance and suppressed naloxone-induced withdrawal jumping in mice made acutely tolerant to and dependent on morphine (50, 52). Morphine tolerance was also found to be

prevented by H7 in a chronic mouse model (122). These results suggested that a phosphorylation event contributes to the expression of narcotic tolerance and dependence *in vivo*.

To measure μ receptor phosphorylation, we developed an assay using γ-^{32}P-ATP labeling of an epitope-tagged μ opioid receptor (EE-μ) in permeabilized, stably transfected HEK 293 cells (HEK-EE-μ) (25). The μ opioid receptor was found to be continuously phosphorylated at a basal rate in the absence of agonist, which is enhanced in the presence of morphine (25). Basal μ receptor phosphorylation has been confirmed to occur in other cells with heterologous or native expression of the μ receptor, including brain slices (103, 104), suggesting that this phenomenon is not limited to HEK 293 cells. The kinase involved in basal μ receptor phosphorylation is Ca^{++}-dependent, unaffected by high Na^{+} ion concentrations, and differs in its sensitivity to inhibitors from the major regulatory kinases, such as PKA, PKC, GRKs, and Ca^{++}-calmodulin dependent kinase-II (88). Thus far, H7 was identified as the only effective inhibitor of basal μ receptor phosphorylation, in correlation with its ability to suppress the naloxone-induced cAMP overshoot *in vitro*, and morphine tolerance and naloxone-induced withdrawal jumping *in vivo* (50, 52, 88). These results do not preclude a role for other kinases that may also contribute to μ receptor phosphorylation.

During our previous studies in SH-SY5Y and HEK-μ cells (27, 50, 52, 96), we noticed that the addition of IBMX to the cell medium prevented the naloxone-induced cAMP overshoot in morphine pretreated cells, without blocking the acute inhibition of cAMP accumulation nor the spontaneous cAMP overshoot. The latter was also unaffected by IBMX in CHO cells transfected with the μ opioid receptor (124). This result suggested that either

the naloxone-induced cAMP overshoot depends on intact signaling of the μ receptor through cAMP phosphodiesterase, or that IBMX has a direct effect on μ receptor phosphorylation similar to that of H7, or both. We show here that IBMX indeed inhibits basal μ receptor phosphorylation and reverses morphine tolerance and naloxone-induced withdrawal jumping *in vivo*.

EXPERIMENTAL PROCEDURES

Materials

3-Isobutyl-1-methylxanthine (IBMX) was obtained from Sigma Chemicals, St.Louis, MO. The rat μ opioid receptor cDNA in vector pRC/CMV (20), provided by Dr. Lei Yu, Indiana University, was tagged with the epitope EYMPME, immediately downstream of the Met initiation codon (EE-μ) (25).

Cell culture

The human neuroblastoma cell line, SH-SY5Y, was grown as previously reported (35, 50). The plasmids encoding μ and EE-μ receptors were transfected into HEK 293 cells, and stably transfected clonal cell lines were established as described (HEK-μ and HEK-EE-μ) (25). The cell lines used expressed ~$5x10^5$ sites per cell. Culture over longer time periods resulted in a gradual diminution of the naloxone-induced cAMP overshoot, relative to the spontaneous cAMP overshoot. Therefore, fresh cell cultures were plated every 6-8 weeks from the same stock.

Receptor binding assay

Receptor expression was examined with 2 nM [^{3}H]-diprenorphine in intact cell monolayers as described (25).

Assay of cyclic AMP levels

Washed cell monolayers were incubated at 37^{o}C for 15 min in the presence of 1 μM prostaglandin E1 (SH-SY5Y) or for 7 min with 10 μM forskolin (HEK-μ and HEK-EE-μ), and cAMP was determined by RIA as described previously (25, 50)

Drug pretreatment protocol

SH-SY5Y cells were pretreated with retinoic acid (5 μM) for 6 days to induce partial neuronal differentiation (35). Cells were pretreated with 1 μM morphine for 0-12 hours. Immediately before the cAMP accumulation assay, cells were washed twice with medium containing 5% serum and twice more with serum-free medium, requiring 10 min for effective agonist removal. A study with ^{3}H-morphine tracer established that greater than 99.7% of the ^{3}H-morphine added was removed by this washing procedure. A similar washing procedure was also found sufficient to remove residual morphine in CHO cells transfected with the μ receptor (124). cAMP levels were determined subsequently, as described above.

Phosphorylation and immunoprecipitation of the epitope tagged μ receptor. HEK-EE-μ cells were pretreated with drugs as indicated, and phosphorylation of EE-μ was determined in washed, permeabilized cells as described (25). Briefly, digitonin permeabilized cells were labeled with 0.2 mCi [g-^{32}P]-ATP (200 μM, 0.5 ml) at 25^{o}C for 15 minutes in the presence or absence of 1 μM morphine or kinase inhibitors. The incubation medium consisted of DMEM medium (containing Ca^{++} required for basal μ receptor kinase activity and Na^{+} ions to suppress intracellular kinase activities unrelated to basal μ receptor phosphorylation, which was insensitive to Na^{+}) (25, 88). Labeled cells were washed and homogenized in the presence of 100 nM diprenorphine and protease inhibitors (25). The 30,000xg membrane pellet was solubilized in 10 mM CHAPS, containing 100 nM diprenorphine. Solubilized EE-μ was immunoprecipitated with anti-EE mAb , subjected to SDS-PAGE, and autoradiographed. The identity of the labeled EE-μ band was confirmed by Western blotting (25).

Naloxone-induced withdrawal jumping and morphine tolerance in mice. Animal experiments were performed exactly as described previously for testing the effect of H7 and H8 (50, 52). Briefly, male ICR mice (20-30 g, Harland Industries, Cleveland, Ohio), in groups of 10 each, were injected with 100 mg/kg morphine sulfate subcutaneously (s.c.). Naloxone-induced withdrawal jumping was determined after 4 hours, by injecting increasing doses of naloxone intraperitoneally (i. p.). Tolerance to morphine was established by obtaining morphine dose-response curves in untreated mice and in separate groups of mice pretreated with a single s.c. dose of 100 mg/kg morphine sulfate 5 hours previously. These time intervals were selected by previous control experiments (50, 52). To test the acute effects of IBMX on

morphine antinociception, morphine sulfate (i. c. v. and s. c.) dose response curves were determined with and without i.c.v. injection of 60 or 100 nmoles IBMX 30 min before the morphine dose, using the warm water (55 °C) tail-flick test (50, 52).

RESULTS

Effects of IBMX on the naloxone-induced cAMP overshoot in SH-SY5Y and HEK-μ cells.

Pretreatment of HEK-μ cells with morphine, followed by removal of the drug by washing, did not result in any detectable tolerance to morphine (control ED50 29 ± 11 nM, Emax 71 ± 5%; morphine pretreated (1 μM for 12 hours) ED50 29 ± 11nM, Emax 76 ± 6%), possibly because of the relatively high number of receptor sites expressed in the HEK-μ cells. Therefore, this *in vitro* model was unsuitable for the study of narcotic tolerance. On the other hand, pretreatment with 1 μM morphine led to a spontaneous cAMP overshoot in SH-SY5Y cells and HEK-μ cells, as previously reported (Fig. 5.1 A and B) (27, 50, 96). Moreover, addition of 1-10 μM naloxone to the thoroughly washed cells caused an additional increase of the cAMP levels (Fig.1A and B), again in agreement with similar results in SH-SY5Y cells (27, 50, 96). The ED50 value for this inverse naloxone-induced cAMP overshoot was ~1 nM both in SH-SY5Y cells (50) and in HEK-μ cells (data not shown).

Addition of 500 μM IBMX to the cAMP assay medium, a concentration usually employed to suppress cAMP phosphodiesterase, greatly increased cAMP levels in SH-SY5Y cells while its effect on cAMP accumulation in HEK-μ cells was relatively small (Fig. 5.1, open bars, control and IBMX Post-treat.). Further, IBMX did not suppress the spontaneous cAMP overshoot observed

after agonist removal (Fig. 5.1, shaded bars), in agreement with another study employing CHO cells transfected with the μ receptor (124). However, in both cell lines, IBMX abolished the naloxone-induced cAMP overshoot (Fig. 5.1, black bars). This experiment was repeated with 10, 100, and 500 μM IBMX added acutely to morphine pretreated HEK-μ cells. The naloxone-induced cAMP overshoot was again undetectable at 100 and 500 μM IBMX, while 10 μM IBMX reduced the naloxone-induced cAMP overshoot from 82 ± 16% above the spontaneous cAMP overshoot in the control to 51± 8% ($P<0.01$). Therefore, IBMX added to the cAMP assay medium after morphine pretreatment prevented the naloxone-induced cAMP overshoot with an EC50 in the range of 10 μM.

Pretreatment of both SH-SY5Y and HEK-μ cells with 1 μM morphine plus 500 μM IBMX also prevented the naloxone-induced cAMP overshoot observed after drug removal (Fig. 5.1A and B, IBMX Pre-treat.). This effect of IBMX was observed even though both morphine and IBMX were carefully removed by washing the cell monolayers four times before the cAMP assay.

Effect of IBMX on basal μ receptor phosphorylation in HEK-EE-μ cells.

The opioid receptor was found to be phosphorylated at a basal rate which is further enhanced by morphine, added either during the labeling period or to the pretreatment medium (Fig. 5.2A) (see also (25, 88)). Addition of 500 μM IBMX suppressed basal μ receptor phosphorylation by >90% when added directly to the permeabilized HEK-EE-μ cells during the 15 min ^{32}P labeling period. We therefore determined the ability of IBMX at three concentrations (10, 100, and 500 μM) to inhibit basal μ receptor

phosphorylation, either when added acutely to the labeling medium, or together with 1 μM morphine during an 8 hour pretreatment period, followed by removal of both drugs by washing. Acute addition of 10 μM IBMX reduced μ receptor labeling by ~50% of the control, whereas higher doses suppressed labeling by >90% (Fig. 5.2B). When added to the pretreatment medium, IBMX suppressed basal μ phosphorylation by 90% at 10 μM and by 95% at 100 μM. At 500 μM IBMX in the pretreatment medium, μ receptor ^{32}P labeling was no longer detectable.

Effect of IBMX on morphine antinociception, antinociceptive tolerance to morphine, and naloxone-induced withdrawal jumping.

We first tested the effect of IBMX given intracerebro-ventricularly (i.c.v.) on acute antinociception by subcutaneous (s.c.) doses of morphine sulfate. There was no measurable change of the morphine dose-response curve (morphine sulfate (95% confidence limits) A50 8.5 (6.5-11) mg/kg; 100 nmole IBMX: morphine sulfate A50 7,5 (4.5-12) (Fig. 5.3A). When morphine and IBMX were given concomitantly by the i.c.v. route, doses of 60 and 100 nmole IBMX also did not affect antinociception by morphine (data for 60 nmole IBMX experiment: control morphine sulfate A50 3.3 (2.4-4.7) nmole; morphine sulfate plus IBMX A50 3.1 (1.6-5.9) mg/kg). Therefore, IBMX given i.c.v. had no detectable effect on the antinociceptive effects of morphine given s.c. or i.c.v. in the tail-flick assay.

The effects of IBMX on antinociceptive tolerance were tested 5 hours after a single s.c. dose of 100 mg/kg morphine sulfate. Given i.c.v. at 100 nmoles 30 minutes before the second s.c. morphine dose, IBMX significantly

reversed the antinociceptive tolerance (Fig. 5.3B). The A50 values for morphine sulfate were (95% confidence limit): control mice 8.5 mg/kg (6.5-11), tolerant mice 24 mg/kg (19-30), tolerant mice given IBMX 15 mg/kg (13-18).

Pretreatment of mice with 100 mg/kg morphine sulfate s.c. resulted in withdrawal jumping induced by naloxone injected i.p 4 hours after the morphine dose. This effect was dose dependent (Fig. 5.3C). Injection of IBMX, given i.c.v. 30 min before the naloxone dose, reduced withdrawal jumping significantly in a dose dependent fashion, with maximum effects seen at 100 nmoles (Fig. 5.3C).

DISCUSSION

The protein kinase inhibitor H7 was previously shown to prevent the naloxone-induced cAMP overshoot in morphine pretreated SH-SY5Y cells without suppressing the spontaneous cAMP overshoot (50), and to block basal μ receptor phosporylation (27, 50, 96). These results suggested that H7 prevented the formation of a proposed sensitized, or constitutively active receptor state, termed μ*, in morphine pretreated cells (50), and further, that μ* is phosphorylated. In the present study, IBMX was also found to prevent the naloxone-induced cAMP overshoot (Fig. 5.1), without affecting the spontaneous cAMP overshoot. We therefore considered the possibility that this cAMP phosphodiesterase inhibitor suppresses basal μ receptor phosphorylation. Using g-^{32}P-ATP labeling in permeabilized HEK-EE-μ cells (25), we indeed observed strong inhibition of basal μ receptor phosphorylation by IBMX, with an IC50 near or below 10 μM. This result

indicates that IBMX serves as an inhibitor of a protein kinase responsible for basal μ receptor phosphorylation, with a potency commensurate to that required for inhibiting cAMP phosphodiesterase (3.4 μM) (125). IBMX had already been shown to have effects other than inhibition of cAMP phosphodiesterase, e.g., antagonism of adenosine receptors (125), and inhibition of sustained voltage-dependent Ca^{++} currents (126) and of Gi proteins at higher doses (127). Moreover, IBMX has multiple effects *in vivo* that are difficult to reconcile by considering only its known biochemical mechanisms (128, 129) Whether inhibition of a receptor kinase can account for some of these *in vivo* effects remains to be seen.

We have previously established that morphine pretreatment of HEK-EE-μ cells enhanced, and pretreatment with H7, abolished subsequent basal μ receptor phosphorylation (27, 88, 96). These effects were seen even though the pretreatment agents were removed before ^{32}P labeling occurred. We therefore proposed a long-lasting positive feedback loop between the μ receptor and its cognate kinases: the phosphorylated receptor further stimulates the receptor kinase and thus maintains a basal phosphorylation reaction in the absence of agonist (27, 88, 96). Once the phosphorylation reaction is interrupted, e.g., by H7, basal phosphorylation was abolished and did not recover rapidly (88). We therefore tested whether IBMX pretreatment would have the same effect as H7 pretreatment. This was indeed observed (Fig. 5.2B), even though IBMX was removed before labeling. Since the basal μ receptor phosphorylation observed under our assay conditions is not mediated by PKA (it is less sensitive or insensitive to H8, HA1004, and the PKA inhibitor peptide PKI (5-24)) (88), any effect of IBMX by raising cAMP levels and PKA activity is unlikely to account for this observation. Moreover,

in HEK-μ cells, IBMX had only a relatively small effect on forskolin stimulated cAMP levels, relative to the large effect seen in SH-SY5Y cells (Fig. 5.1). We conclude that IBMX disrupted basal μ receptor phosphorylation and the positive feedback cycle, in the same fashion as did H7 (88). This action correlated with the ability of both agents to prevent the naloxone-induced cAMP overshoot in SH-SY5Y cells.

Nevertheless, the behavior of IBMX differed from that of H7. First, H7 added acutely to the cAMP assay incubation, had little or no effect on the naloxone-induced cAMP overshoot (50) whereas IBMX abolished it. Second, H7 pretreatment, while effective in SH-SY5Y cells, had variable and incomplete effects on the naloxone-induced cAMP overshoot in HEK-μ cells (data not shown), whereas IBMX was effective under all conditions. Since both agents largely inhibited basal μ receptor phosphorylation at the same concentrations (e.g., 100 μM), it follows that IBMX acts on additional targets, such as cAMP phosphodiesterase. The latter has been shown to be regulated by the δ opioid receptor (17), and we have previously suggested a similar pathway for the μ opioid receptor (33). The potential role of regulation of cAMP phosphodiesterase by the μ receptor in narcotic tolerance and dependence requires further investigation.

As a purine analog, and thus an analog of ATP, it is possible that IBMX inhibits additional kinases that may also play a role in μ receptor phosphorylation. Our ^{32}P labeling assay was designed to measure only the unique phosphorylating activity responsibel for basal μ receptor phosphorylation, but there is evidence that other kinases also contribute to μ receptor phosphorylation (103, 104). However, results in our laboratory (Kassack and Sadée, unpublished) indicate that 250 μM IBMX and 100 μM H7

had no effect on GRK2 (βARK1) activity, a candidate kinase for agonist stimulated receptor phosphorylation. Our assay conditions were optimized to measure basal μ phosphorylating activity while suppressing other intracellular kinases by including high Na^+ concentrations (88). Therefore, any role of other kinases which may contribute to μ receptor phosphorylation in the intact cell, cannot be assessed from our results. Multiple phosphorylation reactions are likely to occur and to contribute to μ receptor regulation.

We have previously shown that H7 rapidly suppresses naloxone induced withdrawal jumping and reverses morphine tolerance in a mouse model, while having no effect on morphine antinociception in untreated animals (25, 50). In contrast, H8 was inactive in these tests correlating with its lack of *in vitro* effect upon pretreatment on μ receptor phosphorylation (88) and the naloxone-induced cAMP overshoot (25, 50). Because of this *in vitro/in vivo* correlation established for H7, and H8 as the negative control, we asked whether IBMX would also reverse morphine tolerance and naloxone-induced withdrawal jumping if administered using an identical protocol. When given i.c.v., the animals tolerated doses of IBMX up to 100 nmoles without visible adverse effects, and IBMX failed to affect morphine antinociception (Fig. 5.3A). Failure of IBMX to affect acute morphine effects occurred regardless of the route of morphine administration (s.c. or i.c.v.), suggesting that differential distribution of the two drugs is unlikely to account for this negative result in the control experiments. Yet, IBMX significantly reversed morphine tolerance within 30 min (Fig. 5.3B) and effectively suppressed naloxone-induced withdrawal jumping (Fig. 5.3C). Therefore,

IBMX served to distinguish the acute and chronic effects of morphine in this mouse model.

Without considering inhibition of μ receptor phosphorylation, one would have expected that IBMX exacerbates withdrawal jumping because it increases cAMP levels by blocking cAMP phosphodiesterase. Indeed, IBMX administered peripherally was previously shown to caused a quasi-narcotic withdrawal syndrome upon naloxone challenge (128). Moreover, Collier and Francis (29) demonstrated that IBMX given s.c. one hour before testing morphine (24 hr)-pretreated mice enhanced naloxone-induced withdrawal jumping. This result was attributed to a possible rise in cAMP levels of target neurons. Clearly, IBMX displays dual effects on morphine dependence which vary with the experimental conditions.

Our results establish that IBMX is an effective inhibitor of basal μ receptor phosphorylation. This previously unknown function of IBMX as a possible protein kinase inhibitor may account for some of its behavioral effects. IBMX and H7 represent two agents with drastically different pharmacological properties. Our finding that both inhibit basal μ receptor phosphorylation, prevent naloxone induced withdrawal jumping in morphine dependent mice, and partially reverse morphine tolerance, supports the hypothesis that basal μ receptor phosphorylation plays a role in narcotic tolerance and dependence. These compounds may therefore provide leads in the development of effective therapy of narcotic addiction.

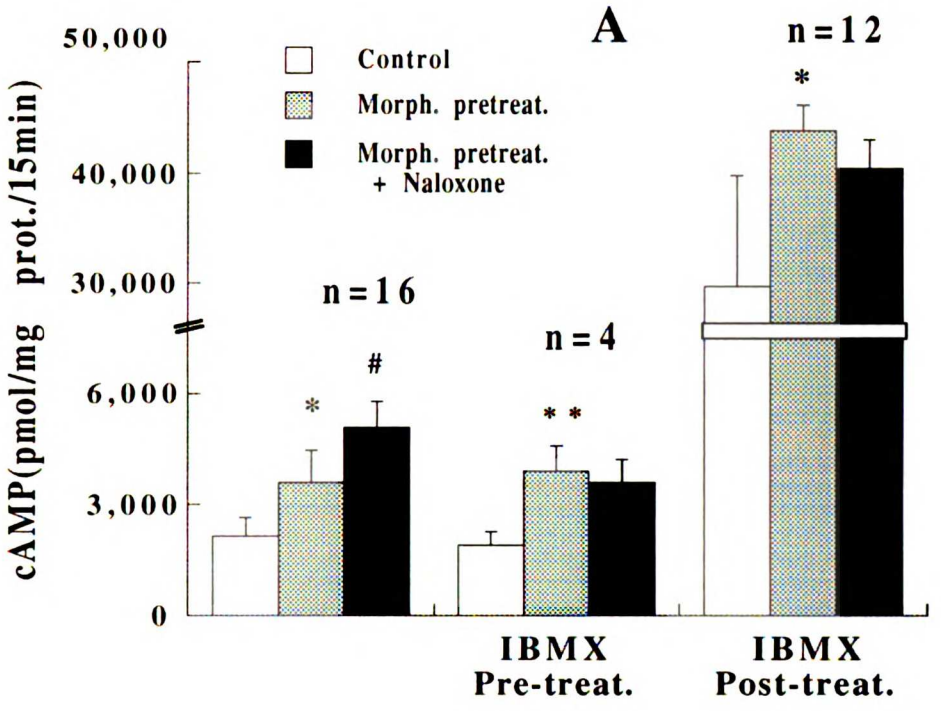


Figure 5.1A. Effect of IBMX on the spontaneous and the naloxone-induced cAMP overshoot. *Panel A.* SH-SY5Y cells were either untreated (control) or pretreated with 1 μM morphine for 12 hours, and thoroughly washed. Accumulation of cAMP, stimulated by 1 μM prostaglandin E1 (27), was then determined over 15 min in the absence or presence of 1 μM naloxone (spontaneous and naloxone-induced cAMP overshoot, respectively). IBMX (500 μM) was added either acutely to the cAMP accumulation medium (IBMX Post-treat.), or during the 12 hour pretreatment period (IBMX Pre-treat.). In the latter case, both morphine and IBMX were removed before the cAMP accumulation was initiated. * $P<0.05$; ** $P<0.01$

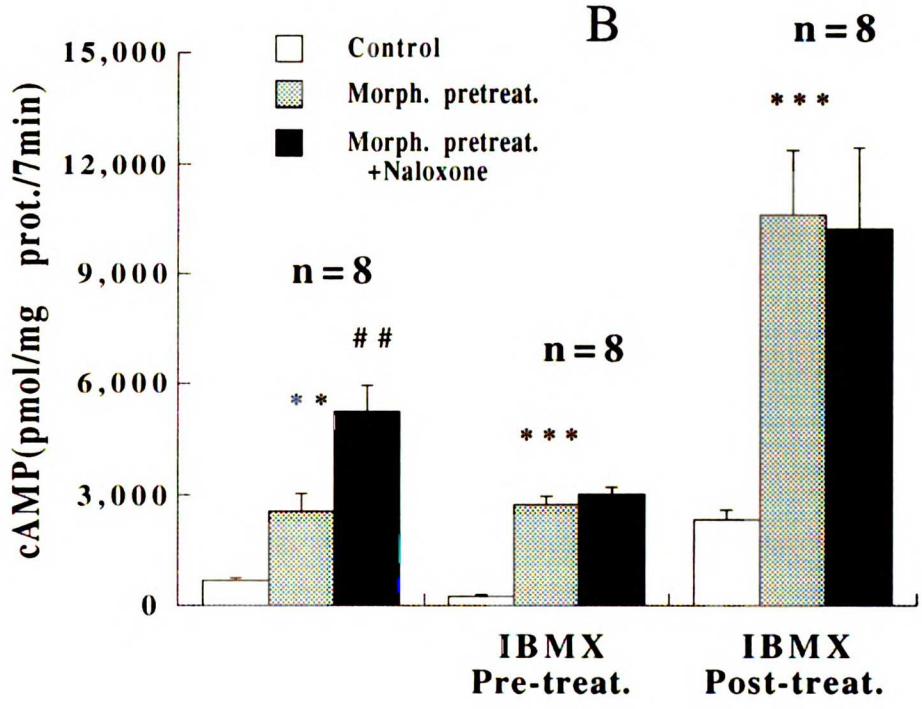


Figure 5.1B. Effect of IBMX on the spontaneous and the naloxone-induced cAMP overshoot. *Panel B.* IBMX effects in HEK-μ cells. The experiment was identical to that shown in Panel A, except that HEK-μ cells were used, and cAMP accumulation was determined over 7 min in the presence of 10 μM forskolin. * $P<0.05$; ** $P<0.01$

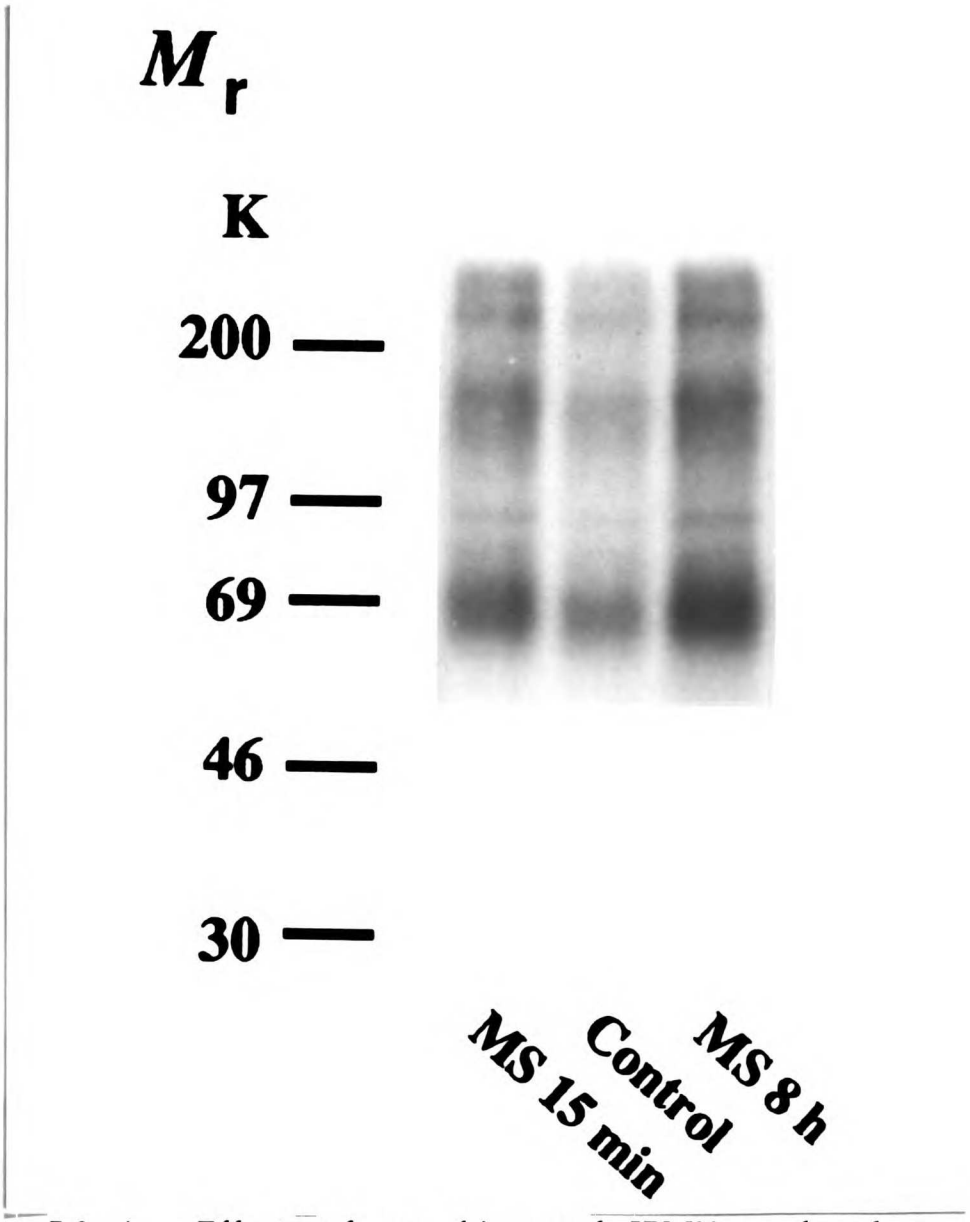


Figure 5.2 A. Effects of morphine and IBMX on basal μ receptor phosphorylation. Basal phosphorylation was measured in permeabilized HEK-EE-μ cells by labeling with γ-^{32}P-ATP for 15 min in the absence of any agonist (25). The labeling medium included Ca^{++} and Na^{+} ions under conditions optimizing receptor labeling by the kinase(s) responsible for basal μ receptor phosphorylation (25, 88). *Panel A.* Basal μ receptor phosphorylation (control) stimulated by 1 μM morphine added during the ^{32}P labeling period or the 8 hr preincubation period, followed by washing before labeling (see also (25, 88)).

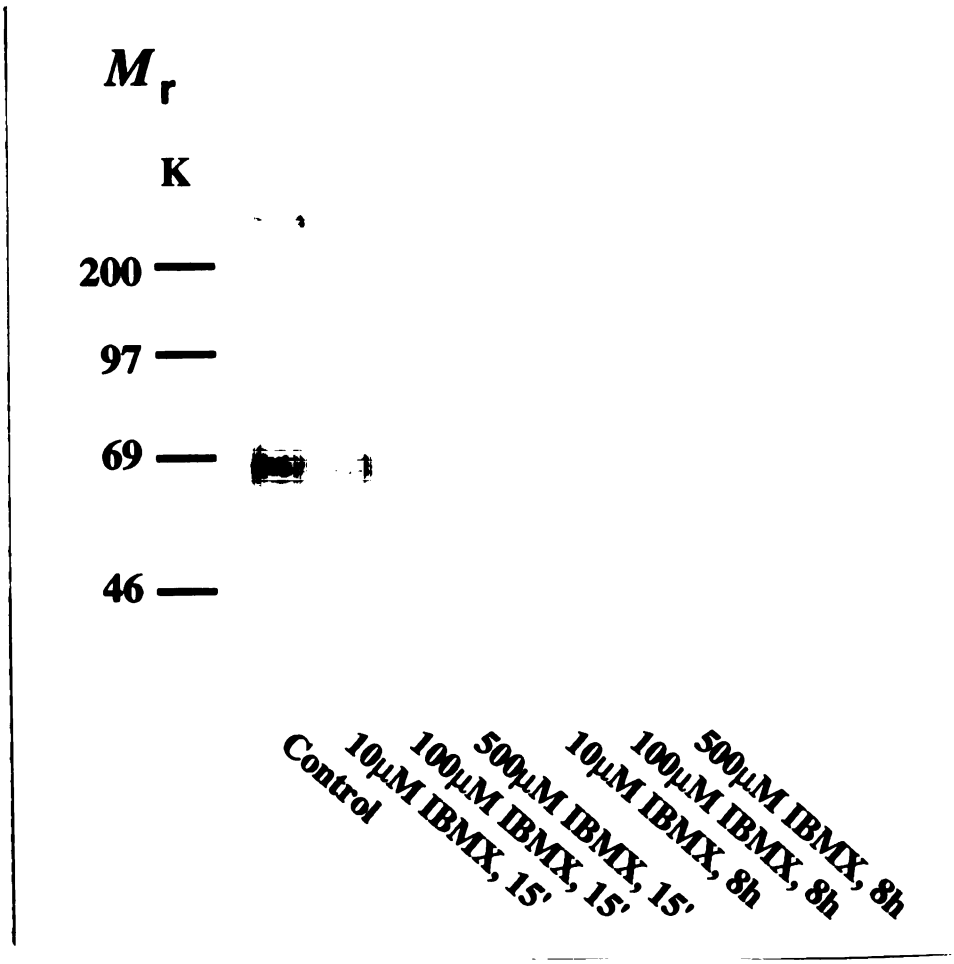


Figure 5.2B. Effects of morphine and IBMX on basal μ receptor phosphorylation. Basal phosphorylation was measured in permeabilized HEK-EE-μ cells by labeling with γ-^{32}P-ATP for 15 min in the absence of any agonist (25). The labeling medium included Ca^{++} and Na^{+} ions under conditions optimizing receptor labeling by the kinase(s) responsible for basal μ receptor phosphorylation (25, 88). *Panel B.* Effect of IBMX on basal μ opioid receptor phosphorylation. IBMX was added either during a 6 hour preincubation period together with 1 μM morphine sulfate, followed by washout before labeling, or during the 15 min labeling period.

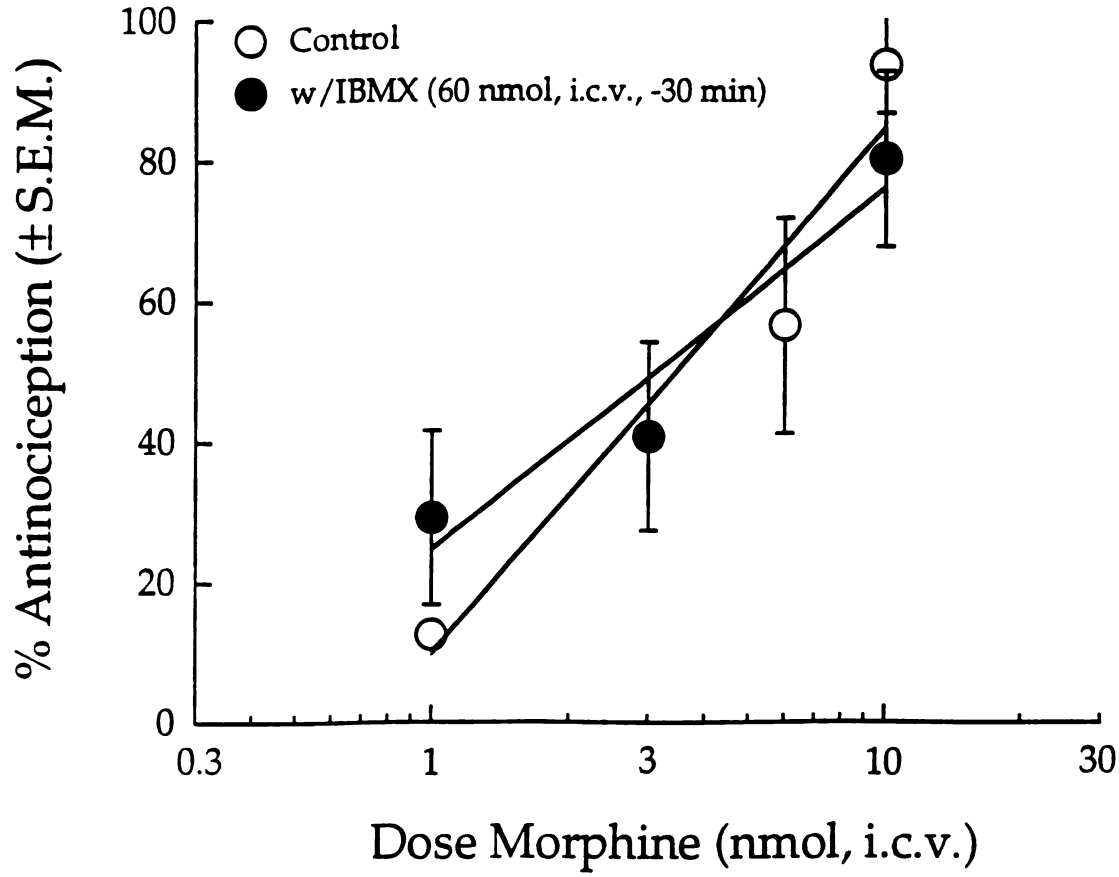


Figure 5.3 A. Effect of IBMX on morphine antinociception, antinociceptive tolerance, and dependence. *Panel A.* Effect of i.c.v. IBMX (100 nmoles) on antinociception by morphine sulfate given s.c., using the warm water (55° C) tail-flick assay (52, 88).

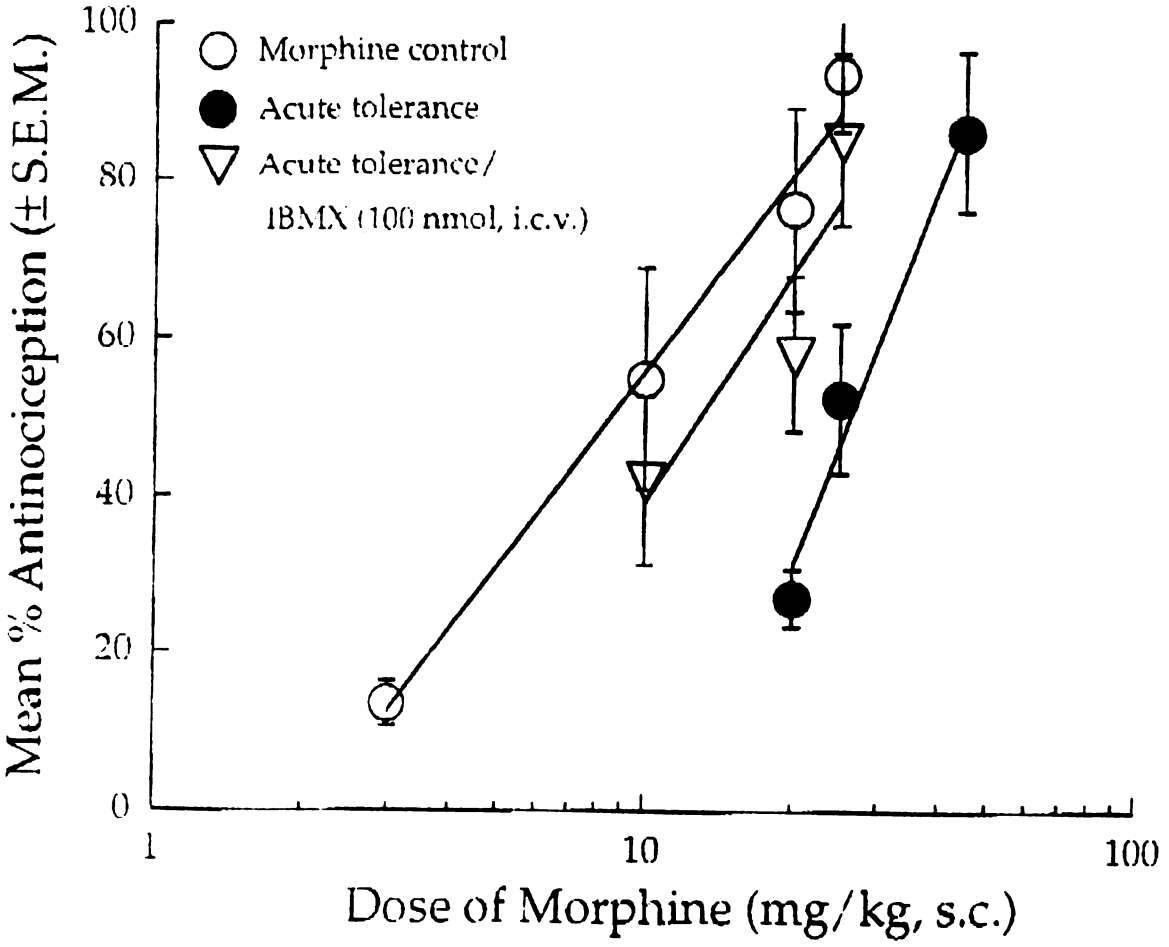


Figure 5.3 B. Effect of IBMX on morphine antinociception, antinociceptive tolerance, and dependence. *Panel B.* Effect of IBMX on antinociceptive tolerance to morphine. To induce tolerance, mice were injected s.c. with a single dose of 100 mg/kg morphine sulfate, and an s.c. morphine dose-response curve was established after 5 hours, using the warm water tail-flick assay. IBMX (100 nmole) was injected i.c.v. 30 minutes before determining the dose-response curve. The control group received a placebo dose in lieu of the first morphine dose.

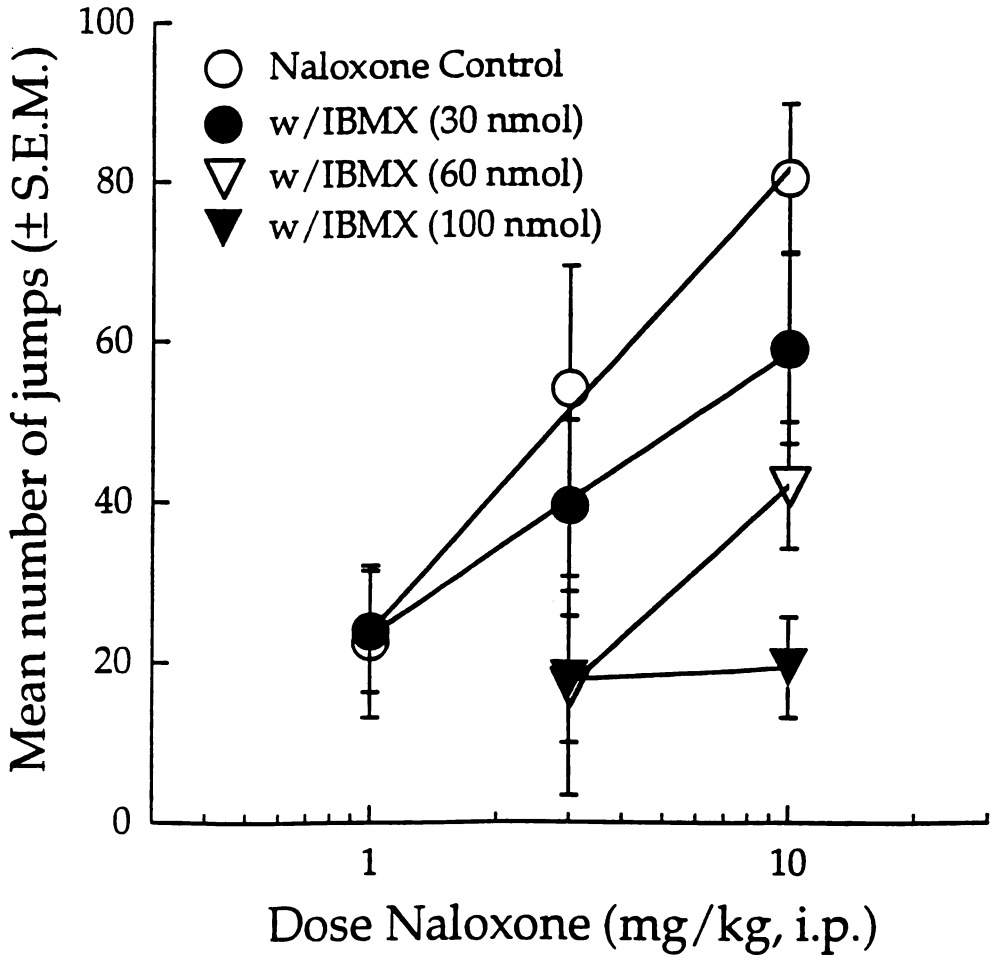


Figure 5.3 C. Effect of IBMX on morphine antinociception, antinociceptive tolerance, and dependence. *Panel C.* Effect of IBMX on naloxone-induced withdrawal jumping in morphine dependent mice. Mice were made dependent on morphine by a single s.c. injection of 100 mg/kg morphine sulfate. Separate groups of mice were used for each point. Doses of naloxone were injected i.p. 4 hours after the morphine dose, at which time naloxone induced jumping was at a maximum. IBMX was injected i.c.v. 30 min before the naloxone dose, as previously described for H7 (52, 88).

REFERENCES

1. Casy, A. F., & Parfitt, R. T. (1986). Opioid analgesics (1st ed.). New York: Plenum Press.

2. Archer, S. (1993). Chemistry of nonpeptide opioids. In A. Herz (Eds.), Opioids I (pp. 241-277). Berlin: Springer-Verlag.

3. Pert, C. b., & Snyder, S. H. (1973). Opiate receptor: demonstration in nervous tissue. *Science* 179, 1011-1014.

4. Simon, E. J., Hiller, J. M., & Edelman, I. (1973). Stereospecific binding of the potent narcotic analgesic [3H]etorphine to rat-brain homogenate. *Proc. Natl. Acad. Sci. USA* 70, 1947-1949.

5. Terenius, L. (1973). Stereospecific interaction between narcotic analgesics and a synaptic plasma membrane fraction of rat cerebral cortex. *Acta. Pharmacol. Toxicol.* 32, 317-320.

6. Hughes, J., Smith, T. W., Kosterlitz, H. W., Fothergil, L. A., Morgan, B. A., & Morris, H. R. (1975). Identification of two related peptides from the brain with potent opiate agonist activity. *Nature* 258, 577-579.

7. Blume, A. J. (1978). Interactions of ligands with opiate receptors of brain membranes: regulation by ions and nucleotides. *Pro. Natl. Acad. Sci. USA* 75, 1713-1717.

8. Childers, S. R., & Snyder, S. H. (1979). Guanine nucleotides differentiate agonist and antagonist interactions with opiate receptor. *Life Sci.* 23, 759-762.

9. Koski, G., & Klee, W. (1981). Opiate inhibit adenylate cyclase by stimulating GTP hydrolysis. *Proc. Natl. Acad. Sci. USA* 78(7), 4185-4189.

10. Yu, V., Richards, M., & Sadée, W. (1986). A Human neuroblastoma cells line expresses μ and δ Opioid receptor sites. *J. Biol. Chem.* 261, 1065-1070.

11. Chou, W. S., Ho, A. K. S., & loh, H. H. (1971). Effect of acute and chronic morphine and nrepinephrine on brain adenylate cyclase activity. *Proc. Wet Pharmacol. Sco.* 14, 42-46.

12. Sharma, S. K., Nirenberg, M., & Klee, W. A. (1975). Morphine receptors as regulators of adenylate cyclase activity. *Proc. Natl. Acad. Sci. USA* 72(2), 590-594.

13. William, J. T., Egan, T. M., & North, D. (1982). Enkephalin opens potassium channel on mammalian cnetral neurons. *Nature* 229, 74-77.

14. North, R., & Williams, J. T. (1983). Opiate activation of potassium conductance inhibitory calcium action potentials in rat locus coeruleus neuron. *Brit. J. Pharmacol.* 80, 225-228.

15. Seward, E., Hammond, C., & Henderson, G. (1991). μ-opioid-receptor-mediated inhibition of the N-type calcium-channel current. *Proc. R. Soc. Lond.* 244, 129-135.

16. Smart, D., Smith, G., & G., L. D. (1994). Mu-Opioid receptor stimulation of inositol (1,4,5)trisphosphate formation via a pertussis toxin-sensitive G protein. *J. Neurochem.* 62(3), 1009-14.

17. Law, P. Y., & Loh, H. H. (1993). δ-opioid receptor activates cAMP phosphodiesterase activities in neuroblastoma x glioma NG108-15 hybrid cells. *Mol. Pharmacol.* 43, 684-693.

18. Evans, C. J., Keith Jr, d. E., Morrison, H., Magendzo, K., & Edwards, R. H. (1992). Cloning of a delta opioid receptor by functional expression. *Science* 258, 1952-1955.

19. Kieffer, B. L., Befort, K., Gaveriaux-Ruff, C., & Hirth, C. G. (1992). The delta-opioid receptor: isolation of a cDNA by expression cloning and pahrmacological characterization. *Proc. Natl. Acad. Sci. USA* 89, 12048-12052.

20. Chen, Y., Mestek, A., Liu, J., Hurley, J. A., & Yu, L. (1993). Molecular cloning and functional expression of a μ-opioid receptor from rat brain. *Mol. Pharmacol.* 44, 8-12.

21. Wang, J.-B., Imai, Y., Eppler, C. M., Spivak, C. E., & Uhl, G. R. (1993). μ opiate receptor: cDNA cloning and expression. *Proc. Nat. Acad. Sci. USA* 90, 10230-10234.

22. Li, S., Zhu, J., Chen, C., Chen, Y. W., Deriel, J. K. A., B., & Liu-Chem, L. Y. (1993). molecular cloning and expression of a rat kappa opioid receptor. *Biochem. J.* 295(3), 629-633.

23. Meng, F., Xie, G. X., Thompson, R. C., Mansour, A., Goldstein, A., Watson, S. J., & Akil, H. (1993). Cloning and pharmacological characterization of a rat kappa opioid receptor. *Proc. Natl. Acad. Sci. USA* 90(21), 9954-9958.

24. Raynor, K., kong, H., Chen, Y., Yasuda, K., Yu, L., Bell, G. I., & Reisine, T. (1994). Pharmacological characterization of the cloned k-, d-, and μ-opioid receptors. *Mol. Pharmacol.* 45, 330-334.

25. Arden, J. R., Segredo, V., Wang, Z., Lameh, J., & Sadée, W. (1995). Phosphorylation and agonist specific intracellular trafficking of an epitope-tagged μ-opioid receptor expressed in HEK 293 cells. *J. Neurochem.* 65, 1636-1645.

26. Chakrabarti, S., Law, P., & Loh, H. H. (1995). Neuroblatoma Neuro2A cells stably expressing a cloned μ-opioid receptor: a specific cellular model to study acute and chronic effects of morphine. *Mol. Brain Res.* 30, 269-278.

27. Wang, Z., Arden, J. R., Segredo, V., & Sadée, W. (1994). Agonist induced constitutive activation of the opioid receptor by phosphorylation. *Regul. Pept.* 54(1), 323-324.

28. Way, E. L., Loh, H. H., & Shen, F. (1969). Simultaneous quantitative assessment of morphine tolerance and physical dependence. *J. Pharmacol. Exp. Ther.* 167(1), 1-8.

29. Collier, H. O. J., & Francis, D. L. (1975). Morphine abstience is associated with increased brain cyclic AMP. *Nature* 253, 159-162.

30. Collier, H. O. J., Francis, D. L., & Schneider, C. (1972). Modification of morphine withdrawal by drugs interacting with humoral mechanisms: some contradictions and their interpretation. *Nature* 237, 220-223.

31. Law, P. Y., Hom, D. S., & Loh, H. H. (1983). Opiate receptor down-regulation and desensitization in neuroblastoma x glioma NG108-15 hybrid

cells are two separate cellular adaptation processes. *Mol. Pharmacol.* 24, 413-424.

32. Wang, Z., & Sadée, W. (1993). Constitutive μ Opioid Receptor Activity in Human Neroblastoma SK-N-SH Cells. *Regul. Pept.* S1(283-284).

33. Yu, V., & Sadée, W. (1988). Efficacy and tolerance of narcotic analgesics at the mu opioid receptor in diferentiated human neuroblastoma cells. *J. Pharmacol. Exp. Ther.* 245, 350-355.

34. Puttfarcken, P. S., Werling, L. L., & Cox, B. M. (1988). Effects of chronic morphine exposure on opioid inhibition of adenylyl cyclase in 7315c cell membrane: a useful model for the study of tolerance at μ receptors. *Mol. Pharmacol.* 33, 520-527.

35. Yu, V., Eiger, S., Duan, D. S., Lameh, J., & Sadée, W. (1990). Regulation of cyclic AMP by the mu-opioid receptor in human neuroblastoma SH-SY5Y cells. *J. Neurochem.* 55, 1390-1396.

36. Traynor, J. R., & Nahorski, S. R. (1995). Modulation bu μ-opioid agonists of guanosine-5'-O-(3-[35S]thio)triphosphate binding to membranes from human neuroblastoma SH-SY5Y cells. *Mol. Pharmacol.* 47, 848-854.

37. Sharma, S. K., Klee, W. A., & Nirenberg, M. (1975). Dual regulation of adenylate cyclase accounts for narcotic dependence and tolerance. *Proc. Natl. Acad. Sci. USA* 72(8), 3092-3096.

38. Collier, H. O. J. (1980). Cellular site of opiate dependence. *Nature* 283(625-629).

39. Rothman, R. B. (1990). High affinity dopamine reuptake inhibitors as potential coaine antagonists: a strategy for drug development. *Life Sci.* 46, PL17-21.

40. Nestler, E. (1992). Molecular Mechanism of Drug Addiction. *J. Neurosci.* 12, 2439-2450.

41. Self, D. W., & Nestler, E. J. (1994). Molecular mechanisms of drug reinforcement and addiction. *Annu. Rev. Neurosci.* 18, 463-495.

42. Lenoir, D., Barg, J., & Simantov, R. (1983). Down-regulation of opiate receptors in serum-free cultures of aggregating fetal brain cells. *Life Sci.* 33(suppl 1), 337-340.

43. Vachon, L., Costa, T., & Herz, A. (1987). Opioid receptor desensitization in NG 108-15 cells: Differential effects of a full and a partial agonist on the opioid-dependent GTPase. *Biochem. Pharmacol.* 36(18), 2889-2897.

44. Vachon, L., Costa, T., & Herz, A. (1987). GTPase and adenylate cyclase desensitize at different rates in NG108-15 cells. *Mol. Pharmacol.* 31, 159-168.

45. Loh, H. H., & Smith, A. P. (1990). Molecular characterization of opioid receptors. *Annu. Rev. Pharmacol. Toxicol.* 30, 123-147.

46. Cone, R. I., Lameh, J., & W., S. (1991). Rapid agonist-induced loss of ^{125}I-β-endorphin opioid receptor sites in NG108-15, but not SK-N-SH neuroblastoma cells. *Life Sci.* 49, 147-152.

47. Ammer, H., & Schulz, R. (1993). Coupling of prostaglandin E1 receptors to the stimulatory GTP-binding protein Gs is enhanced in neuroblastoma x

glioma (NG108-15) hybrid cells chronically exposed to an opioid. *Mol. Pharmacol.* 43, 556-563.

48. Vogel, Z., Attali, B. B., & Simantov, R. (1990). differential effect of μ, δ, and κ ligands on G protein α subunits in cultured brain cells. *J. Neurosci. Res.* 27, 106-111.

49. Harada, H., Ueda, H., Wada, Y., Katada, T., Ui, M., & Satoh, M. (1989). Phosphorylation of μ-opioid receptors-a putative mechanism of selective uncoupling of receptors-Gi interaction, measured with low K_m GTPase and nucleotide-sensitive agonist binding. *Neurosci. Lett.* 100, 221-226.

50. Wang, Z., Bilsky, E. J., Porreca, F., & Sadée, W. (1994). Contitutive μ opioid receptor activation as a regulatory mechanism underlying narcotic tolerance and dependence. *Life Sci.* 54(20), 339-350.

51. Wang, Z., Bilsky, E., Porreca, F., & Sadée, W. 3-Isobutyl-1-methylxanthine inhibits basal μ receptor phosphorylation and reverse morphine tolerance and dependence in mice. *(submitted).*

52. Bilsky, E. J., Bernstein, R. N., Wang, Z., Sadée, W., & Porreca, F. (1996). Effect of naloxone and CTAP and the protein kinase inhibitors H7 and H8 on acute morphine dependence and antinociceptive tolerance in mice. *J. Pharmacol. Exp. Ther..* 277(1) 484-490.

53. Sadée, W., Wang, Z., Arden, J. R., & Segredo, V. (1994). Constitutive activation of the μ-opioid receptor: a novel Paradigm of receptor regulation in narcotic analgesia, tolerance, and dependence. *Analgesia* 1(1), 11-14.

54. Nestler, E. J. (1993). Cellular responses to chronic treatment with drugs of abuse. *Crit. Rev. Neurobiol.* 7(1), 23-39.

55. Alreja, M., & Aghajanian, G. K. (1991). Pacemaker activity of locus coeruleus neurons: whole-cell recordings in brain slices show dependence on cAMP and protein kinase A. *Brain Res.* 556(2), 339-43.

56. Werling, L. L., McMahon, P. N., & Cox, B. M. (1989). Selective changes in mu opioid receptor properties induced by chronic morphine exposure. *Proc. Natl. Acad. Sci. U SA* 86(16), 6393-7.

57. Wong, C. S., Su, Y. F., Watkins, W. D., & Chang, K. J. (1992). Continuous intrathecal opioid treatment abolishes the regulatory effects of magnesium and guanine nucleotides on mu opioid receptor binding in rat spinal membranes. *J. Pharmacol. Exp. Ther.* 262(1), 317-26.

58. Parolaro, D., Patrini, G., Giagnoni, G., Massi, P., Groppetti, A., & Parenti, M. (1990). Pertussis toxin inhibits morphine analgesia and prevents opiate dependence. *Pharmacol. Biochem. Behav.* 35(1), 137-41.

59. Crain, S. M., & Shen, K. F. (1992). After chronic opioid exposure sensory neurons become supersensitive to the excitatory effects of opioid agonists and antagonists as occurs after acute elevation of GM1 ganglioside. *Brain Res.* 575(1), 13-24.

60. Crain, S. M., & Shen, K. F. (1992). After GM1 ganglioside treatment of sensory neurons naloxone paradoxically prolongs the action potential but still antagonizes opioid inhibition. *J. Pharmacol. Exp. Ther.* 260(1), 182-6.

61. Cruciani, R. A., Dvorkin, B., Morris, S. A., Crain, S. M., & Makman, M. H. (1993). Direct coupling of opioid receptors to both stimulatory and inhibitory guanine nucleotide-binding proteins in F-11 neuroblastoma-sensory neuron hybrid cells. *Proc. Natl. Acad. Sci. USA* 90(7), 3019-23.

62. Gintzler, A. R., & Xu, H. (1991). Different G proteins mediate the opioid inhibition or enhancement of evoked [5-methionine]enkephalin release. *Proc. Natl. Acad. Sci. USA* 88(11), 4741-5.

63. Grevert, P., & Goldstein, A. (1978). Endorphins: naloxone fails to alter experimental pain or mood in humans. *Science* 199(4333), 1093-5.

64. Zadina, J. E., Chang, S. L., Ge, L. J., & Kastin, A. J. (1993). Mu opiate receptor down-regulation by morphine and up-regulation by naloxone in SH-SY5Y human neuroblastoma cells. *J. Pharmacol. Exp. Ther.* 265(1), 254-62.

65. Grumbach, L., Shelofsky, M., & Boston, J. J. (1974). The kinetics of physical dependence to morphine in the rat. *Pharmacology* 11(1), 38-51.

66. Thomas, J. M., & Hoffman, B. B. (1989). Chronic somatostatin treatment induces enhanced forskolin-stimulated cAMP accumulation in wild-type S49 mouse lymphoma cells but not in protein kinase-deficient mutants. *Mol. Pharmacol.* 34, 116-124.

67. Hidaka, H., Inagaki, M., Kawamoto, S., & Sasaki, Y. (1984). Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochem.* 23, 5036-5041.

68. Federman, A. D., Conklin, B. R., Schrader, K. A., Reed, R. R., & Bourne, H. R. (1992). Hormonal stimulation of adenylyl cyclase through Gi-protein beta gamma subunits. *Nature* 356(6365), 159-61.

69. Herbert, J. M., Augereau, J. M., Gleye, J., & Maffrand, J. P. (1990). Chelerythrine is a potent and specific inhibitor of protein kinase C. *Biochem. Biophys. Res. Commun.* 172(3), 993-9.

70. Hui, K. K., & Yu, J. L. (1989). Effects of protein kinase inhibitor, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine, on beta-2 adrenergic receptor activation and desensitization in intact human lymphocytes. *J. Pharmacol. Exp. Ther.* 249(2), 492-8.

71. Toews, M. L., Liang, M., & Perkins, J. P. (1987). Agonists and phorbol esters desensitize beta-adrenergic receptors by different mechanisms. *Mol. Pharmacol.* 32(6), 737-42.

72. Cowan, A. (1976). Use of the mouse jumping test for estimating antagonistic potencies of morphine antagonists. *J. Pharm. Pharmacol.* 28(3), 177-82.

73. Pelton, J. T., Kazmierski, W., Gulya, K., Yamamura, H. I., & Hruby, V. J. (1986). Design and synthesis of conformationally constrained somatostatin analogues with high potency and specificity for mu opioid receptors. *J. Med. Chem.* 29(11), 2370-5.

74. Porreca, F., Mosberg, H. I., Hurst, R., Hruby, V. J., & Burks, T. F. (1984). Roles of mu, delta and kappa opioid receptors in spinal and supraspinal mediation of gastrointestinal transit effects and hot-plate analgesia in the mouse. *J. Pharmacol. Exp. Ther.* 230(2), 341-8.

75. Schutz, W., & Freissmuth, M. (1992). Reverse intrinsic activity of antagonists on G protein-coupled receptors. *Trends Pharmacol. Sci.* 13(10), 376-80.

76. Costa, T., & Herz, A. (1989). Antagonists with negative intrinsic activity at delta opioid receptors coupled to GTP-binding proteins. *Proc. Natl. Acad. Sci. USA* 86(19), 7321-7325.

77. Costa, T., Ogino, Y., Munson, P. J., Onaran, H. O., & Rodbard, D. (1992). Drug efficacy at guanine nucleotide-binding regulatory protein-linked receptors: thermodynamic interpretation of negative antagonism and of receptor activity in the absence of ligand. *Mol. Pharmacol.* 41(3), 549-60.

78. Lefkowitz, R. J. (1993). G-protein-coupled receptors. Turned on to ill effect [news; comment]. *Nature* 365(6447), 603-4.

79. Lefkowitz, R. J., Cotecchia, S., Samama, P., & Costa, T. (1993). Constitutive activity of receptors coupled to guanine nucleotide regulatory proteins. *Trends Pharmacol. Sci.* 14(8), 303-7.

80. Kutsuwada, T., Kashiwabuchi, N., Mori, H., Sakimura, K., Kushiya, E., Araki, K., Meguro, H., Masaki, H., Kumanishi, T., Arakawa, M., & et, a. l. (1992). Molecular diversity of the NMDA receptor channel [see comments]. *Nature* 358(6381), 36-41.

81. Jaffe, J. H., & Martin, W. R. (1990). In A. G. Gilman, T. W. Rall, A. S. Nies, & P. Taylor (Eds.), The Pharmacological Basis of Therapeutics (pp. 485-573). New York: Pergamon Press.

82. Hausdorff, W. P., Caron, M. G., & J., L. R. (1990). Turning off the signal: desensitization of β-adrenergic receptor function. *FASEB J.* 4, 2881-2889.

83. Pawson, T. (1995). Protein modules and signalling networks. *Nature* 373(6515), 573-80.

84. Coughlin, S. R. (1994). Expanding horizons for receptors coupled to G proteins: diversity and disease. *Curr. Opin. Cell. Biol.* 6(2), 191-7.

85. Pei, G., Tiberi, M., Caron, M. G., & Lefkowitz, R. J. (1994). An approach to the study of G-protein-coupled receptor kinases: an in vitro-purified membrane assay reveals differential receptor specificity and regulation by G beta gamma subunits. *Proc. Natl. Acad. Sci. USA* .91(9), 3633-6.

86. Gorman, C., Padamanbhan, R., & Howard, B. (1983). High efficiency DNA-mediated transformation of primate cells. *Science* 221, 551-553.

87. Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248-254.

88. Wang, Z., Arden, J., & Sadée, W. (1996). Basal phosphorylation of μ opioid receptor is agonist modulated and Ca++-dependent. *FEBES Lett.* 387, 53-57.

89. Raymond, J. R. (1990). Protein kinase C induces phosphorylation and desensitization of the human 5-HT_{1A} receptor. *J. Biol. Chem.* 266, 14747-14753.

90. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., & Rutter, W. J. (1979). Isolation of biologically active ribonucleic acid from source enriched in ribonuclease. *Biochemistry* 18, 5294-5299.

91. Nestler, E. J. (1994). Molecular neurobiology of drug addiction. *Neuropsychopharmacology* 11(2), 77-87.

92. Zadina, J. E., Harrison, L. M., Ge, L. J., Kastin, A. J., & Chang, S. L. (1994). Differential regulation of mu and delta opiate receptors by morphine, selective agonists and antagonists and differentiating agents in SH-SY5Y human neuroblastoma cells. *J. Pharmacol. Exp. Ther.* 270(3), 1086-96.

93. Caterina, M. J., Devreotes, P. N., Borleis, J., & Hereld, D. (1995). Agonist-induced loss of ligand binding is correlated with phosphorylation of cAR1, a G protein-coupled chemoattractant receptor from Dictyostelium. *J. Biol. Chem.* 270(15), 8667-72.

94. Toll, L. (1995). Intact cell binding and the relation to opioid activities in SH-SY5Y cells. *J. Pharmacol. Exp. Ther.* 273(2), 721-7.

95. Mullaney, I., Shah, B. H., Wise, A., & Milligan, G. (1995). Expression of the human beta 2-adrenoceptor in NCB20 cells results in agonist activation of adenylyl cyclase and agonist-mediated selective down-regulation of Gs alpha. *J. Neurochem.* 65(2), 545-53.

96. Sadée, W., & Wang, Z. (1995). Agonist induced constitutive receptor activation as anovel regulatory mechanism: Mu receptor regulation. *Advances Exp. Med. Biol.* 373, 85-90.

97. Wang, D. X., Lu, X. Q., & Qin, B. Y. (1995). Dihydroetorphine is a mu-receptor-selective ligand. *J. Pharm. Pharmacol.* 47(8), 669-673.

98. Pippig, S., Andexinger, S., & Lohse, M. J. (1995). Sequestration and recycling of beta 2-adrenergic receptors permit receptor resensitization. *Mol. Pharmacol.* 47(4), 666-676.

99. Giannini, E., & Boulay, F. (1995). Phosphorylation, dephosphorylation, and recycling of the C5a receptor in differentiated HL60 cells. *J. Immunol.* 154(8), 4055-4064.

100. Herz, A. (Ed.). (1993). Opioids I and II. Berlin: Springer-Verlag.

101. Shen, K. F., & Crain, S. M. (1994). Antagonists at excitatory opioid receptors on sensory neurons in culture increase potency and specificity of opiate analgesics and attenuate development of tolerance/dependence. *Brain Res.* 636, 286-297.

102. Wang, L., & Gintzler, A. R. (1994). Bimodal opioid regulation of cyclic AMP formation: implications for positive and negative coupling of opiate receptors to adenylyl cyclase. *J. Neurochem.* 63(5), 1726-30.

103. Wang, J. B., Pei, G., & Uhl, G. R. (1995). Phosphorylation of expressed human μ opiate receptors. *Abstr. Soc. Neurosci.* 21(2), 1357.

104. Patterson, T. A., Appleyard, S. M., & Chavkin, C. (1995). Opioid receptors are basely phosphorylated in guinea pig brain slices by PKC and PKA. *Abstracts, Soc. Neurosci.* 21(2), 1356.

105. Westphal, R. S., Backstrom, J. R., & Sanders, B. E. (1995). Increased basal phosphorylation of the constitutively active serotonin 2C receptor accompanies agonist-mediated desensitization. *Mol. Pharmacol.* 48(2), 200-5.

106. Lefkowitz, R. J. (1993). G protein-coupled receptor kinases. *Cell* 74(3), 409-12.

107. Haga, K., Kameyama, K., Haga, T., Kikkawa, U., Shiozaki, K., & Uchiyama, H. (1996). Phosphorylation of human m1 muscarinic acetylcholine receptors by G protein-coupled receptor kinase 2 and protein kinase C. *J. Biol. Chem.* 271(5), 2776-82.

108. Hanks, S. K., Quinn, A. M., & Hunter, T. (1988). The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science* 241(4861), 42-52.

109. Aloyo, V. J. (1995). Modulation of mu opioid binding by protein kinase inhibitors. *Biochem. Pharmacol.* 49(1), 17-21.

110. Shi, W., Osawa, S., Dickerson, C. D., & Weiss, E. R. (1995). Rhodopsin mutants discriminate sites important for the activation of rhodopsin kinase and Gt. *J. Biol. Chem.* 270(5), 2112-9.

111. Kameyama, K., Haga, K., Haga, T., Moro, O., & Sadee, W. (1994). Activation of a GTP-binding protein and a GTP-binding-protein-coupled receptor kinase (beta-adrenergic-receptor kinase-1) by a muscarinic receptor m2 mutant lacking phosphorylation sites. *Eur. J. Biochem.* 226(2), 267-76.

112. Akers, R. F., Lovinger, D. M., Colley, P. A., Linden, D. J., & Routtenberg, A. (1986). Translocation of protein kinase C activity may mediate hippocampal long-term potentiation. *Science* 231(4738), 587-9.

113. Klann, E., & Shelton, K. R. (1990). A lead-associated nuclear protein which increases in maturing brain and in differentiating neuroblastoma 2A

cells exposed to cyclic AMP-elevating agents. *Brain Res. Dev. Brain Res.* 57(1), 71-5.

114. Meyer, T., Hanson, P. I., Stryer, L., & Schulman, H. (1992). Calmodulin trapping by calcium-calmodulin-dependent protein kinase. *Science* 256(5060), 1199-202.

115. Harada, H., Ueda, H., Katada, T., Ui, M., & Satoh, M. (1990). Phosphorylated mu-opioid receptor purified from rat brains lacks functional coupling with Gi1, a GTP-binding protein in reconstituted lipid vesicles. *Neurosci. Lett.* 113(1), 47-49.

116. Seamon, K. B., Padgett, W., & Daly, J. W. (1981). Forskolin: unique diterpene activator of adenylate cyclase in membranes and in intact cells. *Proc. Natl. Acad. Sci. USA* 78(6), 3363-3367.

117. Bergsbaken, C. L., Sommers, S. L., & Law, P. Y. (1993). Effect of forskolin and isobutylmethylxanthine on delta-opioid receptor activity in neuroblastoma x glioma NG108-15 cells. *J. Pharmacol. Exp. Ther.* 264(3), 1474-83.

118. Glantz, S. A. (1987). Primer of Biostatistics (2nd ed.). MaGraw-Hill Book Co.

119. Chen, Y., & Yu, L. (1994). Differential regulation by cAMP-dependent protein kinase and protein kinase C of the mu opioid receptor coupling to a G protein-activated K+ channel. *J. Biol. Chem.* 269(11), 7839-42.

120. Way, E. L. (1993). Opioid tolerance and physical dependence and their relationship. In A. Herz (Eds.), Opioid (pp. 573-596). Berlin: Springer-Verlag.

121. Lameh, J., Eiger, S., & Sadée, W. (1992). Interaction among μ-opioid receptors and a2-adrenoceptors on SH-SY5Y human neuroblastoma cells. *Eur. J. Pharmacol.* 227, 19-24.

122. Narita, M., Feng, Y., Makimura, M., Hoskins, B., & Ho, I. K. (1994). A protein kinase inhibitor, H-7, inhibits the development of tolerance to opioid antinociception. *Eur. J. Pharmacol.* 271(2-3), 543-5.

123. Morgan, A. J., Murray, K. J., & Challiss, R. A. (1993). Comparison of the effect of isobutylmethylxanthine and phosphodiesterase-selective inhibitors on cAMP levels in SH-SY5Y neuroblastoma cells. *Biochem. Pharmacol.* 45(12), 2373-80.

124. Avidor, R. T., Bayewitch, M., Levy, R., Matus, L. N., Nevo, I., & Vogel, Z. (1995). Adenylylcyclase supersensitization in mu-opioid receptor-transfected Chinese hamster ovary cells following chronic opioid treatment. *J. Biol. Chem.* 270(50), 29732-8.

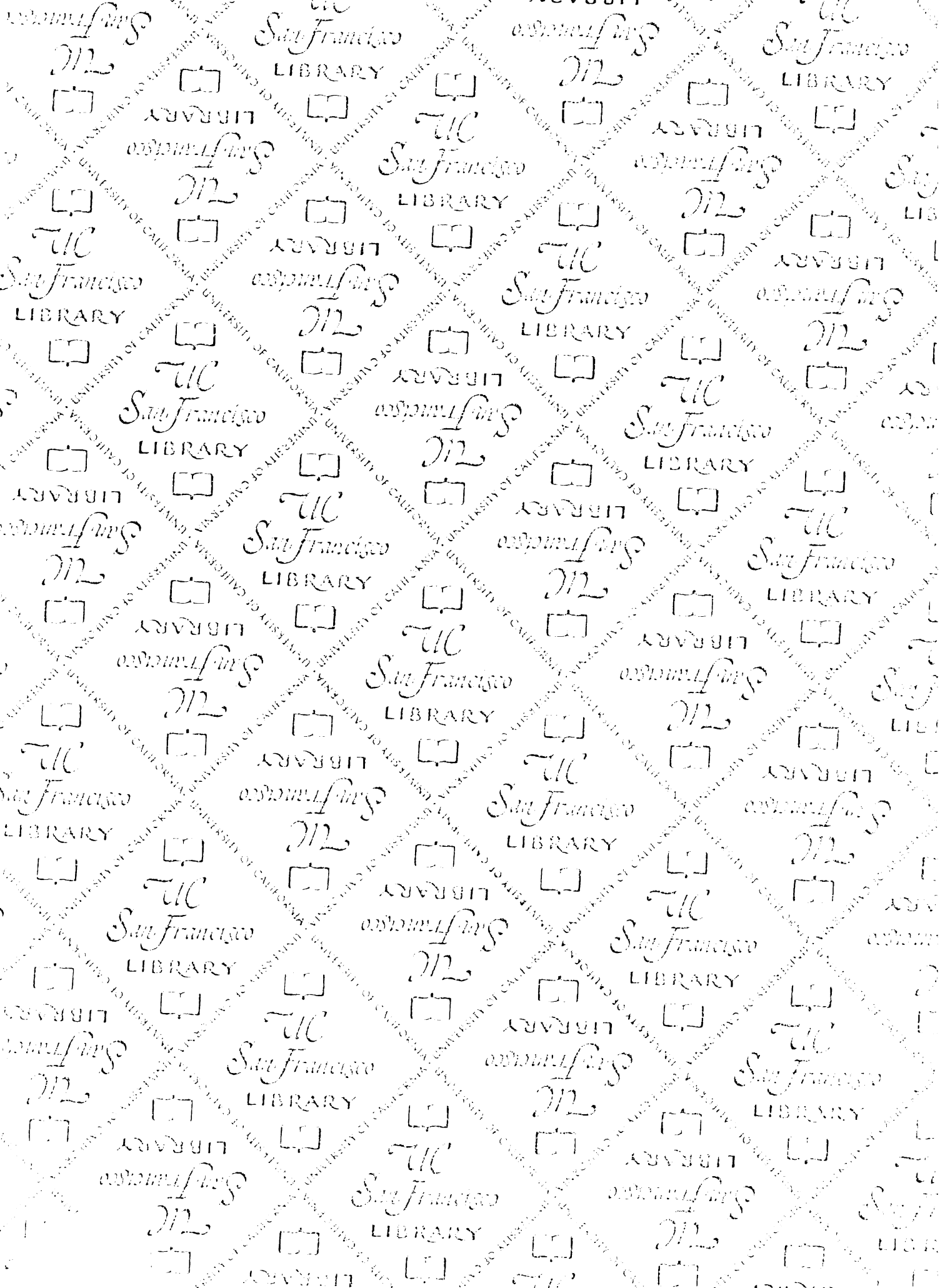
125. Parsons, W. J., Ramkumar, V., & Stiles, G. L. (1988). Isobutylmethylxanthine stimulates adenylate cyclase by blocking the inhibitory regulatory protein, Gi. *Mol. Pharmacol.* 34(1), 37-41.

126. Simasko, S. M., & Yan, S. (1993). 3-Isobutyl-1-methylxanthine inhibits sustained calcium current independently of cyclic AMP in neuronal and endocrine cells. *Mol. Pharmacol.* 44(3), 622-7.

127. Horrobin, D. F., Manku, M. S., Franks, D. J., & Hamet, P. (1977). Methyl xanthine phosphodiestrase inhibitors behave as prostaglandin antagonists in a perfused rat nmesenteric artery preparation. *Prostagland* 13, 33-40.

128. Holtzman, S. G. (1989). Phosphodiesterase inhibitors potentiate opiate-antagonist discrimination by morphine-dependent rats. *Pharmacol. Biochem. Behav.* 33(4), 875-9.

129. Nicholson, D., Reid, A., & Sawynok, J. (1991). Effects of forskolin and phosphodiesterase inhibitors on spinal antinociception by morphine. *Pharmacol. Biochem. Behav.* 38(4), 753-8.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