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Involvement of cAMP/cAMP-Dependent Protein Kinase Signaling Pathway in Regulation of Na^+,K^+-ATPase upon Activation of Opioid Receptors by Morphine

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ABSTRACT

The depolarization of neurons induced by impairment of Na^+,K^+-ATPase activity after long-term opiate treatment has been shown to involve the development of opioid dependence. However, the mechanisms underlying changes in Na^+,K^+-ATPase activity after opioid treatment are unclear. The best-established molecular adaptation to long-term opioid exposure is up-regulation of the cAMP/cAMP-dependent protein kinase (PKA) signaling pathway; this study, therefore, was undertaken to investigate the role of up-regulation of cAMP/PKA signaling pathway in alteration of the mouse hippocampal Na^+,K^+-ATPase activity. The results demonstrated that short-term morphine treatment dose dependently stimulated Na^+,K^+-ATPase activity. This action could be significantly suppressed by adenylyl cyclase activator 7β-acetoxy-8,13-epoxy-1α,6β,9α-trihydroxylabd-14-en-11-one (forskolin), or the cAMP analog dibutyryl-cAMP. Contrary to short-term morphine treatment, long-term treatment significantly inhibited Na^+,K^+-ATPase activity. Moreover, an additional decrease in Na^+,K^+-ATPase activity was observed by naloxone precipitation. The effects of both short- and long-term morphine treatment on Na^+,K^+-ATPase activity were naltrexone-reversible. The regulation of Na^+,K^+-ATPase activity by morphine was inversely correlated with intracellular cAMP accumulation. *N*-[2-(4-Bromocinnamylamino)ethyl]-5-isoquinoline (H89), a specific PKA inhibitor, mimicked the stimulatory effect of short-term morphine but antagonized the inhibitory effect of long-term morphine treatment on Na^+,K^+-ATPase activity. However, okadaic acid, a protein phosphatase inhibitor, suppressed short-term morphine stimulation but potentiated long-term morphine inhibition of Na^+,K^+-ATPase activity. The regulation of Na^+,K^+-ATPase activity by morphine treatment seemed to associate with the alteration in phosphorylation level but not to be relevant to the change in abundance of Na^+,K^+-ATPase. These findings strongly demonstrate that cAMP/PKA signaling pathway involves regulation of Na^+,K^+-ATPase activity after activation of opioid receptors.

Opioid analgesics are clearly the most efficacious agents currently available for treatment of moderate-to-severe pain. However, their use for long-term pain management is often limited because of the development of tolerance and/or dependence upon prolonged administration. The mechanisms underlying these adaptations to repeated or long-term opiate administration are still poorly understood. To date, the best-established molecular adaptations to long-term drug exposure is up-regulation of the cAMP/PKA signal pathway (Nestler and Aghajanian, 1997), which is thought to play a key role in the development of opioid tolerance and dependence. But how the up-regulation of cAMP/PKA signaling system is functionally involved in opioid tolerance and dependence remains to be further investigated.

Several lines of evidence suggest that treatment with morphine and other opiates enhances neural Na^+,K^+-ATPase activity in vivo (Desaiah and Ho, 1977; Sharma et al., 1998) and in vitro (Masocha et al., 2002; Horvath et al., 2003), whereas long-term morphine treatment leads to the depolarization of neurons in locus ceruleus and myenteric plexus of the guinea pig by the reduction of electrogenic activity of the Na^+,K^+-ATPase, which has been suggested to be linked to opioid tolerance and dependence (Kong et al., 1997, 2001).

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ABBREVIATIONS: PKA, cAMP-dependent protein kinase; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; PTX, pertussis toxin; db-cAMP, dibutyryl-cAMP; OA, okadaic acid; H89, *N*-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline; IBMX, 3-isobutyl-1-methylxanthine; PAGE, polyacrylamide gel electrophoresis; TBST, Tris-buffered saline/Tween 20; Blotto, bovine lacto transfer technique optimizer; IP, immunoprecipitation; MAPK, mitogen-activated protein kinase; LTP, long-term potentiation.

However, the mechanism underlying the modulation of Na^+,K^+-ATP-ase activity by short- and long-term morphine treatment has not yet been determined, and what relationship exists between the up-regulation of cAMP/PKA signaling pathway and changes in Na^+,K^+-ATPase activity upon long-term exposure to opioids is unknown.

The hippocampus is generally thought to be an important brain region associated with drug addiction, and it has been shown to be functionally involved in withdrawal-like behavior mediated by opioid receptors (Fan et al., 1999) and in cocaine-seeking behavior (Vorel et al., 2001). The present study, therefore, was undertaken to investigate how short- and long-term morphine treatments modulate Na^+,K^+-ATPase activity and whether the up-regulation of cAMP/PKA signaling pathway plays a role in modulation of the mouse hippocampal Na^+,K^+-ATPase activity.

Materials and Methods

Materials. Morphine hydrochloride was purchased from Qinghai Pharmaceutical General Factory (Qinghai, China). Dimethyl sulfoxide, SDS, and dithiothreitol (DTT) were purchased from AMRESCO Inc. (Solon, OH). Phenylmethylsulfonyl fluoride (PMSF) and Triton X-100 were obtained from Merck (Darmstadt, Germany). Naltrexone hydrochloride, naloxone hydrochloride, pertussis toxin (PTX), 7β-acetoxy-8,13-epoxy-1α,6β,9α-trihydroxylabd-14-en-11-one (forskolin), dibutyryl-cAMP (db-cAMP), okadaic acid (OA) sodium salt, H89, 3-isobutyl-1-methylxanthine (IBMX), and ouabain hydrate were supplied by Sigma-Aldrich (St. Louis, MO). Protein A/G Plus agarose beads were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The cAMP enzyme immunoassay kit was obtained from Sigma-Aldrich.

Animals and Morphine Treatment. Kunming strain male mice (25–30 g) were obtained from the Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China). Mice were housed in groups and maintained on a 12-h light/dark cycle in a temperature-controlled environment with free access to food and water. All animal treatments were strictly in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. For short-term treatment, animals were treated with a single s.c. injection of morphine at a dose of 10 mg/kg or across a range of doses from 1 to 40 mg/kg for 1 h. Naltrexone was given i.p. 30 min before morphine administration at a dose of 4 mg/kg. PTX was given i.c.v. 5 day before the administration of morphine at a dose of 0.5 μg/mouse as described previously (Parenti et al., 1986). Forskolin, db-cAMP, or OA was administrated i.c.v. 30 min before morphine injection at the doses of 0.1~100 ng, 1~100 ng, and 1~100 pg, respectively. For long-term treatment, mice were treated by morphine (10 mg/kg s.c.) twice per day at 12-h intervals for 10 days as described previously (Pu et al., 2002). In one group, animals were treated concomitantly with naltrexone and morphine; naltrexone was injected i.p. 30 min before morphine administration. In a control group, mice were treated similarly except that normal saline was used as a substitute for morphine. One hour after the final injection of morphine, some animals were decapitated immediately, and the other animals were precipitated with naloxone (2 mg/kg i.p.), and withdrawal behaviors were assessed during a period of 15 min. In some experiments, 1 h after the final injection of morphine or saline, the animals were injected i.c.v. with H89 (1 nmol) or OA (100 pg), and then they were killed by decapitation 30 min later (Pu et al., 2002). After treatments, the animals were sacrificed by decapitation and then the hippocampi were isolated rapidly on ice and stored at −80°C until use.

Membrane Preparation. Plasma membranes were prepared as described previously (Roth et al., 1981) with some modifications. In brief, hippocampal tissues from three mice were homogenized on ice by 20 strokes with a tight-fitting Dounce homogenizer in 1 ml of homogenization buffer, pH 7.4, composed of 5 mM HEPES, 1 mM PMSF, 50 μM $CaCl_2$, 10% (w/v) sucrose, and 1 mM DTT, and centrifuged at 1000*g* for 10 min at 4°C to remove cellular debris and nuclei. The supernatant was centrifuged at 12,000*g* for 20 min at 4°C to yield the crude plasma membranes (P_2 pellets). To remove adhering microsomes, the obtained pellet was washed an additional three times by resuspension and recentrifugation at 14,000*g* for 20 min at 4°C. The final pellet was resuspended on ice in a sufficient amount of 50 mM Tris-HCl buffer, pH 7.4, to give a protein concentration of 0.4 mg/ml, and aliquots were stored at −20°C. To avoid the loss of Na^+,K^+-ATPase activity, the stored plasma membrane pellets were used within 3 days. Membrane protein concentrations were determined by a bicinchoninic acid assay (Beyotime Biotechnology, Haimen, China).

Measurement of Na^+,K^+-ATPase Activity. The Na^+,K^+-ATPase activity was measured as described previously (Esmann, 1988) with slight modifications. In brief, a 100-μl aliquot containing 40 μg of protein was preincubated at 37°C for 10 min with 850 μl of reaction buffer A containing 100 mM NaCl, 20 mM KCl, 2 mM $MgCl_2$, 0.4 mM EGTA, and 50 mM Tris-HCl, pH 7.4. To measure the ouabain-insensitive ATPase, the medium was the same but with 1 mM ouabain and without NaCl and KCl (reaction buffer B). The reaction was initiated by adding 50 μl of ATP disodium solution (final ATP concentration in the medium was 2.5 mM), followed by incubation for 10 min at 37°C. The reaction was terminated by addition of trichloroacetic acid [0.2 ml; 50% (w/v)]. The tube was put on ice for 15 min, followed by centrifugation at 10,000*g* at 4°C for 10 min. Then, 400 μl of supernatant was taken for the assay of liberated inorganic phosphate as described previously (Taussky and Shorr, 1953) with some modifications. In brief, 800 μl of ammonium molybdate solution color reagent was added to the tube containing 400 μl of sample, and the absorbance was read at 700 nm with a UV-visible spectrophotometer (Shimadzu, Tokyo, Japan) after a 5-min incubation at room temperature, using Na_2HPO_4 as standard. Na^+,K^+-ATPase activity was obtained by the difference between total ATPase and ouabain-insensitive Mg^{2+}-ATPase activity (Esmann, 1988).

In Vitro Assay. The crude hippocampal synaptosomes (P_2 pellets) were prepared as described above. The final pellets were suspended in Krebs-Ringer-HEPES medium containing the following: 120 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 25 mM HEPES, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 10 mM glucose, pH 7.4, to give a protein concentration of 1 mg/ml. A 100-μl aliquot was pipetted into the appropriate well of the 24-well plate, 890 μl of the KRH medium was added to the well, and then the plate was preincubated for 15 min at 37°C. Ten microliters of different concentrations of morphine were then added to the appropriate wells followed by an additional incubation at 37°C for 10 min. In some experiments, naltrexone was added to the appropriate wells and incubated for 5 min at 37°C before morphine treatments. After this time, the plate was transferred rapidly to an ice bath to terminate the reaction. The sample in one well was divided equally into two tubes (500 μl/tube) followed by centrifugation at 14,000*g* for 20 min at 4°C. The recovered synaptosomes were resuspended in 950 μl of the assay buffer A or B (see above) and preincubated at 37°C for 10 min. The reaction was initiated by adding 50 μl of ATP disodium solution (final ATP concentration in the medium was 2.5 mM) followed by incubation for 10 min at 37°C. The following procedures for Na^+,K^+-ATPase activity determination were performed as described above.

cAMP Assay. The cytosolic fraction for cAMP assay was prepared according to the method described previously (Liu et al., 2004) with some modifications. In brief, hippocampal tissues from three mice were homogenized on ice by 20 strokes with a tight-fitting Dounce homogenizer with 1 ml of homogenization buffer containing 50 mM Tris-HCl, pH 7.4, 1 mM EGTA, 1 mM DTT, and 500 μM IBMX. An aliquot of the homogenate was taken for protein concentration determination as described above. The homogenate was centrifuged at

20,000*g* for 20 min at 4°C. The resulting supernatant was collected on ice and then placed in a 90–100°C water bath for 5 min. After centrifugation at 3000*g* at 4°C for 10 min, the supernatant was collected and frozen at −80°C until use or immediately assayed for cAMP using the enzyme immunoassay kit, according to the manufacturer's instructions. In brief, 100 μl of the preacetylated samples and 100 μl of cAMP standards was added to the appropriate wells of goat anti-rabbit IgG microplate, and then 50 μl of the blue cAMP conjugates and the yellow cAMP antibodies were sequentially added. The plate was incubated at room temperature for 2 h on a plate shaker at ~500 rpm. The contents of the wells were emptied and washed three times by adding 200 μl of wash solution. After the final wash, the wells were aspirated, and the plate was firmly taped to remove any remaining wash buffer. Two hundred microliters of the substrate solution was added to every well and incubated at room temperature for 1 h without shaking. Fifty microliters of the stop solution was added to every well, and the optical density was read immediately at 405 nm. The intracellular cAMP concentration was expressed as picomoles per milligram of protein.

Immunoblotting Assay. For immunoblotting assay, the crude plasma membranes were prepared as described for Na^+,K^+-ATPase activity assay with the exception of the homogenization buffer containing 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 500 μM IBMX, 50 mM NaF, 2 μg/ml aprotinin, and 5 μg/ml leupeptin. The final pellet was suspended on ice in a sufficient amount of this homogenization buffer to give a protein concentration of 3 mg/ml. An equal volume of 2× sample buffer (100 mM Tris-HCl, pH 6.8, 200 mM DTT, 4% SDS, 20% glycerol, and 0.2% bromphenol blue) was added, boiled at 100°C for 10 min, and stored at −20°C until use. Electrophoresis was performed by using the Mini-Protean 3 apparatus (Bio-Rad, Hercules, CA). In brief, equal quantities of protein (24 μg) were separated by SDS-polyacrylamide gel electrophoresis (PAGE) on 8% SDS polyacrylamide gel for approximately 90 min at 120 V, and then electroblotted at 4°C onto nitrocellulose membrane (0.45-μm pore size; Bio-Rad). The membrane was rinsed briefly in Tris-buffered saline/Tween 20 (TBST) (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, and 0.1% Tween 20) and blocked at room temperature in a solution (Blotto) containing 5% fat-free dry milk in TBST for 120 min. Blocking solution was replaced with Blotto containing the 1/600 dilution of the primary antibody against the α1 or α3 subunit of mouse Na^+,K^+-ATPase (Santa Cruz Biotechnology), and the membrane was incubated overnight at 4°C, followed by washing in TBST for 15 min. Washing was repeated twice more. The membrane was then incubated for 120 min at room temperature in the appropriate secondary antibody (horseradish peroxidase conjugated donkey anti-goat IgG; Biodesign, Melbourne, Australia) diluted 1/300 in Blotto. After three 15-min extensive washes in TBST, the antibody binding was detected using an enhanced chemiluminescence method (Pierce Chemical, Rockford, IL) according to the manufacturer's instructions.

Protein Phosphorylation Assay. The crude plasma membranes were prepared as described for Na^+,K^+-ATPase activity assay. Protein phosphorylation assay was performed as described previously with some modifications (Wang et al., 2003). In brief, the final pellets were solubilized in ice-cold immunoprecipitation (IP) buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2 mM EDTA, 30 mM NaF, 30 mM $Na_4O_7P_2$, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml leupeptin, 4 μg/ml aprotinin, and 1% Triton X-100), and incubated with rotation at 4°C for 2 h. Insoluble material was removed by centrifugation at 12,000*g* for 20 min at 4°C, and the concentration of protein in the supernatant was determined as described above. An equal amount of protein (200 μg) from the supernatants was incubated with rotation overnight at 4°C with 3 μg of goat polyclonal Na^+-K^+-ATPase α1 or α3 subunit antibody. A saturating amount of protein A/G Plus agarose beads (prewashed with IP buffer three times) was added and incubated with rotation at 4°C for 2 h. The beads were washed three times with ice-cold IP buffer by centrifugation at 8000*g* for 5 min at 4°C. An equal volume of 2× sample buffer (100 mM Tris-HCl, pH 6.8, 200 mM DTT, 4% SDS, 20% glycerol, and 0.2% bromphenol blue) was added and boiled at 100°C for 10 min. The samples were separated by 8% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with rabbit polyclonal anti-pan phosphorylated protein antibody at a dilution of 1/250 (Zymed Laboratories, South San Francisco, CA) as described under *Immunoblotting Assay*.

p44/42 Mitogen-Activated Protien Kinase (MAPK) Phosphorylation Assay. The hippocampus was homogenized on ice in 50 mM Tris-HCl, pH 7.2, 1% Triton X-100, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM PMSF, 10 μg/ml leupeptin, and 4 μg/ml aprotinin. The lysate was allowed to stand for 30 min on ice and was centrifuged at 14,000*g* for 20 min at 4°C. The protein concentration of the supernatant was determined as described above. An equal volume of 2× sample buffer (100 mM Tris-HCl, pH 6.8, 200 mM DTT, 4% SDS, 20% glycerol, and 0.2% bromphenol blue) was added and boiled at 100°C for 10 min. The samples were separated by 12% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with rabbit polyclonal anti-phosphorylated p44/42 MAPK antibody at a dilution of 1/1000 (Cell Signaling Technology Inc., Beverly, MA). The membrane was stripped and reprobed with rabbit polyclonal anti-p42 MAPK antibody at a dilution of 1/1000 (Santa Cruz Biotechnology, Inc.).

PKA Activity Assay. PKA activity was determined essentially according to the method described by Pu et al. (2002). The hippocampus was homogenized on ice in homogenization buffer (25 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM DTT, and 100 μM leupeptin). The homogenate was centrifuged at 20,000*g* for 5 min at 4°C. The resulting supernatant was assayed for PKA activity using PepTag nonradioactive PKA assay kit (Promega, Madison, WI) according to the manufacturer's instructions. All reaction components were added on ice in a final volume of 25 μl of the following mixture: 5 μl of PepTag PKA reaction buffer, 5 μl of PepTag A1 peptide (0.4 μg/μl), 5 μl of cAMP (5 μM), and 5 μl (0.2 μg/μl) of sample homogenate. The mixture was incubated for 30 min at room temperature. Then, the reaction was terminated by placing the tube in a boiling water bath for 10 min, and the samples were loaded onto the 0.8% agarose gel for electrophoresis. Before loading samples, 1 μl of 80% glycerol was added to the sample to ensure that it remained in the well. PKA-specific peptide substrate used in this experiment was PepTagA1 peptide, L-R-R-A-S-L-G (Kemptide). The assay was based on the changes in the net charge of the fluorescent PKA substrates before and after phosphorylation. This change allowed the phosphorylated and unphosphorylated versions of the substrate to be rapidly separated on an agarose gel at neutral pH. The phosphorylated species migrated toward the positive electrode, whereas the nonphosphorylated substrate migrated toward the negative electrode. After photographing, the intensity of fluorescence of phosphorylated peptides, which reflected the activity of PKA, was quantified by spectrophotometry.

Statistical Analysis. All data were represented the mean ± S.D. from at least three independent experiments, and the results of each experiment performed in duplicate were from three animals in each group. Statistical comparisons between two experimental groups were made by unpaired Student's *t* test. When more than two groups were compared, a one-way analysis of variance followed by Newman-Keuls test was used. Differences with a *P* value less than 0.05 were considered statistically significant.

Results

Short-Term Morphine Treatment Increased Na^+,K^+-ATPase Activity, and This Effect Was Naltrexone- and PTX-Reversible. Injection of morphine (s.c.) produced a dose-dependent increase in ouabain-sensitive Na^+,K^+-ATPase activities in the mouse hippocampus, with maximal effect at dose of 10 mg/kg (Fig. 1A). To determine whether observed morphine effects are direct or indirect, the stimulation of

hippocampal synaptosomal Na^+,K^+-ATPase activity by morphine was detected. As shown in Fig. 1B, in vitro morphine treatment also increased the hippocampal synaptosomal Na^+,K^+-ATPase activity in a dose-dependent manner. The maximal stimulation occurred at the concentration of 10 μM. The stimulatory effect of short-term morphine treatment on the hippocampal Na^+,K^+-ATPase activity was opioid receptor antagonist naltrexone- and $G_{i/o}$ protein blocker PTX-reversible. Naltrexone, given i.p. 30 min before morphine administration at dose of 4 mg/kg or given in vitro to hippocampal synaptosome at a dose of 10 μM, fully antagonized the stimulatory effect of short-term morphine treatment on Na^+,K^+-ATPase activity in the hippocampus, and obvious alteration in Na^+,K^+-ATPase activity was not observed in the animals treated with naltrexone alone (Fig. 2, A and B). Likewise, PTX (0.5 μg i.c.v.) also significantly inhibited the morphine-induced increase in Na^+,K^+-ATPase activity when injected 5 days before short-term morphine administration (Fig. 2C). In addition, a slight but significant decrease in Na^+,K^+-ATPase activity also occurred in mice that were treated with PTX alone (Fig. 2C). Opioid receptors are coupled through PTX-sensitive G proteins to activate the p42/p44 mitogen-activated protein kinase (MAPK) pathway (for review, see Law et al., 2000). To validate that injection of PTX i.c.v. 5 days before morphine could diffuse sufficiently to the hippocampus to elicit its effect, PTX treatment on morphine-induced activation of p42/p44 MAPK in the hippocampus was investigated. Injection of PTX 5 days before morphine abolished morphine-activated p42/p44 MAPK phosphorylation in the hippocampus (Fig. 2D).

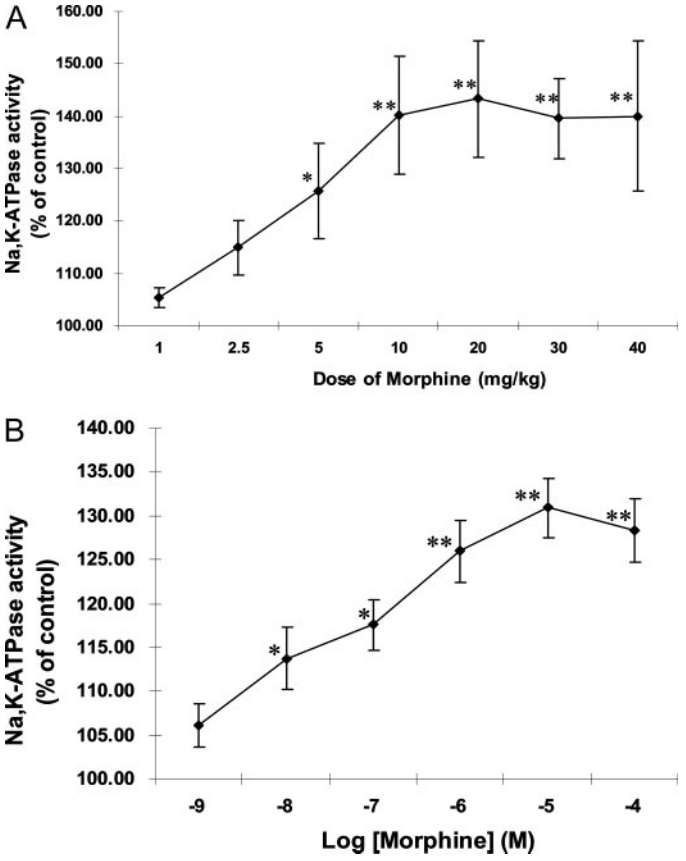

Fig. 1. In vivo and in vitro short-term morphine stimulation of ouabain-sensitive Na^+,K^+-ATPase activity in the mouse hippocampus in a dose-dependent manner. A, mice were treated with saline or increasing doses of morphine for 1 h. After treatment, animals were decapitated, and the hippocampus was quickly isolated on ice and membrane was prepared. B, crude synaptosomes (P_2 pellets) prepared from the hippocampus of naive mice were treated with different concentrations of morphine (10^{-9}~10^{-4} M) for 10 min at 37°C. Na^+,K^+-ATPase activity was measured as described under *Materials and Methods*. Values of each point are presented as the mean ± S.D. of three separate experiments performed in duplicate. *, $P < 0.05$; **, $P < 0.01$ compared with vehicle-treated control animals or nonmorphine-treated crude synaptosomes.

Decrease in Hippocampal cAMP Concentration in Mice Undergoing Short-Term Morphine Treatment May Be Related to Increase in Na^+,K^+-ATPase Activity. Short-term activation of opioid receptors by opiates is widely documented to inhibit cAMP accumulation and PKA activation. To determine the relationship between alteration of intracellular cAMP concentration and Na^+,K^+-ATPase activity, hippocampal cAMP concentrations in mice treated with morphine for 1 h were measured. As was expected, short-term treatment of mice with morphine (10 mg/kg s.c.) significantly decreased cAMP concentrations in the hippocampus compared with saline-treated control group, and pretreatment of naltrexone could abolish this decrease in cAMP concentration (Fig. 3A). To further determine the role of alteration of cAMP concentration in changes in Na^+,K^+-ATPase activities, the effects of forskolin, an activator of adenylyl cyclase, and db-cAMP, an analog of cAMP, on short-term morphine treatment-induced enhancement of Na^+,K^+-ATPase activity were examined. Forskolin and db-cAMP both activate cAMP/PKA signaling pathway by elevation of cAMP concentration. First, the effects of injection of forskolin or db-cAMP i.c.v. on hippocampal cAMP levels were detected. As expected, injection of forskolin (10 ng) or db-cAMP (100 ng) both pronouncedly increased hippocampal cAMP concentrations (Fig. 3B). Moreover, forskolin or db-cAMP also significantly increased hippocampal PKA activity (Fig. 3C). Next, the effects of injection of forskolin or db-cAMP i.c.v. on hippocampal Na^+,K^+-ATPase activity were measured. Both forskolin and db-cAMP exhibited a significant inhibition of basal Na^+,K^+-ATPase activity in a dose-dependent manner, with maximal inhibition at the dose of 10 ng for forskolin and 100 ng for db-cAMP, respectively (data not shown). Concomitant administration with morphine, forskolin (10 ng i.c.v.), or db-cAMP (100 ng i.c.v.) significantly suppressed morphine-stimulated enzyme activity in the hippocampus (Fig. 3, D and E). The results indicate that reduction of cAMP concentration may contribute to enhancement of Na^+,K^+-ATPase activity upon short-term activation of opioid receptors, suggesting that cAMP concentration is inversely correlated with Na^+,K^+-ATPase activity.

H89, a Selective Inhibitor of PKA, Mimicked but OA, a Protein Phosphatase Inhibitor, Reversed the Stimulatory Effect of Short-Term Treatment with Morphine on Na^+,K^+-ATPase Activity. To determine whether alteration of PKA activity involved morphine-induced increase in Na^+,K^+-ATPase activity, the effect of H89, a specific PKA inhibitor, on basal Na^+,K^+-ATPase activity was investigated. Indeed, a single injection of H89 (1 nmol i.c.v.) mimicked the action of short-term morphine stimulation of Na^+,K^+-ATPase activity, displaying a significant increase in Na^+,K^+-ATPase activity of the hippocampus relative to saline-treated control group (Fig. 4A), indicative of reduction of PKA activity involvement of enhancement of Na^+,K^+-ATPase activity. To investigate whether alteration of phos-

phorylation level of Na^+,K^+-ATPase was involved in enhancement of Na^+,K^+-ATPase activity after short-term morphine treatment, the effect of OA, a protein phosphatase inhibitor, on morphine-mediated changes in Na^+,K^+-ATPase activity was assessed. OA can increase the phosphorylation levels of Na^+,K^+-ATPase via the blockade of dephosphorylation of rat renal Na^+,K^+-ATPase (Li et al., 1998). Preliminary study showed that OA (1~100 pg) dose dependently inhibited the hippocampal Na^+,K^+-ATPase activity (data not shown). Therefore, a dose of 100 pg was used to assessed the effect of OA on short-term morphine-mediated changes in Na^+,K^+-ATPase activity. OA, given i.c.v. 30 min before morphine administration at a dose of 100 pg, markedly attenuated short-term morphine-stimulated Na^+,K^+-ATPase activity in the hippocampus compared with the group receiving short-term morphine treatment alone (Fig. 4B), suggesting that reduction of phosphorylation levels of Na^+,K^+-ATPase may be implicated in enhancement of Na^+,K^+-ATPase activity induced by short-term morphine treatment.

Long-Term Morphine Treatment Decreased Na^+,K^+-ATPase Activity but Increased cAMP Concentration. It has been well established that long-term exposure to opiates leads to a marked increase in cAMP concentration (Liu and Anand, 2001). To further confirm the modulation of Na^+,K^+-ATPase activity by cAMP, the effects of long-term morphine treatment and naloxone-precipitated morphine withdrawal on Na^+,K^+-ATPase activity and intracellular cAMP concentration were tested. One group of animals was administrated with 10 mg/kg morphine twice per day for 10 consecutive days. The other group of animals was treated identically except that animals were precipitated with naloxone after long-term morphine treatment and withdrawal syndromes were observed. The mice receiving long-term morphine treatment followed by naloxone precipitation demonstrated withdrawal behaviors such as jumping, diarrhea, wet dog shakes, and lost body weight (data not shown). In agreement with previous studies, long-term treatment of mice with morphine for 10 days markedly enhanced hippocampal cAMP con-

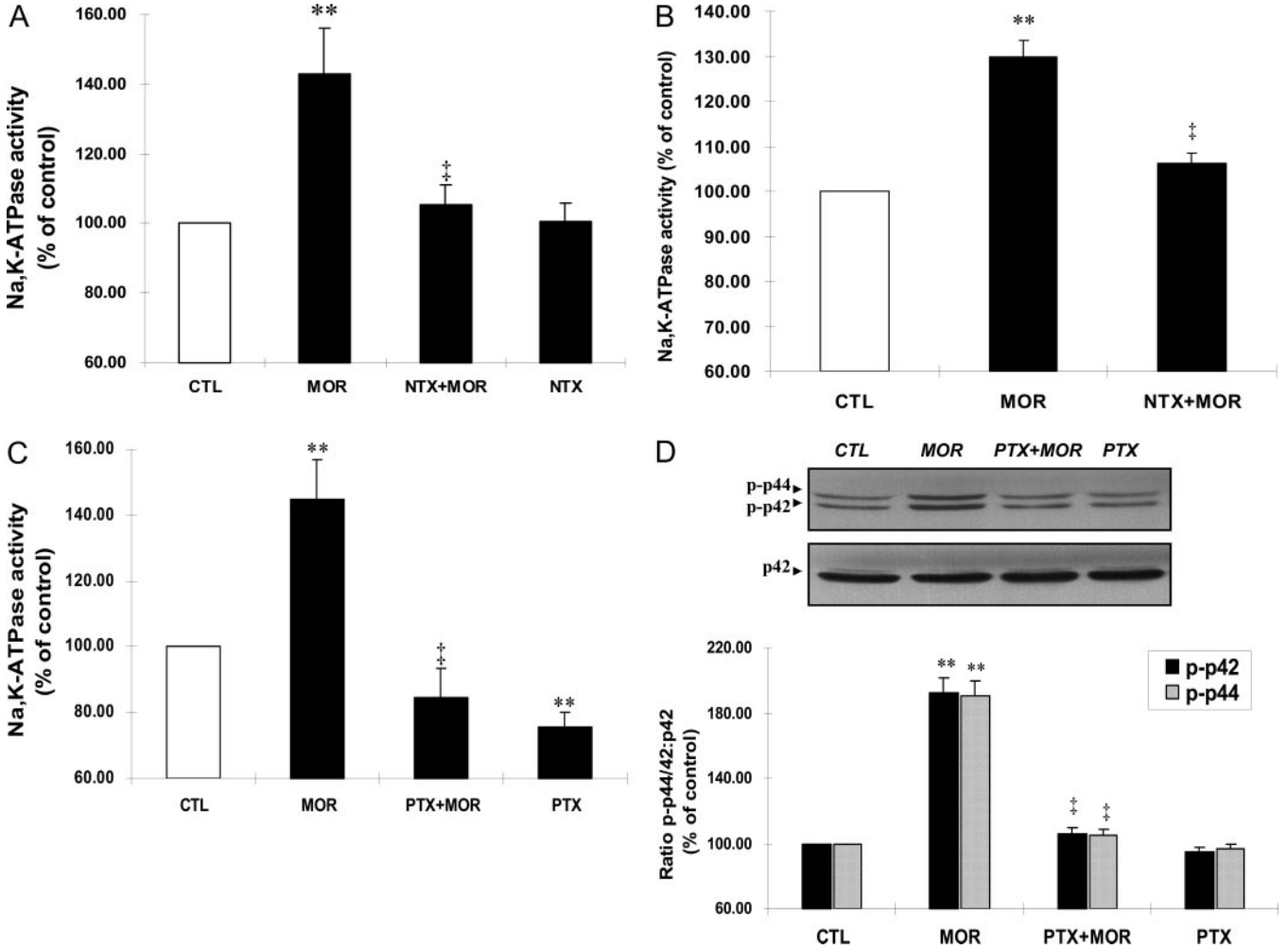

Fig. 2. Reversal of the stimulatory effect of short-term morphine treatment on hippocampal Na^+,K^+-ATPase activity by naltrexone and PTX. A, mice were treated with morphine (10 mg/kg s. c.) or saline (10 ml/kg s.c.) or concomitantly treated with naltrexone (4 mg/kg i.p.) or saline (10 ml/kg i.p.). Naltrexone or saline was injected 30 min before morphine administration. B, hippocampal crude synaptosomes were incubated with 10^{-5} M naltrexone for 5 min at 37°C and then treated with 10^{-5} M morphine for 10 min at 37°C. C and D, animals were treated with morphine (10 mg/kg s.c.) or saline (10 ml/kg s.c.) or concomitantly treated with PTX (0.5 μg i.c.v.) or vehicle [50% (v/v) glycerol, 50 mM Tris, pH 7.5, 10 mM glycine, and 0.5 M NaCl; 5 μl i.c.v.). PTX or vehicle was administrated 5 days before morphine treatment. Na^+,K^+-ATPase activity (A–C) and p44/p42 MAPK phosphorylation (D) were measured as described under *Materials and Methods*. D, top, representative immunoblots for phosphorylated p44/p42 MAPK (top) and total p42 MAPK (bottom). Bottom, quantitative estimation by scanning densitometry of ratio of phosphorylated p44/p42 MAPK to total p42 MAPK. Data represent the mean ± S.D. of three separate experiments performed in duplicate. **, $P < 0.01$ compared with vehicle-treated control mice; ‡, $P < 0.01$ compared with mice treated with morphine alone. CTL, control; MOR, morphine; and NTX, naltrexone.

centration (Fig. 5B). In contrast to the short-term single morphine treatment, long-term morphine treatment significantly decreased Na^+,K^+-ATPase activity in the hippocampus compared with saline control group (Fig. 5A). An additional reduction of Na^+,K^+-ATPase activity and enhancement of cAMP concentration was found upon naloxone-precipitated withdrawal in long-term morphine-treated mice relative to non–naloxone-precipitated group (Fig. 5, A and B). The attenuation of Na^+,K^+-ATPase activity and enhancement of intracellular cAMP concentration by long-term morphine treatment were prevented by concomitant administration of naltrexone (Fig. 5, A and B). Naltrexone, coadministered with morphine, also significantly suppressed long-term morphine treatment-induced withdrawal behav-

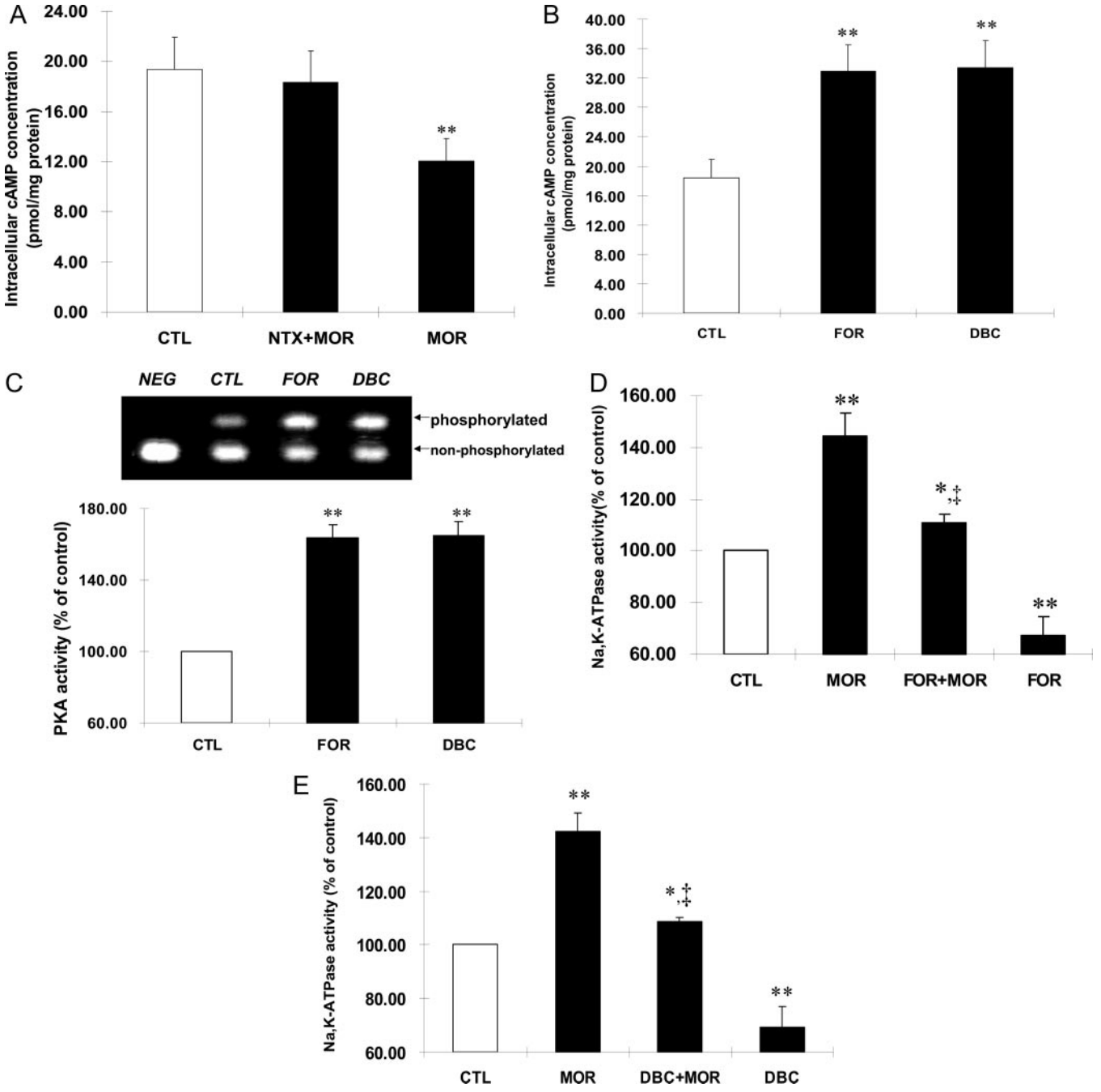

Fig. 3. Enhancement of hippocampal cAMP level and PKA activity and inhibition of short-term morphine-induced enhancement of Na^+,K^+-ATPase activity by forskolin and db-cAMP. A, D, and E, mice were treated with morphine (10 mg/kg s.c.) or saline (10 ml/kg s.c.) or concomitantly treated with naltrexone (4 mg/kg i.p.), forskolin (10 ng i.c.v.), or db-cAMP (100 ng i.c.v.) or vehicle. Naltrexone, forskolin, and db-cAMP were injected 30 min before morphine administration. B and C, mice were treated with forskolin (10 ng in 5 μl i.c.v.) or db-cAMP (100 ng in 5 μl i.c.v.) or vehicle [0.2% DMSO (v/v) in saline; 5 μl i.c.v.] for 30 min. Animals were sacrificed, and the hippocampi were quickly isolated on ice. Na^+,K^+-ATPase activity (D and E), intracellular cAMP concentration (A and B), and PKA activity (C) were measured as described under *Materials and Methods*. C, top, representative gel electrophoresis of PKA activity assays. Bottom, quantitative determination of the PKA activity by spectrophotometry. Data are expressed as the mean ± S.D. of three separate experiments performed in duplicate. *, $P < 0.05$; **, $P < 0.01$ compared with vehicle-treated control mice; ‡, $P < 0.01$ compared with mice treated with morphine alone. CTL, control; MOR, morphine; NTX, naltrexone; FOR, forskolin; DBC, db-cAMP; and NEG, negative control.

iors (data not shown). These results suggest that increase in cAMP concentration in mouse hippocampus may be related to the reduction of Na^+,K^+-ATPase activity after long-term morphine treatment and that changes in Na^+,K^+-ATPase activity may be one of the potential mechanisms underlying development of morphine dependence.

H89 Antagonized but OA Enhanced the Inhibitory Effect of Long-Term Morphine Treatment on Na^+,K^+-ATPase Activity. To determine whether the alteration of PKA activity and phosphorylation level of Na^+,K^+-ATPase was also associated with modulation of Na^+,K^+-ATPase activity by long-term morphine treatment, the effects of H89 or OA treatment on long-term morphine-induced reduction of Na^+,K^+-ATPase activity were detected. Application of H89 (1 nmol i.c.v.) to reduce the up-regulation of PKA activity induced by long-term morphine treatment significantly reversed the inhibitory effect of long-term morphine treatment on Na^+,K^+-ATPase activity (Fig. 6A), suggesting that enhancement of PKA activity was involved in reduction of Na^+,K^+-ATPase activity by long-term morphine treatment. However, administration of OA (100 pg i.c.v.) enhanced the inhibitory effect of long-term morphine treatment on Na^+,K^+-ATPase activity compared with morphine-treated alone group (Fig. 6B), suggesting that enhancement or maintenance of phosphorylation process of Na^+,K^+-ATPase in hippocampus was associated with reduction of Na^+,K^+-ATPase activity by long-term morphine treatment. Together, the findings suggested that reduction of Na^+,K^+-ATPase activity after long-term morphine treatment may attributed to up-regulation of cAMP/PKA signaling pathway.

Alteration of the Phosphorylation Levels but Not Protein Expression Abundance of Na^+,K^+-ATPase Involved the Modulation of Na^+,K^+-ATPase Activity by Morphine. Phosphorylation is a primary regulatory mechanism for activity of Na^+,K^+-ATPase (Bertorello et al., 1991; Cheng et al., 1997). The results shown above suggested that the PKA-mediated phosphorylation may involve in the regulation of Na^+,K^+-ATPase activity in the hippocampus by short- and long-term morphine administration. Because it has been reported that the $\alpha 3$ subunit seems to be expressed

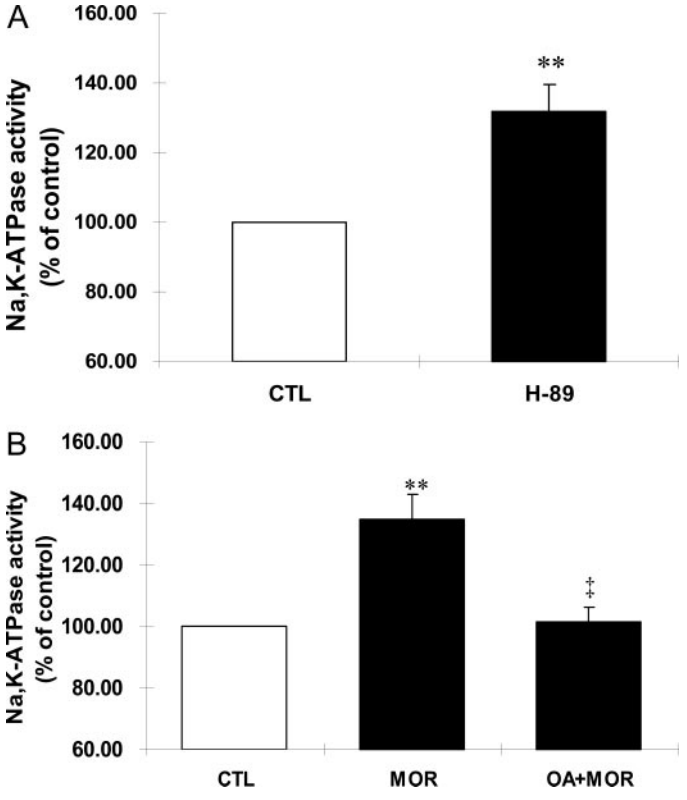

Fig. 4. H89 mimicked the stimulatory effect of short-term morphine on Na^+,K^+-ATPase activity, but OA suppressed the stimulatory effect of short-term morphine on Na^+,K^+-ATPase activity. A, mice were treated with H89 (1 nmol in 5 μl i.c.v.) or saline (5 μl i.c.v.) for 30 min. B, mice were treated with morphine (10 mg/kg s.c.) or saline (10 ml/kg) or concomitantly treated with OA (100 pg in 5 μl i.c.v.) or saline (5 μl i.c.v.) for 1 hr. OA was injected 30 min before morphine administration. After treatment, the hippocampus was quickly isolated, and membrane was prepared. Na^+,K^+-ATPase activity was measured as described under *Materials and Methods*. Data are expressed as the mean ± S.D. of three separate experiments performed in duplicate. **, $P < 0.01$ compared with vehicle-treated control mice; ‡, $P < 0.01$ compared with mice treated with morphine alone. CTL, control; and MOR, morphine.

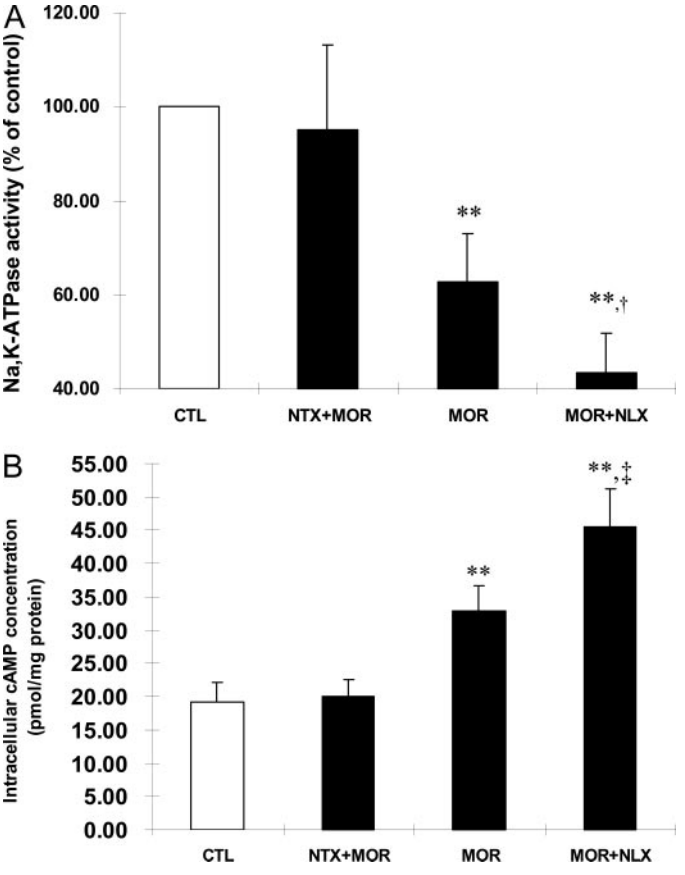

Fig. 5. Long-term morphine treatment decreased Na^+,K^+-ATPase activity but increased intracellular cAMP accumulation, and the effects were reversed by concomitant administration of naltrexone, and withdrawal morphine by naloxone precipitation led to an additional reduction of Na^+,K^+-ATPase activity and enhancement of cAMP concentration. Mice were continuously treated with morphine (10 mg/kg s.c.) or saline (10 ml/kg s.c.) or combination morphine with naltrexone (4 mg/kg i.p.) or saline (10 ml/kg i.p.) for 10 consecutive days. One hour after the final morphine or saline administration, one group of animals was sacrificed, and the hippocampi were isolated on ice. The other group of animals was treated identically with the exception that the mice were precipitated with naloxone (2 mg/kg i.p.) before sacrifice. Na^+,K^+-ATPase activity (A) and cAMP levels (B) were determined as described under *Materials and Methods*. Data are expressed as the mean ± S.D. of three independent experiments performed in duplicate. **, $P < 0.01$ compared with vehicle-treated mice; †, $P < 0.05$; ‡, $P < 0.01$ compared with mice treated with morphine alone. CTL, control; MOR, morphine; NLX, naloxone; and NTX, naltrexone.

predominantly in brain and plays a key role in the function of Na^+,K^+-ATPase (McGrail et al., 1991; Wang et al., 2003), next, the effect of short- and long-term morphine treatment on the phosphorylation of $\alpha 3$ subunit of Na^+,K^+-ATPase was assessed using the antibody against the enzyme $\alpha 3$ subunit, and the antibody (anti-pan) recognizing serine-, threonine-, and tyrosine-phosphorylated proteins. As shown in Fig. 7A, short-term morphine administration (10 mg/kg s.c.) resulted in a significant reduction of the total phosphorylation levels of the $\alpha 3$ subunit of Na^+,K^+-ATPase in the hippocampus. Pretreatment of naltrexone (4 mg/kg i.p.) could abolish the decrease in the basal total phosphorylation of the $\alpha 3$ subunit of the enzyme. In contrast to short-term morphine treatment, long-term morphine treatment led to marked enhancement of the phosphorylation levels of the $\alpha 3$ subunit of the enzyme in the hippocampus (Fig. 7B). Concurrent administration of naltrexone (4 mg/kg i.p.) fully antagonized the enhancement in phosphorylation of the enzyme by long-term morphine treatment. Likewise, H89 (1 nmol i.c.v.) also significantly suppressed the increase of phosphorylation of the enzyme by inhibiting up-regulation of PKA activity induced by long-term morphine treatment. The identical results were also obtained by using the antibody (anti-pan) recognizing serine-, threonine-, and tyrosine-phosphorylated proteins first and then by using the antibody against the enzyme $\alpha 3$ subunit (data not shown). Besides determination of phosphorylation of $\alpha 3$ subunit of Na^+,K^+-ATPase, the effects of short- and long-term morphine treatments on phosphorylation of $\alpha 1$ subunit were also detected. As shown in Fig. 7C, neither short- nor long-term morphine treatment could significantly regulate the basal phosphorylation of $\alpha 1$ subunit. The results suggest that alteration in phosphorylation level of Na^+,K^+-ATPase $\alpha 3$ but not $\alpha 1$ subunit was involved in modulation of Na^+,K^+-ATPase by morphine.

A reduction of the $\alpha 3$ subunit abundance in guinea pig myenteric neurons was also shown after long-term exposure to morphine by previous study (Biser et al., 2002). To determine whether changes in the expression abundance of Na^+,K^+-ATPase after morphine treatment also involved alteration of Na^+,K^+-ATPase activity in the hippocampus, two subunits ($\alpha 1$ and $\alpha 3$) of Na^+,K^+-ATPase were measured in the hippocampus by Western blot analyses. An apparent change in the abundance of the two subunits of Na^+,K^+-ATPase in the hippocampus was not observed by either short- or long-term morphine treatment (Fig. 8), and this was supported by previous study (Cheng and Aperia, 1998).

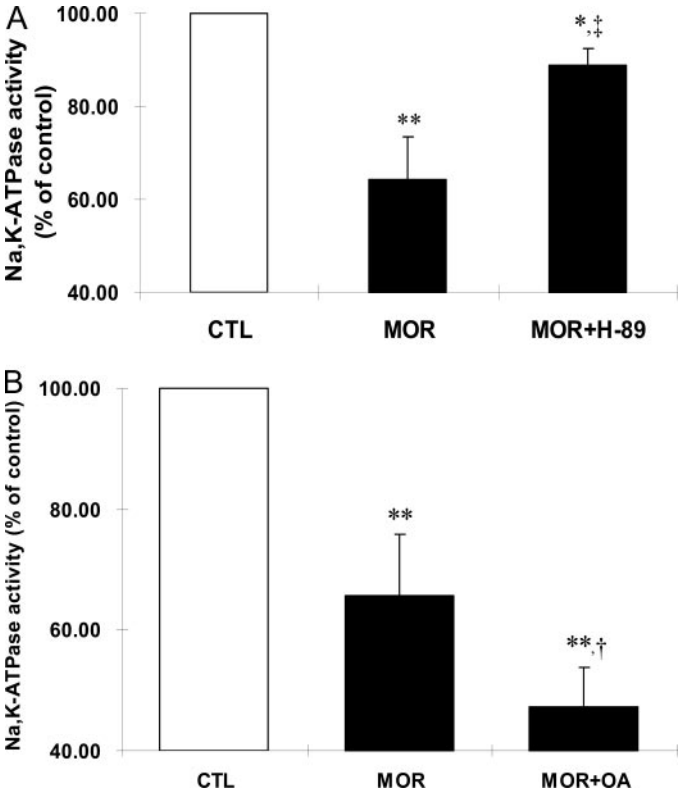

Fig. 6. Antagonism by H89 and potentiation by OA of the inhibitory effect of long-term morphine on Na^+,K^+-ATPase activity. A, reversal by H89 of inhibition of Na^+,K^+-ATPase activity induced by long-term morphine treatment. B, potentiation by OA of inhibition of Na^+,K^+-ATPase activity mediated by long-term morphine treatment. Mice were continuously treated with morphine (10 mg/kg s.c.) or saline (10 ml/kg s.c.) for 10 days. One hour after the termination of long-term morphine treatment, animals were given with H89 (1 nmol in 5 μl i.c.v.) or OA (100 pg in 5 μl i.c.v.) or saline (5 μl i.c.v.). Thirty minutes later, mice were decapitated, and the hippocampi were quickly isolated and membrane was prepared. Na^+,K^+-ATPase activity was measured as described under *Materials and Methods*. Data are expressed as the mean ± S.D. of three independent experiments performed in duplicate. *, $P < 0.05$; **, $P < 0.01$ compared with vehicle-treated control mice. †, $P < 0.05$ and ‡, $P < 0.01$ compared with morphine-treated mice. CTL, control; and MOR, morphine.

Discussion

Opioid receptors belong to the superfamily of G protein-coupled receptors that produce their effects by activation of pertussis toxin-sensitive $G_{i/o}$ proteins. Activation of opioid receptors by agonists leads to the regulation of several intracellular effectors, including the inhibition of adenylyl cyclase activity (Childers, 1991), the closing of voltage-gated Ca^{2+} channels (Piros et al., 1995), and the opening of inwardly rectifying K^+ channels (Henry et al., 1995). Among these intracellular effectors, adenylyl cyclase has been demonstrated to play a crucial role in opioid receptor-mediated functions and adverse effects. Short-term opiate exposure inhibits adenylyl cyclase activity and reduces cAMP production, whereas long-term opiate exposure leads to superactivation of adenylyl cyclase, manifested by a rebound of cAMP production upon administration of an opioid receptor antagonist or the abrupt cessation of the long-term opioid treatment (Childers, 1991; Nestler and Aghajanian, 1997). cAMP system has been shown to play multiple roles in the development of opioid dependence. The present study demonstrated a new effect of cAMP system upon activation of opioid receptors: stimulation of Na^+,K^+-ATPase activity upon activation by short-term morphine treatment and inhibition of Na^+,K^+-ATPase activity after activation by long-term morphine treatment in the hippocampus. The stimulation or inhibition of Na^+,K^+-ATPase activity in response to differential morphine treatments was because of reduction or enhancement of intracellular cAMP concentration, which led, at least in part, to changes in basal phosphorylation levels of Na^+,K^+-ATPase by cAMP/PKA signaling pathway.

The findings that opioid receptors can modulate Na^+,K^+-ATPase activity via cAMP/PKA signaling pathway are supported by several observations. First, both the stimulation of Na^+,K^+-ATPase by short-term morphine treatment and inhibition of Na^+,K^+-ATPase by long-term morphine treatment were naltrexone-reversible, indicating that the regula-

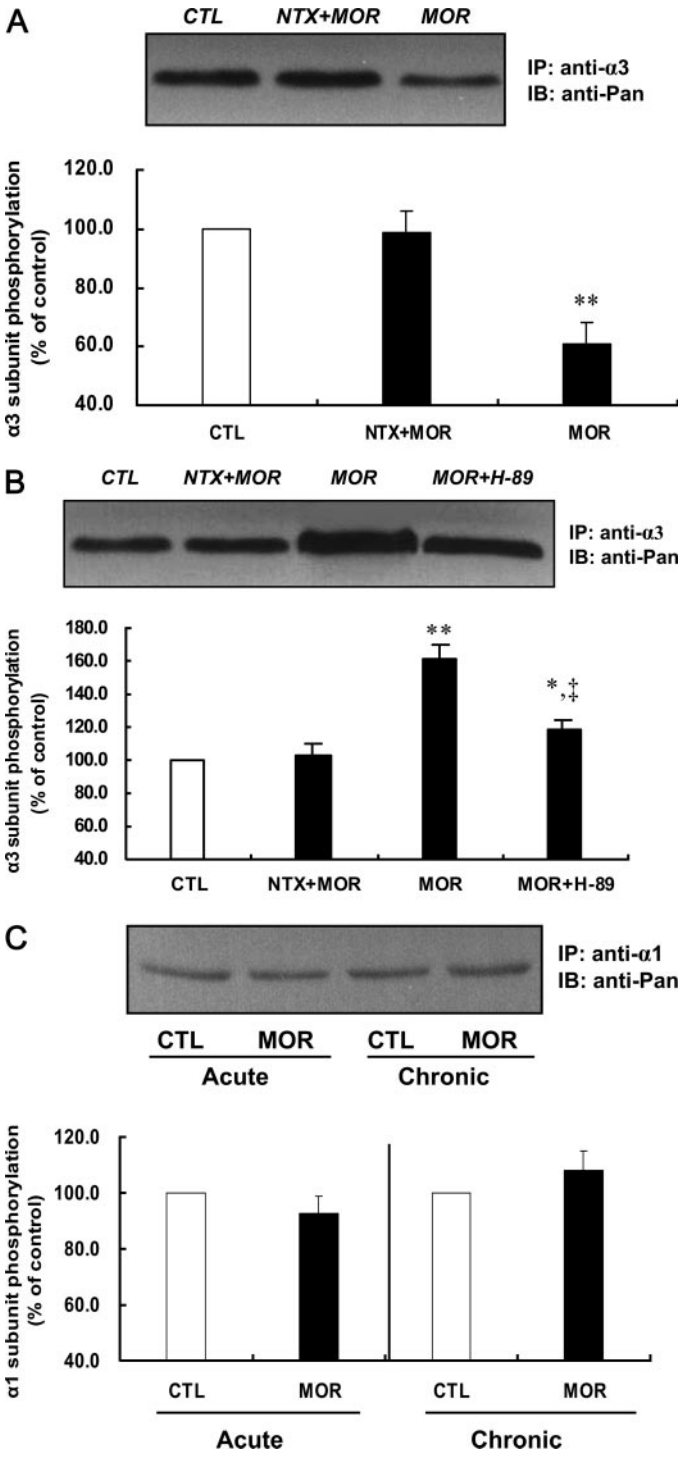

Fig. 7. Reduction by short-term morphine treatment and enhancement by long-term morphine treatment of the phosphorylation levels of α3 but not α1 subunit of Na^+,K^+-ATPase in the hippocampus. A, mice were treated with morphine (10 mg/kg s.c.) or saline (10 ml/kg s.c.) or concomitantly treated with naltrexone (4 mg/kg i.p.) or saline (10 ml/kg i.p.) for 1 h. Naltrexone and saline were administrated 30 min before morphine treatment. One hour after morphine treatment, mice were decapitated, and the hippocampi were isolated on ice. B, mice were continuously treated with morphine (10 mg/kg s.c.) or saline (10 ml/kg s.c.) or concomitantly treated with naltrexone (4 mg/kg i.p.) or saline (10 ml/kg i.p.) for 10 days. One hour after the final morphine or saline treatment, mice were treated with H89 (1 nmol in 5 μl i.c.v.) or saline (5 μl i.c.v.) for 30 min, and then the hippocampi were isolated on ice. C, mice were treated with morphine or saline for 1 h or 10 days as described in A and B. Phosphorylation assay was performed as described under *Materials and Methods.*

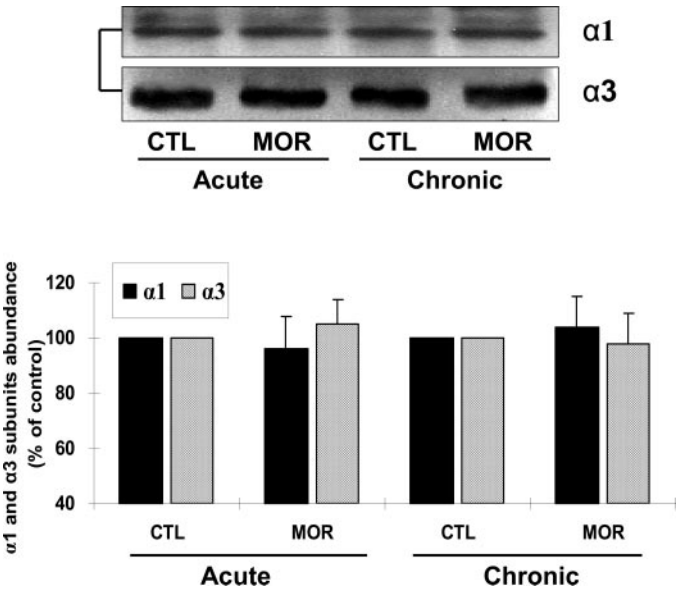

Fig. 8. Effect of short- and long-term morphine treatment on the expression of α1 and α3 subunits of the mouse hippocampal Na^+,K^+-ATPase. Equal quantities of protein prepared from short- and long-term morphine-treated mice were separated by SDS-PAGE and then incubated with the primary antibody against the α1 or α3 subunit of mouse Na^+,K^+-ATPase (antibody was diluted 1:600) overnight at 4°C. The blots were then incubated for 120 min at room temperature with horseradish peroxidase-conjugated donkey anti-goat IgG diluted 1:300 in Blotto. The antibody binding was detected using an enhanced chemiluminescence method following the manufacturer's instructions. Top, representative immunoblots for the α1 and α3 subunits of Na^+,K^+-ATPase. Bottom, quantitative estimation (by scanning densitometry) of the expression of α1 and α3 subunits of Na^+,K^+-ATPase. CTL, control; and MOR, morphine.

tion of Na^+,K^+-ATPase activity by short- or long-term morphine treatment was mediated by opioid receptors. Second, the regulation of Na^+,K^+-ATPase activity by short- or long-term morphine treatment was inversely correlated with intracellular cAMP accumulation. Short-term morphine treatment enhanced Na^+,K^+-ATPase activity but reduced cAMP concentration. The direct evidence to support the inverse relationship between cAMP concentration and Na^+,K^+-ATPase activity was that increase in cAMP concentration in hippocampus by concomitant administration of forskolin and db-cAMP markedly suppressed short-term morphine-induced enhancement of Na^+,K^+-ATPase activity. Contrary to short-term morphine treatment, long-term morphine treatment reduced Na^+,K^+-ATPase activity but enhanced cAMP concentration. Moreover, in long-term morphine-treated mice, naloxone precipitation induced a further reduction of Na^+,K^+-ATPase activity in hippocampus because it resulted in an additional increase of cAMP concentration. The findings convincingly demonstrated that cAMP was involved in modulation of Na^+,K^+-ATPase activity. Third, the selective PKA inhibitor H89 mimicked the stimu-

The α3 or α1 subunit of the enzyme was immunoprecipitated by the anti-α3 or anti-α1 antibody, respectively, and then was probed with the anti-pan antibody. A to C, top, representative immunoblots for the phosphorylated α3 and α1 subunits of Na^+,K^+-ATPase. Bottom, quantitative estimation by scanning densitometry of the total phosphorylation levels of the α3 and α1 subunits of Na^+,K^+-ATPase. Data were expressed as a percentage of the control, and values represent the mean ± S.D. of three independent experiments. *, $P < 0.05$; **, $P < 0.01$ compared with vehicle-treated control mice; ‡, $P < 0.01$ compared with morphine-treated mice. CTL, control; IB, immunoblotting; MOR, morphine; and NTX, naltrexone.

latory effect of short-term morphine on Na^+,K^+-ATPase activity but antagonized the inhibitory effect of long-term morphine on Na^+,K^+-ATPase activity. Importantly, H89 significantly inhibited long-term morphine-induced the phosphorylation of Na^+,K^+-ATPase, supporting that alteration of PKA activity was implicated in the regulation of Na^+,K^+-ATPase activity upon activation of opioid receptors. A significant increase in PKA activity in rat hippocampus after long-term morphine treatment has been reported by previous study (Pu et al., 2002). On the other hand, the protein phosphatase inhibitor OA suppressed the stimulatory effect of short-term morphine on Na^+,K^+-ATPase activity but potentiated the inhibitory effect of long-term morphine on Na^+,K^+-ATPase activity, suggesting that the phosphorylation level of Na^+,K^+-ATPase is inversely correlated with Na^+,K^+-ATPase activity. This is consistent with previous observations that phosphorylation of Na^+,K^+-ATPase is associated with inhibition of the enzyme activity (Bertorello et al., 1991; Cheng et al., 1997; Li et al., 1998). The direct evidence to support involvement of changes in basal phosphorylation level of Na^+,K^+-ATPase in regulation of Na^+,K^+-ATPase activity was that short-term morphine administration resulted in significant reduction of the total phosphorylation levels of the $\alpha 3$ subunit of Na^+,K^+-ATPase in the hippocampus, whereas long-term morphine treatment led to marked enhancement of the phosphorylation levels of the $\alpha 3$ subunit. Naltrexone could reverse the attenuation of phosphorylation of the $\alpha 3$ subunit of Na^+,K^+-ATPase by short-term morphine and enhancement of phosphorylation of the $\alpha 3$ subunit by long-term morphine. It is noteworthy that the regulation of phosphorylation of $\alpha 3$ subunit seems to be selective, because neither short- nor long-term morphine treatment significantly regulated the phosphorylation of the $\alpha 1$ subunit of Na^+,K^+-ATPase. There are several possible explanations for the different effects of morphine on the phosphorylation of the $\alpha 3$ and $\alpha 1$ subunits of Na^+,K^+-ATPase. First, the $\alpha 1$-low and $\alpha 3$-high expressions in the hippocampus (McGrail et al., 1991; Pietrini et al., 1992; Fig. 8) may be associated with this difference. For example, a selective inhibition of $\alpha 3$ subunit by dopamine has been observed in the rat retina that predominantly expresses $\alpha 3$ subunit (Shulman and Fox, 1996). Second, the different phosphorylation of $\alpha 3$ and $\alpha 1$ subunits might also be related to their different sensitivity to regulation by neurotransmitters because of their intrinsic structural difference as proposed by Sweadner (1985). In addition, at subcellular level, the $\alpha 3$ but not $\alpha 1$ subunit may be segregated with various signaling proteins (e.g., kinases, phosphatase, and receptors.) necessary for mediating second messenger regulation of activity (Nishi et al., 1999). Together, these results strongly demonstrate that cAMP/PKA signaling pathway is involved in regulation of Na^+,K^+-ATPase activity after activation of opioid receptors. It should be noted that although the present study showed involvement of PKA-mediated alteration of the phosphorylation of Na^+,K^+-ATPase in regulation of Na^+,K^+-ATPase by morphine, further work is needed to elucidate whether PKA acts on Na^+,K^+-ATPase directly or effects through the ancillary proteins.

Phosphorylation of the catalytic α-subunit of Na^+,K^+-ATPase by protein kinases has been reported to be associated with triggering for endocytosis of Na^+,K^+-ATPase in other tissue (Chibalin et al., 1998a,b). Although the present study demonstrated that neither short- nor long-term morphine treatment could significantly change the abundance of $\alpha 1$ or $\alpha 3$ subunit of Na^+,K^+-ATPase protein, this result did not exclude the possibility of occurrence of endocytosis and subsequent intracellular trafficking upon phosphorylation by PKA because different preparations (e.g., crude membranes versus clathrin-coated vesicle and endosomes) or distinct experimental conditions (e.g., time used for drug treatment) were applied in this study.

There are two well established adaptations to long-term opioid exposure, which are linked to opioid tolerance and dependence (for review, see Taylor and Fleming, 2001). One adaptation is the up-regulation of cAMP cascade, which was suggested to represent a cellular correlate of opioid withdrawal and has been used to define a state of dependence (Sharma et al., 1975; Childers, 1991; Nestler and Aghajanian, 1997). The other adaptation is the depolarization of neuronal cells as a result of reduction in Na^+,K^+-ATPase function, which has been demonstrated to associate the development of opioid tolerance and dependence (Kong et al., 1997, 2001). Although up-regulation of cAMP cascade and depolarization of neuronal membrane potential both are suggested to associate with the development of opioid tolerance and dependence, future work is needed to determine whether two adaptations are independent mechanisms of tolerance and dependence induction or are different steps in the same mechanism. The present study demonstrated that up-regulation of cAMP cascade would induce the phosphorylation of $\alpha 3$ subunit of Na^+,K^+-ATPase, leading to diminution of Na^+,K^+-ATPase activity, which may contribute to the depolarization of neuron, thereby resulting in opioid tolerance and dependence. Up-regulation of cAMP/PKA signaling pathway upon long-term exposure to opioid modulates many effectors, from the phosphorylation of receptors, ion channels, and intracellular messenger proteins to the phosphorylation and activation of cAMP response element-binding protein, a transcription factor that regulates the expression of numerous genes (for review, see Nestler and Aghajanian, 1997; Liu and Anand, 2001), the present results demonstrated a new adaptation: the phosphorylation of Na^+,K^+-ATPase. This may give an insight into the mechanisms underlying opioid tolerance and dependence.

Na^+,K^+-ATPase (pump) is ubiquitous in neurons and widely distributed in the mammalian brain (Hauger et al., 1985). It maintains Na^+ and K^+ gradient across cell membrane by returning Na^+ and K^+ to their resting transmembrane levels after bursts of activity and contributes to the cell resting membrane potential. It also has secondary effects on processes involving monovalent cation gradients such as Na^+-Ca^{2+} exchanger (Blaustein and Lederer, 1999). Impairment of the Na^+,K^+ pump reduces intracellular K^+ and increases intracellular Na^+, consequently leading to the membrane depolarization and enhancement of intracellular free Ca^{2+} because of activation of voltage-gated Ca^{2+} channels and reverses operation of the Na^+-Ca^{2+} exchanger (Blaustein and Lederer, 1999). It has been reported that Na^+,K^+-ATPase is involved in synaptic plasticity (Glushchenko and Izvarina, 1997; Ross and Soltesz, 2001; Reich et al., 2004) and plays a role in the process of learning and memory (Christian et al., 2004; Wyse et al., 2004). Impairment of Na^+,K^+-ATPase was proposed to be responsible for memory loss and other cognitive disturbance caused by brain injury (Ross and Soltesz, 2000; Reich et al., 2004). Inhibition

of Na^+,K^+-ATPase with dihydroouabain, a ouabain analog, produced a long-lasting suppression of field excitatory postsynaptic potentials in rat hippocampal CA1 cells, which leads to depotentiation of long-term potentiation (LTP) (Reich et al., 2004). LTP is thought to be a possible neuronal mechanism underlying learning and memory (Bliss and Collingridge, 1993). It has been proposed that drug dependence is an aberrant form of learning mediated by maladaptive recruitment of certain memory systems in brain (Robbins and Everitt, 1999). Long-term use of opiates has been reported to potentially attenuate hippocampal LTP (Pu et al., 2002). Diminution of Na^+,K^+-ATPase activity induced by up-regulation of cAMP/PKA signaling pathway after long-term exposure to opioids may be a potential mechanism by which opioids lead to maladaptive changes in hippocampal LTP, thereby contributing to the development of opioid dependence. Future work will be required to address this issue.

In summary, the present study demonstrated that both short-term and long-term morphine treatment could modulate Na^+,K^+-ATPase activity in the mouse hippocampus by cAMP/PKA signaling pathway. The regulation of Na^+,K^+-ATPase activity by morphine treatment seemed to associate with the alteration in phosphorylation level of Na^+,K^+-ATPase. The results suggest that diminution of Na^+,K^+-ATPase mediated by up-regulated cAMP/PKA cascade may be a potential mechanism by which long-term exposure to morphine results in opioid dependence.

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References

Bertorello AM, Aperia A, Walaas SI, Nairn AC, and Greengard P (1991) Phosphorylation of the catalytic subunit of Na^+,K^+-ATPase inhibits the activity of the enzyme. *Proc Natl Acad Sci USA* **88:**11359–11362.

Biser PS, Thayne KA, Fleming WW, and Taylor DA (2002) Na^+,K^+-ATPase alpha-subunit subunit distribution and abundance in guinea-pig longitudinal muscle/myenteric plexus after exposure to morphine. *Brain Res* **931:**186–193.

Blaustein MP and Lederer WJ (1999) Sodium/calcium exchange: its physiological implications. *Physiol Rev* **79:**763–854.

Bliss TVP and Collingridge GL (1993) A synaptic model of memory: long-term potentiation in the hippocampus. *Nature (Lond)* **361:**31–39.

Cheng X-J, Fisone G, Aizman O, Aizman R, Levenson R, Greengard P, and Aperia A (1997) PKA-mediated phosphorylation and inhibition of Na^+,K^+-ATPase in response to beta-adrenergic hormone. *Am J Physiol* **273:**C893–C901.

Cheng X-J and Aperia A (1998) Forskolin-induced down-regulation of Na^+,K^+-ATPase activity is not associated with internalization of the enzyme. *Acta Physiol Scand* **164:**39–46.

Chibalin AV, Pedemonte CH, Katz AI, Feraille E, Berggren P-O, and Bertorrello AM (1998a) Phosphorylation of the catalytic α subunit constitutes a triggering signal for Na^+,K^+-ATPase endocytosis. *J Biol Chem* **273:**8814–8819.

Chibalin AV, Zierath JR, Katz AI, Berggren P-O, and Bertorello AM (1998b) Phosphatidylinositol 3-kinase-mediated endocytosis of renal Na^+,K^+-ATPase α subunit in response to dopamine. *Mol Biol Cell* **9:**1209–1220.

Childers SR (1991) Opioid receptor-coupled second messenger systems. *Life Sci* **48:**1991–2003.

Christian B, McConnaughey K, Bethea E, Brantley S, Coffey A, Hammond L, Harrell S, Metcalf K, Muehlenbein D, Spruill W, et al. (2004) Chronic aspartame affects T-maze performance, brain cholinergic receptors and Na^+,K^+-ATPase in rats. *Pharmacol Biochem Behav* **78:**121–127.

Desaiah D and Ho IK (1977) Effect of morphine on mice brain ATPase activities. *Biochem Pharmacol* **26:**89–92.

Esmann M (1988) ATPase and phosphatase activity of Na^+,K^+-ATPase: molar and specific activity, protein determination. *Methods Enzymol* **156:**105–115.

Fan G-H, Wang L-Z, Qiu H-C, Ma L, and Pei G (1999) Inhibition of calcium/calmodulin-dependent protein kinase II in rat hippocampus attenuates morphine tolerance and dependence. *Mol Pharmacol* **56:**39–45.

Glushchenko TS and Izvarina NL (1997) Na^+,K^+-ATPase activity in neurons and glial cells of olfactory cortex of rat brain during the development of long-term potentiation. *Neurosci Behav Physiol* **27:**49–52.

Hauger R, Luu HM, Meyer DK, Goodwin FK, and Paul SM (1985) Characterization of "high-affinity" [^{3}H]ouabain binding in the rat central nervous system. *J Neurochem* **44:**1709–1715.

Henry DJ, Grandy DK, Lester HA, Davidson N, and Chavkin C (1995) κ-Opioid receptors couple to inwardly rectifying potassium channels when coexpressed by *Xenopus* oocytes. *Mol Pharmacol* **47:**551–557.

Horvath G, Agil A, Joo G, Dobos I, Benedek G, and Baeyens JM (2003) Evaluation of endomorphin-1 on the activity of Na^+,K^+-ATPase using in vitro and in vivo studies. *Eur J Pharmacol* **458:**291–297.

Kong J-Q, Leedham JA, Taylor DA, and Fleming WW (1997) Evidence that tolerance and dependence of guinea pig myenteric neurons to opioids is a function of altered electrogenic sodium-potassium pumping. *J Pharmacol Exp Ther* **280:**593–599.

Kong J-Q, Meng J, Biser PS, Fleming WW, and Taylor DA (2001) Cellular depolarization of neurons in the locus ceruleus region of the guinea pig associated with the development of tolerance to opioids. *J Pharmacol Exp Ther* **298:**909–916.

Law PY, Wong YH, and Loh HH (2000) Molecular mechanisms and regulation of opioid receptor signaling. *Annu Rev Pharmacol Toxicol* **40:**389–430.

Li D, Cheng SX, Fisone G, Caplan MJ, Ohtomo Y, and Aperia A (1998) Effects of okadaic acid, calyculin A and PDBu on state of phosphorylation of rat renal Na^+,K^+-ATPase. *Am J Physiol* **275:**F863–F869.

Liu J-G and Anand KJS (2001) Protein kinases modulate the cellular adaptations associated with opioid tolerance and dependence. *Brain Res Rev* **38:**1–19.

Liu J-G, Rovnaghi CR, Garg S, and Anand KJS (2004) Opioid receptor desensitization contributes to thermal hyperalgesia in infant rats. *Eur J Pharmacol* **491:**127–136.

Masocha W, Gonzalez LG, Baeyens JM, and Agil A (2002) Mechanisms involved in morphine-induced activation of synaptosomal Na^+,K^+-ATPase. *Brain Res* **957:**311–319.

McGrail K, Phillips JM, and Sweadner KJ (1991) Immunofluorescent localization of three Na^+,K^+-ATPase isozymes in the rat central nervous system: both neurons and glia can express more than one Na^+,K^+-ATPase. *J Neurosci* **11:**381–391.

Nestler EJ and Aghajanian GK (1997) Molecular and cellular basis of addiction. *Science (Wash DC)* **278:**58–62.

Nishi A, Fisone G, Snyder GL, Dulubova I, Aperia A, Nairn AC, and Greengard P (1999) Regulation of Na^+,K^+-ATPase isoforms in rat neostriatum by dopamine and protein kinase C. *J Neurochem* **73:**1492–1501.

Parenti M, Tirone F, Giagnoni G, Pecora N, and Parolaro D (1986) Pertussis toxin inhibits the antinociceptive action of morphine in the rat. *Eur J Pharmacol* **124:**357–359.

Pietrini G, Matteoli M, Banker G, and Caplan MJ (1992) Isoforms of the Na^+,K^+-ATPase are present in both axons and dendrites of hippocampal neurons in culture. *Proc Natl Acad Sci USA* **89:**8414–8418.

Piros ET, Prather PL, Loh HH, Law PY, Evans CJ, and Hales TG (1995) Ca^{2+} channel and adenylyl cyclase modulation by cloned-opioid receptors in GH_3 cells. *Mol Pharmacol* **47:**1041–1049.

Pu L, Bao G-B, Xu N-J, Ma L, and Pei G (2002) Hippocampal long-term potentiation is reduced by chronic opiate treatment and can be restored by re-exposure to opiates. *J Neurosci* **22:**1914–1921.

Reich CG, Mason SE, and Alger BE (2004) Novel form of LTD induced by transient, partial inhibition of the Na,K-pump in rat hippocampal CA1 cells. *J Neurophysiol* **91:**239–247.

Robbins TW and Everitt BJ (1999) Drug addiction: bad habits add up. *Nature (Lond)* **398:**567–570.

Ross ST and Soltesz I (2000) Selective depolarization of interneurons in the early posttraumatic dentate gyrus: involvement of the Na^+,K^+-ATPase. *J Neurophysiol* **83:**2916–2930.

Ross ST and Soltesz I (2001) Long-term plasticity in interneurons of the dentate gyrus. *Proc Natl Acad Sci USA* **98:**8874–8879.

Roth BL, Laskowaki MB, and Coscia CJ (1981) Evidence for distinct subcellular sites of opiate receptors. *J Biol Chem* **256:**10117–10123.

Sharma P, Kaur G, Bhardwaj SK, and Kaur G (1998) Role of opioidergic and monoaminergic neurotransmission in the GnRH release mechanism of EBP-primed OVX rats. *Brain Res Bull* **47:**81–86.

Sharma SK, Klee WA, and Nirenberg M (1975) Dual regulation of adenylate cyclase accounts for narcotic dependence and tolerance. *Proc Natl Acad Sci USA* **72:**3092–3096.

Shulman LM and Fox DA (1996) Dopamine inhibits mammalian photoreceptor Na^+,K^+-ATPase via a selective effect on the $\alpha 3$ isozyme. *Proc Natl Acad Sci USA* **93:**8034–8039.

Sweadner KJ (1985) Enzymatic properties of separated isozymes of the Na^+,K^+-ATPase. Substrate affinities, kinetic cooperativity and ion transport stoichiometry. *J Biol Chem* **260:**11508–11513.

Taussky HH and Shorr E (1953) A microcalorimetric method for the determination of inorganic phosphorus. *J Biol Chem* **202:**675–685.

Taylor DA and Fleming WW (2001) Unifying perspectives of the mechanisms underlying the development of tolerance and physical dependence to opioids. *J Pharmacol Exp Ther* **297:**11–18.

Vorel SR, Liu X, Hayes RJ, Spector JA, and Gardner EL (2001) Relapse to cocaine-seeking after hippocampal theta burst stimulation. *Science (Wash DC)* **292:**1175–1178.

Wang XQ, Xiao AY, Sheline C, Hyrc K, Yang A, Goldberg MP, Choi DW, and Yu SP (2003) Apoptotic insults impair Na^+,K^+-ATPase activity as a mechanism of neuronal death mediated by concurrent ATP deficiency and oxidant stress. *J Cell Sci* **116:**2099–2110.

Wyse AT, Bavaresco CS, Reis EA, Zugno AI, Tagliari B, Calcagnotto T, and Netto CA (2004) Training in inhibitory avoidance causes a reduction of Na^+,K^+-ATPase activity in rat hippocampus. *Physiol Behav* **80:**475–479.

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