Basal phosphorylation of μ opioid receptor is agonist modulated and Ca^{2+}-dependent

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Received 4 March 1996; revised version received 11 April 1996

Abstract **The μ opioid receptor was shown to be phosphorylated at a basal rate in the absence of agonist, measured in permeabilized HEK293 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^{2+} dependent; however, several inhibitors of protein kinase C and Ca^{2+}-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.**

Key words: Mu opioid receptor; Receptor phosphorylation; Ca^{2+} dependent kinase; Narcotic dependence and tolerance

1. 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 HEK293 cells transfected with an epitope tagged μ opioid receptor (HEK-EE-μ) [1]. 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 [2,3], suggesting that this phenomenon is not limited to HEK293 cells. Similarly high basal levels of receptor phosphorylation were previously observed with a constitutively active β_2 receptor mutant [4], and with the serotonin 5-HT_{2C} wild-type receptor which displays basal signaling activity [5]. 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 [6]. Basal μ receptor phosphorylation, i.e. the rate in the absence of any agonist, was further enhanced in the presence of morphine [1], as observed upon agonist stimulation of the constitutively active β_2 receptor mutant [4].

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 [6]. This sensitization to naloxone was prevented by the general kinase inhibitor H7, but not its congener H8 [6]. 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 [6,7]. Compound H7, but not its congener H8, reversed morphine tolerance and dependence [6,7], in parallel to in vitro results in SH-SY5Y cells [6]. 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^{2+} dependent, modulated by morphine pretreatment, and suppressed by H7.

2. Materials and methods

2.1. 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, La Jolla, 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 [8], 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) [1].

2.2. Cell culture

The plasmid encoding the EE-μ receptor was transfected into HEK293 cells, and stably transfected clonal cell lines were established as described [1]. The cell line with the highest receptor expression initially contained 5×10^6 sites per cell for HEK-EE-μ [1]. As a result of gradual loss of μ receptor sites during culture, experiments were performed with cultures expressing $5–10\times10^5$ sites per cell.

2.3. Receptor binding and cAMP assays

Receptor expression was examined with 2 nM [^{3}H]diprenorphine in intact cell monolayers, and cAMP levels by radioimmunoassay as described [1].

2.4. Drug pretreatment protocol

Cells were pretreated with 1 μM morphine and/or the indicated inhibitors for 0–12 h. 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.

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Abbreviations: GRK, G protein coupled receptor kinase; PKA, protein kinase A; PKC, protein kinase C; HEK, human embryonic kidney cells; EE-μ, epitope tagged μ receptor; HEK-EE-μ, HEK293 cells stably transfected with EE-μ

PII S0014-5793(96)00467-X

2.5. Phosphorylation and immunoprecipitation of the epitope tagged μ receptor

Phosphorylation of EE-μ was determined in washed, permeabilized cells as described [1]. Briefly, digitonin permeabilized cells were labeled with 0.2 mCi [γ-^{32}P]ATP (200 μM, 0.5 ml) at 25°C for 15 min in the presence or absence of morphine or kinase inhibitors. The incubation medium consisted of either DMEM medium (containing Ca^{2+} 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 [1]. The 30 000 × *g* 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 [1]. The identity of the labeled EE-μ band was confirmed by Western blotting [1].

3. Results

3.1. 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 [1]. ^{32}P-labeling of the EE-μ receptor was obtained in DMEM medium which contains 2 mM Ca^{2+} and 150 mM Na^{+} [1].

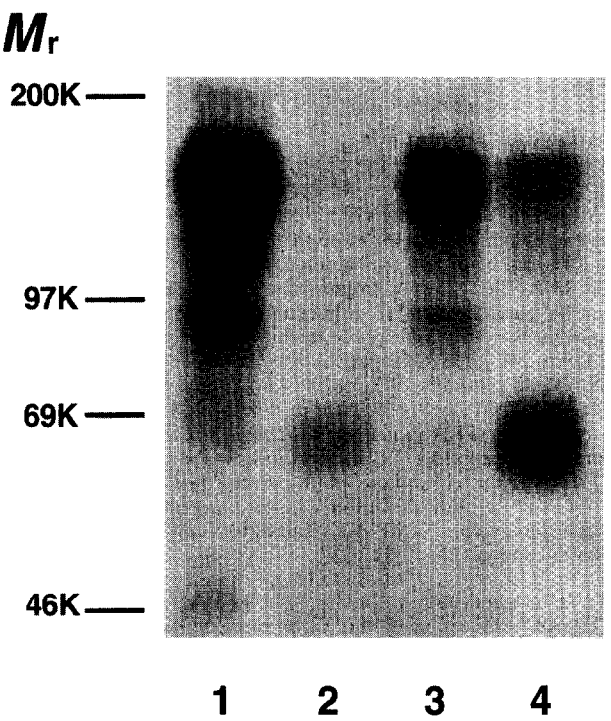

Fig. 1. Phosphorylation of the EE-μ receptor: ionic requirements. HEK-EE-μ cells were permeabilized, labeled with [γ-^{32}P]ATP in the presence of 10 μM morphine, immunoprecipitated, and separated on SDS-PAGE, as described [1]. The ^{32}P-labeling reaction was performed in 10 mM HEPES buffer, pH 7.1, 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^{2+} was confirmed in three additional experiments with varying ionic compositions, both in the presence and in the absence of morphine. Even though the labeling intensity of the 65 kDa band in lane 2 (Ca^{2+}) was lower than that in lane 4 (Ca^{2+} and Na^{+}), the presence of Na^{+} did not enhance labeling in other experiments.

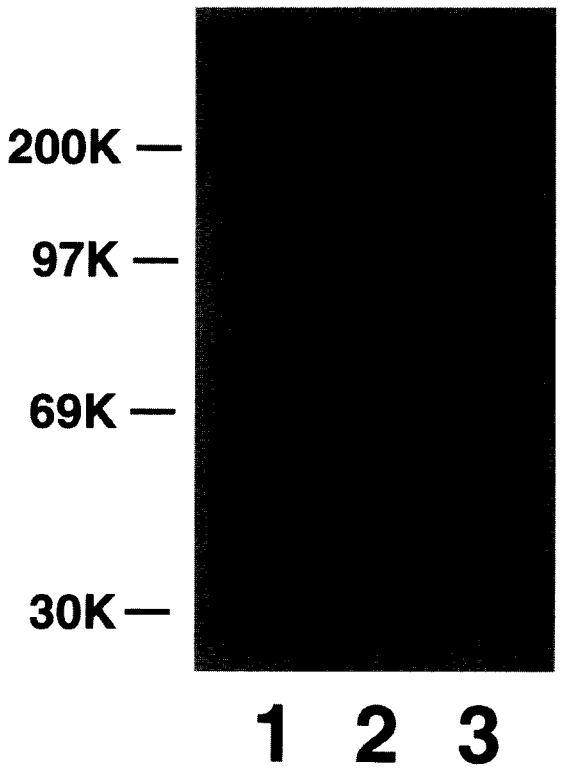

Fig. 2. Basal phosphorylation of the μ receptor: inhibitors H7 and H8. Permeabilized HEK-EE-μ cells were incubated for 15 min with a medium containing 150 mM Na^{+}, 5 μM Ca^{2+}, 200 μM [γ-^{32}P]ATP, and 100 μM H7 or H8. Lane 1: control cells; lane 2: H7; lane 3: H8.

To determine the ionic requirements for this reaction, we incubated the permeabilized cells in medium containing 150 mM K^{+}, 7 mM Mg^{2+}, and 5 mM EGTA to chelate Ca^{2+}, commonly used conditions to study intracellular kinases, including the GRKs [4,9,10]. 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. 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 [1]. The morphine enhanced phosphorylation activity at 65 kDa 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^{2+}) generally reduced detectable phosphorylation, as expected since Na^{+} inhibits many intracellular kinases, including the GRKs (Fig. 1, lane 3). In contrast, the addition of 2 mM Ca^{2+} stimulated μ receptor phosphorylation, yielding a clear band at 65 kb, regardless of the presence or absence of Na^{+} (Fig. 1, lanes 2 and 4). Therefore, to measure μ receptor phosphorylation, we included both Ca^{2+} and Na^{+} ions, the latter to suppress GRKs and extraneous kinase activities. To determine the Ca^{2+} concentration required for basal μ receptor phosphorylation, the reaction was carried out at 5, 50, 500, and 2000 μM Ca^{2+}. Maximal μ receptor phosphorylation

was already attained at 5 μM Ca^{2+}, the lowest Ca^{2+} concentration tested (data not shown).

The results shown in Fig. 1 were obtained in the presence of 10 μM morphine to enhance EE-μ labeling [1]. In the absence of morphine, basal μ receptor phosphorylation also depended upon Ca^{2+} 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.

3.2. *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. 2, lane 2). Compound H8 (100 μM) inhibited basal μ receptor phosphorylation only partially in permeabilized HEK-EE-μ cells ($n = 2$, Fig. 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^{2+} dependent kinases, including protein kinase C (PKC) and Ca^{2+}-calmodulin dependent (CaM) kinases [11]. A representative experiment is shown in Fig. 3. The chosen concentrations of these kinase inhibitors were ~20-fold higher than published IC_{50} values for the respective kinases, except for W7 which was tested at 200 μM, or only ~5-fold the IC_{50} for calmodulin, to avoid nonspecific effects (see legend, Fig. 3). None of these inhibitors strongly suppressed basal μ receptor phosphorylation (Fig. 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

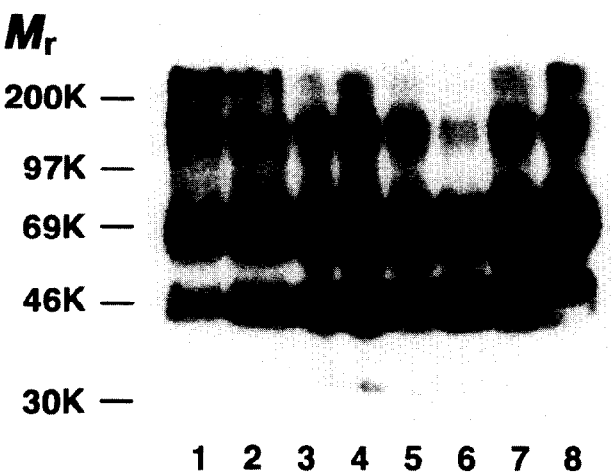

Fig. 3. Basal phosphorylation of the μ receptor: effect of protein kinase inhibitors. HEK-EE-μ cells were labeled as described for Fig. 1. The following inhibitors were added to the labeling medium (the kinases expected to be strongly inhibited are given in parentheses): lane 1: control without any inhibitor; 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).

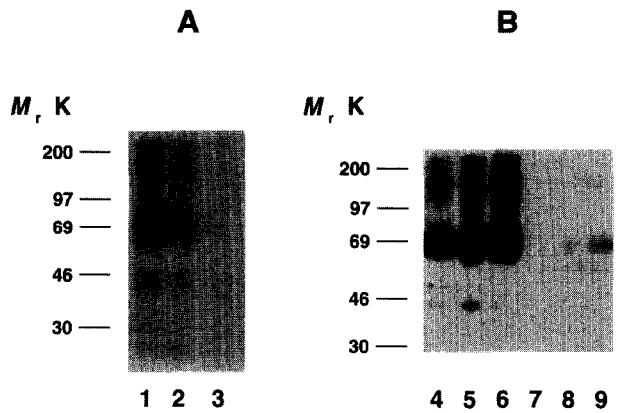

Fig. 4. Effects of morphine, H7, and H8 on μ receptor phosphorylation. HEK-EE-μ cells were labeled as described for Fig. 1. A: Acute treatment of permeabilized cells. Receptor labeling was determined over 15 min in the presence of 10 μM morphine (lane 1), no drug (lane 2), or 100 μM H7 (lane 3). B: Pretreatment of intact cells with morphine, H7 and H8, followed by removal of the drug. Pretreatment of the intact cells was performed for 7 h with no drug (lane 4), 1 μM morphine (lane 5), 1 μM morphine plus 100 μM H8 (lane 6), 1 μM morphine 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 4× washing before permeabilizing the cells. Thus, only the experiment shown in lane 9 contained morphine during ^{32}P-labeling. Each condition in A and B was repeated at least twice, with similar results.

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 IC_{50} concentration for calmodulin inhibition (~40 μM). It should be noted that these experiments were designed for semiquantitative analysis suitable 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 section 2.1).

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

Fig. 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. 4A) (see also Fig. 2, lane 2 and ref. [1]). 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 morphine for 7 h significantly increased subsequent μ receptor phosphorylation nearly 3-fold (Fig. 4B, lane 5, 2.6 ± 0.4-fold, $n = 5$), even

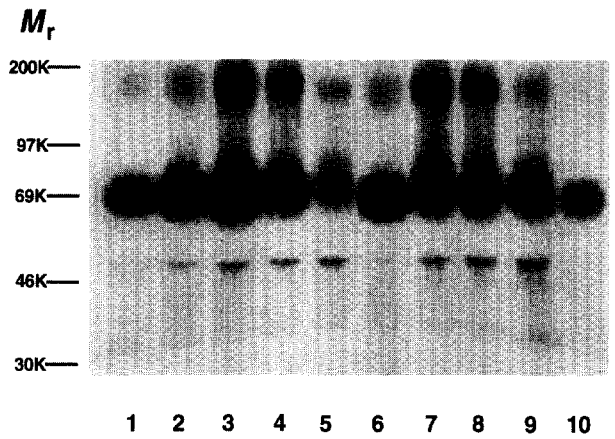

Fig. 5. Dose-response curve and time course of the effects of morphine pretreatment on μ receptor phosphorylation. HEK-EE-μ cells were labeled as described for Fig. 1. For the dose-response experiment, cells were preincubated for 12 h with 0, 10, 100, 1000 nM morphine (lanes 1–4, respectively), followed 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 h (lanes 8–10).

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

Preincubation of intact HEK-EE-μ cells with 100 μM H7 alone for 7 h, 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. 4B, lane 8). The same strong inhibition was seen with a 100 μM H7 preincubation for 2 h. 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. 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. 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).

3.4. 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 [12], 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.

4. 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 [1]. 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 preincubation with a suitable kinase inhibitor (H7).

Basal μ receptor phosphorylation was suppressed by addition of the Ca^{2+} chelator EGTA, and the addition of Ca^{2+} restored the phosphorylating activity. Under these ionic conditions, including Ca^{2+} and Na^+, any role of a known GRK in μ receptor phosphorylation can be excluded, because GRKs are independent of Ca^{2+} and inhibited by Na^+ [9,10]. Moreover, the Ca^{2+} 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^{2+}-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. 2). However, when intact cells were pretreated with H7 and H8, only H7 fully suppressed basal μ receptor phosphorylation, and H8 was inactive (Fig. 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 generally 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 [6].

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 [6]. Following morphine pretreatment, naloxone was shown to cause an increase in cAMP levels even though morphine had been completely removed after 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 [6]. Similarly, inhibitor H7, but not H8

reversed morphine tolerance and naloxone induced withdrawal jumping in mice [6,7]. 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. 1, lane 1), which could have been mediated by a GRK activity. Further, stimulation of PKC with phorbol esters leads to μ receptor phosphorylation [2]. 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. 4). Conversely, morphine enhanced the basal rate which persisted even after agonist removal (Fig. 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. 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 [13,14]. 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 [15]. 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 [16,17]. Similarly, stimulation of Ca^{2+}-calmodulin dependent kinase II results in auto-phosphorylation and enhanced activation [18]. 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.

Acknowledgements: This work was supported by Grant DA04166 from the National Institute on Drug Abuse, Rockville, MD. Z.W. was supported by an NIH predoctoral training grant, GM07175.

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