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## Research Article

# Regulation of the extracellular signal-regulated kinases following acute and chronic opioid treatment

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**Abstract.** The adaptations in extracellular signal-regulated kinase (ERK) pathway activity result in alterations in transcription of several genes that can be essential for development of both opioid tolerance and dependence. In this study, we investigated the effect of acute and prolonged opioid treatment on ERK pathway activity in SH-SY5Y cells. Acute administration of morphine and DAMGO stimulated ERK activity and this stimulation required activation of $Ca^{2+}$/calmodulin-dependent kinase II (CaMKII) and protein kinase C (PKC). In contrast, prolonged morphine treatment decreased the level of phosphorylated ERK. The precipitation of withdrawal further decreased the ERK1/2 activity. The principal finding of these studies is demonstration that the activation of CaMKII and PKC is required for ERK stimulation following acute opioid treatment while in a chronic morphine treatment and withdrawal, the up-regulation of PKC and CaMKII pathways seems to be engaged in the ERK inhibition. These results provide evidence that both opioid administration and opioid withdrawal, affecting similar intracellular pathways, can exert different effects on ERK activity.

**Key words.** Opioids; ERK; SH-SY5Y; protein kinase.

The mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway is a signaling cascade, which plays a crucial role in several cellular regulatory processes [1, 2]. The MAPK/ERK pathway can also integrate second-messenger systems, such as calcium, protein kinase A (PKA), and diacylglycerol (DAG) in activity-dependent regulation of neuronal function [3]. ERKs 1 and 2 (ERK1/2) are the most abundant ERK kinases in neurons [4]. The role of the ERK pathway in neuronal differentiation and survival is well established [5, 6] but several lines of evidence have recently pointed to an involvement of ERK1/2 signaling in the rewarding properties of drugs of abuse [7, 8] and adaptative responses of the central nervous system (CNS). The functional relevance of ERK1/2 to reinforcing properties of morphine has been shown recently by demonstrating the hypersensitivity of ERK1 mutant mice to the rewarding properties of morphine [2]. ERK1/2 catalytic activity is regulated by dual phosphorylation on specific tyrosine and threonine residues which causes their activation. In rat/mouse brain, chronic morphine treatment has been shown to regulate ERK1/2 catalytic activity in a region-specific manner. The chronic, but not acute, administration of morphine has been shown to increase [8–10], to decrease [11, 12] or not to influence ERK catalytic activity [8]. After chronic morphine, a decrease in ERK1/2 phosphorylation level was observed in the nucleus accumbens [11] and also in some other structures of the rat brain [12]. In contrast, in the ventral tegmental area (VTA) – a brain region involved in the rewarding properties of morphine,

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chronic administration of morphine was reported to increase ERK catalytic activity [13]. In the pons/medulla also, the p-ERK immunoreactivity increased remarkably after 7 days of repeated morphine injections [8]. Chronic morphine has also been shown to increase the ERK1 and ERK2 levels in the rats locus ceruleus and caudate/putamen [14].

The MAPK/ERK pathway is among numerous signal transduction pathways that could alter gene expression in distinct brain regions in response to repeated opioid exposure. These drug-induced changes in gene expression are considered to be the main reason for long-lasting alterations in the brain neuronal plasticity responsible for the state of addiction manifested as complex behavioral abnormalities. Phosphorylation of transcription factors via MAPKs kinases leading to changes in target gene expression has been postulated to be involved in the development of opioid dependence. A long, and ever-growing over the past few years list of multiple transcription factors phosphorylated by ERKs indicates the complexity of this regulation of gene expression [for a review see ref. 15].

At the cellular level, ERK can phosphorylate not only nuclear proteins including transcription factors but also some cytosolic proteins. Studies with HEK cells transfected to express the δ opioid receptor showed that activated ERK could phosphorylate cytosolic proteins participating in the process of receptor internalization thereby preventing this process. Moreover, blockade of morphine-mediated ERK activation enabled opiates to strongly induce δ opioid receptor internalization [16]. On the other hand, experiments with CHO cells tranfected to express μ opioid receptor revealed that MAPK activity contributed to the receptor internalization and that blocking of ERK phosphorylation suppressed the receptor desensitization caused by DAMGO exposure [17]. The activation of the ERK cascade in response to opioids has been suggested to trigger initial events leading to phosphorylation and desensitization of the μ opioid receptor [18]. However, μ and δ opioid receptor internalization is not an obligatory requirement for ERK activation [19]. At any rate, ERKs have been shown to be involved in the processes of receptor desensitization (uncoupling of the receptor from its effector) followed by their internalization. Both processes leading to a decrease in functional receptor number on the cell membrane have been suggested to be one of the mechanisms underlying the phenomenon of opioid tolerance.

Although the acute activation of ERK in cultured cells has been reported, we still know relatively little about ERK activity following chronic opioid treatment. This raises the question how chronic morphine action can affect ERK phosphorylation at the cellular level and which molecular mechanisms are involved in the regulation of ERK activity. In the present study, the effects of both acutely administered opioids and of chronic morphine treatment and withdrawal on cellular ERK1/2 phosphorylation were examined in the human neuroblastoma SH-SY5Y cell line. These cells were chosen because they express endogenous μ opioid receptor and develop cellular tolerance and dependence to morphine after prolonged treatment [20]. In the present study, we show that acute activation of μ opioid receptors rapidly induced ERK1/2 phosphorylation, whereas prolonged stimulation was associated with a decrease in ERK1/2 phosphorylation. Moreover, precipitation of withdrawal with an opioid receptor antagonist profoundly augmented the existing decrease in ERK1/2 phosphorylation. Protein kinase C (PKC) and $Ca^{2+}$/calmodulin-dependent kinase II (CaMKII) are responsible for the induction of ERK1/2 phosphorylation following acute opioid exposure but, on the other hand, they seem to be involved in the inhibition of ERK1/2 phorphorylation during prolonged morphine treatment and withdrawal.

## Materials and methods

**Materials.** Morphine sulfate was purchased from POCh Gliwice; Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum were purchased from GIBCO Laboratories; bisindolylmaleimide I, a highly selective cell-permeable PKC inhibitor was purchased from Calbiochem; autocamtide-2 inhibitor peptide, myristoylated, which acts as a highly specific and potent, cell-permeable inhibitor of CaMKII, was purchased from Merck; [D-Ala2, MePhe4, Gly5-ol]enkephalin (DAMGO) and naloxone were obtained from RBI. All other reagents were purchased from Sigma.

**Cell culture.** SH-SY5Y cells were maintained at 37 °C in a humidified $CO_2$ incubator in DMEM containing 10% fetal calf serum and antibiotics. Aproximately 10 h before experiments, complete medium was substituted by serum-reduced DMEM medium containing 0.5% fetal calf serum. Cells remained in the serum-reduced medium until the end of the experiments to avoid activation of ERKs/MAPKs kinases by growth factors. Experiments were carried out after an adaptation period, and reagents were added directly to the medium.

**Western blotting.** Cells were immediately lysed in a warm 2% sodium dodecylsulfate (SDS)-sample buffer. Protein concentration was determined using the BCA Protein Assay Kit (Sigma-Aldrich). Aliquots of crude extracts (containing 5–20 μg of protein) were then subjected to electrophoresis on a 12% SDS-polyacrylamide gel, and proteins were electroblotted onto microporous polyvinylidene difluoride (PVDF) membranes (Boehringer-Mannheim). The membranes

were blocked for 1 h, washed, and incubated overnight with primary antibodies at 4 °C. After washing steps, immunocomplexes were detected using a Chemiluminescence Western Blotting Kit (mouse/rabbit; Boehringer-Mannheim). For immunoblotting, mouse monoclonal antibodies raised against a peptide corresponding to amino acids 196–209 of ERK1/2 of human origin phosphorylated at Tyr-204 (a modification that reflects ERK1/2 activation [21]) were used (Santa Cruz). For the control of total unphosphorylated ERK1/2 expression, rabbit polyclonal antibodies were used (Santa Cruz). The relative protein level in each lane was controlled after transfer by staining the polyacrylamide gels with Coomassie Brilliant Blue G250. To control transfer quality, each blot (membrane) was stained with Ponceau S. Levels of immunoreactivity were visualized and quantified with a Fujifilm LAS-1000 fluoroimager system and Fujifilm software (Image Gauge).

## Results

**ERK1/2 phosphorylation following acute opioid treatment.** Immunoblots of extracts from SH-SY5Y cells demonstrated that the anti-phosphorylated ERK (p-ERK) antibody reacted with two bands of molecular weight 42 and 44 kDa. Incubation with a selective agonist of the μopioid receptor, DAMGO (100 nM–10 μM), or morphine (100 nM–10 μM) for 5 min caused a significant and dose-dependent stimulation of ERK1/2 phosphorylation (fig. 1A, B). Both opioids, morphine and DAMGO, elicited a rapid but transient increase in phosphorylated ERK1/2 levels, which peaked at 5 min (5–10 min in the case of DAMGO) after receptor activation, and decreased to the control levels after 30 min of incubation (fig. 2A, B). Morphine (1 μM) triggered ERK1/2 phosphorylation (5 min, approx. two-fold), albeit to a lesser extent than DAMGO (fig. 1). The different efficacy of DAMGO and morphine in ERK activation is probably caused by different affinity for these ligands. The effect of both opioids was reversed by the opioid receptor antagonist naloxone (fig. 2A, B). Treatment with 3 μM naloxone alone did not produce any significant effect on ERK1/2 phosphorylation (fig. 2B).

**ERK1/2 phosphorylation during prolonged morphine treatment and withdrawal.** In contrast to acute exposure, prolonged (up to 3 days) treatment with morphine significantly decreased ERK1/2 phosphorylation below the levels seen in control cells. Administration of 3 μM naloxone to SH-SY5Y cells exposed to morphine for 72 h profoundly inhibited phosphorylation of ERK1/2 (fig. 3). At 5 min after addition of the opioid receptor antagonist, the ERK1/2 phosphorylation was 2.5-fold when compared with the level seen after prolonged morphine treatment and about 4-fold in comparison with the control untreated cells. The maximal decrease in ERK1/2 phosphorylation level occurred 5 min after naloxone and then, with time, it returned to the level noted after chronic morphine administration.

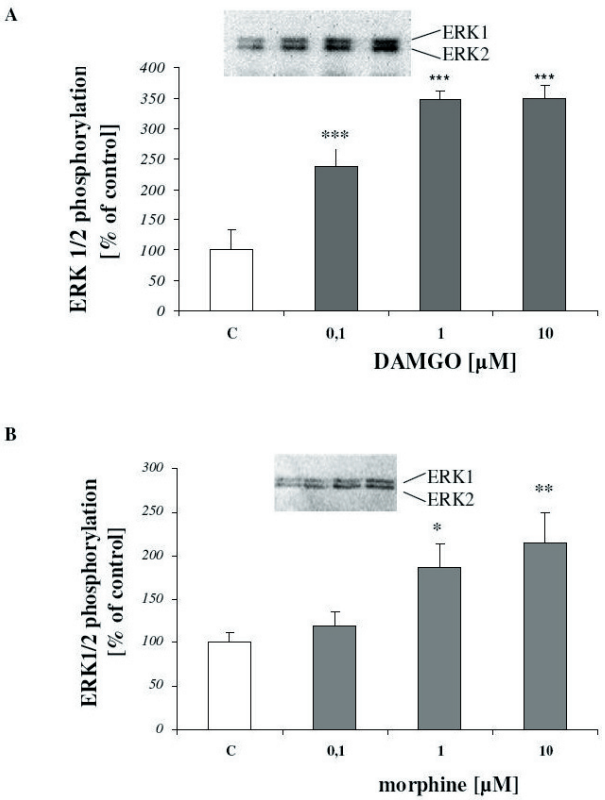


Figure 1. The effect of opioid treatment on ERK1/2 phosphorylation in SH-SY5Y cells. All data are expressed as the means ± SD of three independent experiments. ANOVA followed by Tukey's multiple-comparison test was performed; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared with the control (C). (A) Cells were incubated with increasing concentrations of DAMGO for 5 min. (B) Cells were incubated with increasing concentrations of morphine for 5 min.

**Total ERK expression during chronic morphine treatment.** Unphosphorylated total ERK immunoreactivity after chronic morphine treatment (fig.4) did not show any changes in the expression level of ERK during the entire experimental period (72 h).

**PKC and CaMKII activity is required for acute opioid-induced ERK phosphorylation.** Inhibitors of PKC (bisindolylmaleimide I, 100 nM) and CaMKII (autocamtide-2 inhibitory peptide, myristoylated, 20 μM) abolished opioid-induced ERK1/2 phosphorylation in SH-SY5Y cells (fig. 5A, B). These data indicate that stimulation of μ opioid receptors in SH-SY5Y cells may result in phospholipase C (PLC) activation and subsequently activation of PKC and CaMKII kinases.

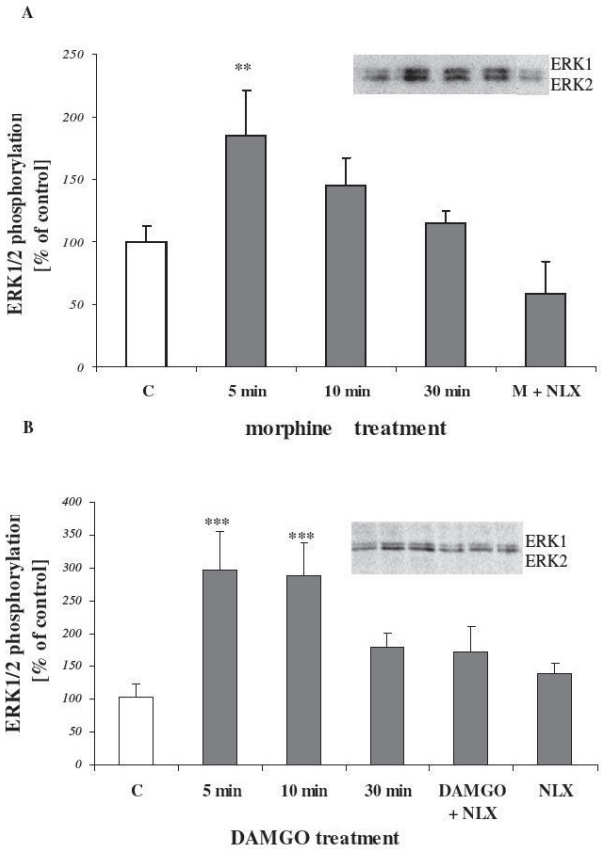


Figure 2. Time courses of opioid-induced ERK1/2 phosphorylation in SH-SY5Y cells. All data are expressed as the means ± SD of three experiments. ANOVA followed by Tukey's multiple-comparison test was performed; $^{**}p < 0.01$, $^{***}p < 0.001$, compared with the control (C). (A) Cells were incubated with 1 μM morphine (M) for the indicated periods of time. Additionally, the effect of the opioid receptor antagonist, naloxone (NLX), on morphine-induced ERK1/2 phosphorylation was tested. The cells were preincubated with naloxone (3 μM, 5 min), then incubated with morphine (1 μM, 10 min). (B) Cells were incubated with 1 μM DAMGO for the indicated periods of time. Additionally, the effect of the opioid receptor antagonist, naloxone (NLX), on DAMGO-induced ERK1/2 phosphorylation was tested. The cells were preincubated with naloxone (3 μM, 5 min) and then incubated with DAMGO (1 μM, 5 min). The last bar presents the effect of naloxone on ERK1/2 phosphorylation.

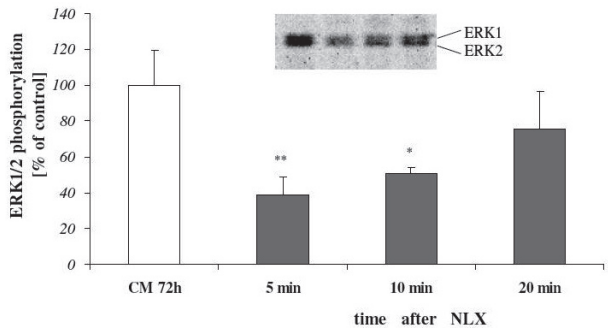


Figure 3. The effect of opioid withdrawal on ERK1/2 phosphorylation in SH-SY5Y cells. The cells were incubated with 1 μM morphine for 72 h (CM 72h) and after that period the withdrawal was precipitated with 3 μM naloxone (NLX). All data are expressed as the means ± SD of three experiments. ANOVA followed by Tukey's multiple-comparison test was performed; $^{*}p < 0.05$, $^{**}p < 0.01$, compared with the prolonged morphine (CM 72h) treatment.

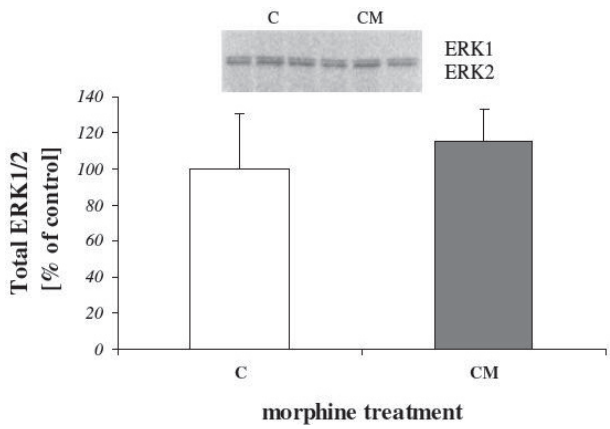


Figure 4. Changes in ERK1/2 expression level during chronic (72 h) morphine treatment. (C), control, untreated cells; CM chronic morphine. All data are expressed as the means ± SD of three experiments.

**Inhibition of PKC or CaMKII during morphine withdrawal superactivates ERK1/2.** In contrast to their effects during acute opioid treatment, inhibitors of PKC and CaMKII (bisindolymaleimide I, 100 nM and autocamtide-2 inhibitor peptide, myristoylated, 20 μM applied for 5 or 20 min) did not decrease but, in fact, increased ERK1/2 phosphorylation level when applied to the cells subjected to chronic morphine action (fig. 6A–C). This was further augmented after the addition of naloxone to SH-SY5Y cells exposed to morphine for 72 h. Both PKC and CaMKII inhibitors caused superactivation of ERK1/2 phosphorylation (fig. 6).

## Discussion

The data presented here provide evidence that μ opioid receptor activation differentially modulates ERK1/2 activity after acute and chronic opioid treatment as well as during morphine withdrawal in SH-SY5Y cells. When given acutely, opioids (morphine and DAMGO) activate ERK1/2, while chronic morphine treatment/withdrawal leads to ERK1/2 dephosphorylation. The observed increase in ERK phosphorylation following acute opioid treatment has also been shown previously by others [18, 22]. The acute administration of a selective μ opioid receptor agonist, DAMGO, exerted a stronger effect than morphine. The different efficacy of DAMGO and morphine in ERK activation is probably caused by different affinity of these ligands for the μ opioid receptor, although this effect may also be connected with the involvement of

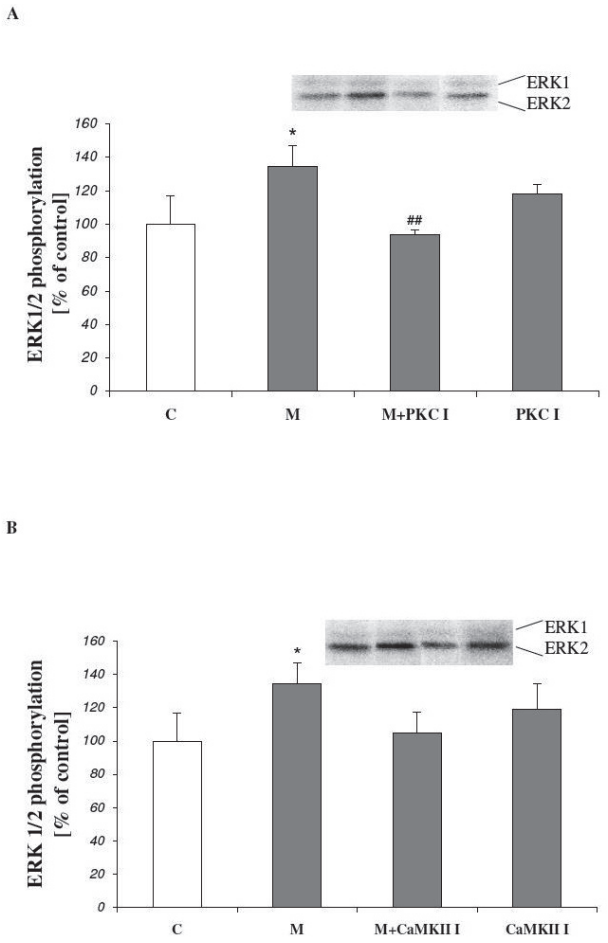


Figure 5. The effect of protein kinase inhibitors on ERK1/2 phosphorylation after acute morphine-treatment. All data are expressed as the means ± SD of three independent experiments. ANOVA followed by Tukey's multiple comparison test was performed, $^{*}p < 0.05$, compared with the control (C). $^{\#\#}p < 0.01$ compared with morphine treatment (M). (A) Cells were preincubated with 100nM PKC I (Bisindolylmaleimide I) for 5 min and then were incubated with 1μM morphine for 10 min (M+PKC I). Control group (C), morphine treatment (M). (B) Cells were preincubated with 20μM CaMKII I (Autocamtide-2 inhibitor peptide, myristoylated) for 5 min and then were incubated with 1μM morphine for 10 min (M+CaMKII I). Control group (C), morphine treatment (M).

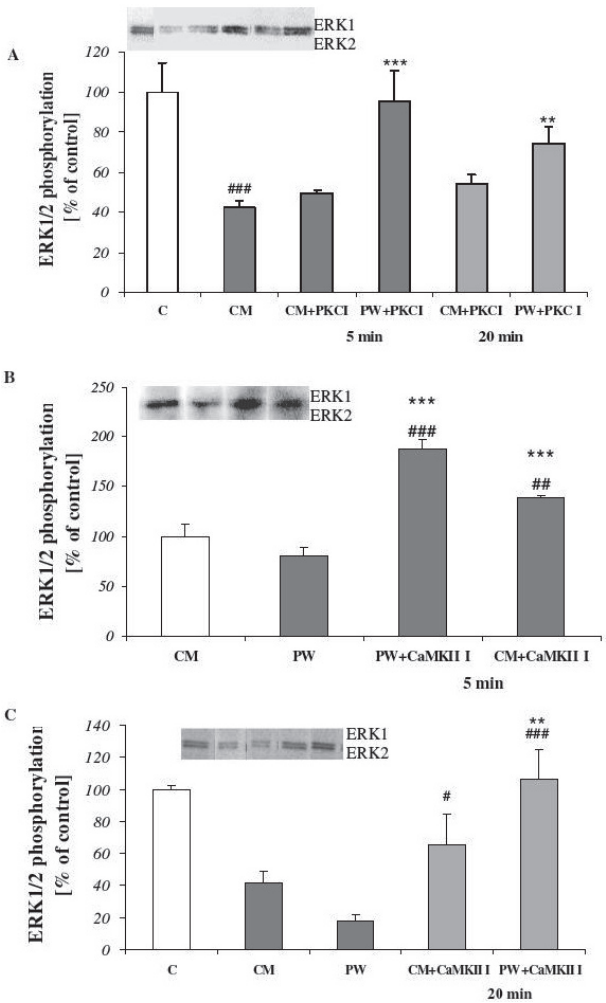


Figure 6. The effect of protein kinase inhibitors on ERK1/2 phosphorylation after chronic morphine (CM) treatment as well as during the naloxone-precipitated withdrawal (PW). All data are expressed as the means ± SD of three independent experiments. (A) Cells were incubated with 1 μM morphine for 72 h and then preincubated with 0.1 μM PKC I (bisindolylmaleimide I) for 5 or 20 min; the withdrawal was precipitated with 3 μM naloxone for 10 min (PW+PKC I). Control untreated group, (C). An ANOVA followed by Tukey's multiple-comparison test was performed; $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the prolonged morphine group (CM); $^{\#\#\#}p < 0.001$ compared with the control group (C). (B) Cells were incubated with 1 μM morphine for 72 h (CM) and then preincubated with 20 μM CaMKII I (Autocamtide-2 inhibitor peptide, myristoylated) for 5 min; the withdrawal was precipitated with 3 μM naloxone for 10 min (PW+CaMKII I). An ANOVA followed by Tukey's multiple-comparison test was performed; $^{***}p < 0.001$ compared with the precipitated withdrawal group (PW); $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the prolonged morphine group (CM). (C) Cells were incubated with 1 μM morphine for 72 h (CM) and then preincubated with 20 μM CaMKII I (autocamtide-2 inhibitor peptide, myristoylated) for 20 min; the withdrawal was precipitated with 3 μM naloxone for 10 min (PW+CaMKII I). An ANOVA followed by Tukey's multiple-comparison test was performed; $^{**}p < 0.01$, compared with the prolonged morphine group (CM); $^{*}p < 0.05$ , $^{***}p < 0.001$ compared with the precipitated withdrawal group (PW).

ERK1/2 in the process of opioid receptor internalization [18]. In fact, several reports have indicated that DAMGO induced μ opioid receptor internalization much more efficiently than morphine [17].

We found that in SH-SY5Y cells, the opioid-induced ERK1/2 phosphorylation was connected with the activation of PKC and CaMKII. These observations are in line with data showing that although acute opioid exposure inhibits cAMP production and $Ca^{2+}$ current [23], at the same time it may activate PLC, which can lead to the activation of PKC and mobilization of $Ca^{2+}$ from intracellular stores [24, 25]. Indeed, in SH-SY5Y cells, μ opioid receptors appear to be coupled to PLC. CaMKII itself is

a major downstream kinase of the inositol 1,4,5-triphosphate (IP3)/$Ca^{2+}$ pathway [26] and also noteworthy that μ opioid receptors co-exist with the CaMKII alpha isoform which is a major CaMKII isoform expressed in the CNS where it can regulate the activity of the opioid receptors [27]. CaMKII was shown to directly phosphorylate Raf-1 in vitro, and thus to control Raf/ERK activity [28]. PKC stimulates MAPK through the activation of the protein kinase Raf [29, 30].

Prolonged treatment of SH-SY5Y cells with morphine attenuated ERK1/2 activity and the precipitation of the withdrawal further decreased the ERK1/2 phosphorylation. The most striking observation of the current study with the cultured cells was the fact that PKC and CaMKII appear to be involved in the inhibition of ERK1/2 phosphorylation or even to be involved in the activation of the ERK1/2 dephosphorylation during prolonged morphine treatment and especially during morphine withdrawal. Inhibition of PKC as well as CaMKII not only slightly increases ERK1/2 activation during chronic morphine exposure but also superactivates ERK1/2 during naloxone-precipitated withdrawal. How is it possible to reconcile the differential actions of PKC and CaMKII on ERK1/2 activation following acute and prolonged opioid treatment? Chronic morphine treatment results in complex adaptations in the intracellular signaling pathways. Up-regulations of adenylyl cyclase (AC), PKA [31] and PKC [32, 33] are among the hallmark features of opioid tolerance/dependence. In turn, sustained PKA activation has been shown to be sufficient to produce prolonged activation of CaMKII via inhibition of protein phosphatase 1, which blocks CaMKII dephosphorylation [34]. Additionally, an increased expression of CaMKII during prolonged morphine exposure has also been demonstrated [35]. Up-regulated PKC and CaMKII may activate at the same time not only stimulatory, ERK-phosphorylating pathways but also distinct inhibitory pathways which generate a decrease in p-ERK level. A special example of such a kind of regulation is a negative feedback, where an up-regulated pathway shuts itself off with a time elapse. Indeed, active MAPKs are known to induce MAPK phosphatases (MKPs), which in turn dephosphorylate ERKs, this being vital for their inactivation [36]. Apart from MAPKs, the PKA pathway alone, up-regulated during chronic opioid treatment, has also been shown to induce MKPs efficiently [37], what together with MKP phosphorylation by MAPKs, leads to their accumulation with time [38]. Additionally, in neurons, PKA was shown to suppress activation of the Ras pathway by an inhibitory phosphorylation of Raf-1 [39, 40]. Consequently, in our model, activators of the PKA pathway (forskolin and 8Br-cAMP) profoundly inhibited ERK1/2 phosphorylation (data not shown). Thus, a cross-talk between the PKA pathway and ERK1/2 pathway seems to be capable of stimulation or inhibition of the MAPK/ERK pathway following acute and chronic opioid treatment, respectively [41].

Another mechanism controlling ERK activity would involve the stress-activated protein kinases (SAPK) pathway. We observed that chronic morphine activates the JNK/SAPK signaling cascade (unpublished data). Sustained JNK activation uncouples ERK activation from MEK, resulting in ERK resistance to activation. Interestingly, Jun-mediated gene transcription is required in this negative cross-talk between the JNK and ERK pathways [42]. Finally, the decrease in ERK activity observed during chronic morphine treatment appears to be a result of the balance established between oppositely acting pathways, a balance which favors inhibitory pathways. These pathways seem to be further up-regulated during withdrawal. In such an adapted system, inhibition of either CaMKII or PKC results both in inhibition of stimulatory, ERK-phosphorylating pathways and inhibition of inhibitory or dephosphorylating pathways, thus allowing ERK activation via so far unidentified CaMKII- and PKC-independent pathways. To understand the obtained results, instead of thinking about the individual pathways, we should, rather, think of a complex signaling network created by many cross-talks resulting in properties that the individual pathways do not have.

Whatever is the nature of the above regulations, PKC and CaMKII seem to act either after the acute opioid signal or during prolonged morphine treatment and withdrawal. Interestingly, the time courses of the ERK1/2 phosphorylation after acute exposure, and dephosphorylation after chronic treatment withdrawal were similar, suggesting that some common pathways may be responsible for these phenomena.

In summary, acute opioids transiently activate ERK1/2 phosphorylation, whereas prolonged morphine administration exerts a long-lasting inhibitory effect on the ERK pathway. Moreover, activation of $Ca^{2+}$/calmodulin and PKC is required for ERK1/2 activation following acute opioid treatment while upon chronic morphine exposure, the up-regulation of PKC and CaMKII pathways seems to be engaged in ERK1/2 inhibition.

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