

Involvement of Dopamine System in Regulation of Na^+, K^+ -ATPase in the Striatum upon Activation of Opioid Receptors by Morphine

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ABSTRACT

The striatum is believed to be a crucial brain region associated with drug reward. Adaptive alteration of neurochemistry in this area might be one potential mechanism underlying drug dependence. It has been proposed that the dysfunction of Na^+, K^+ -ATPase function is involved in morphine tolerance and dependence. The present study, therefore, was undertaken to study the adaptation of the striatal Na^+, K^+ -ATPase activity in response to morphine treatment. The results demonstrated that in vivo short-term morphine treatment stimulated Na^+, K^+ -ATPase activity in a dose-dependent manner. This action could be significantly inhibited by D_2 -like dopamine receptor antagonist *S*(-)-3-chloro-5-ethyl-*N*-[(1-ethyl-2-pyrrolidinyl)methyl]-6-hydroxy-2-methoxybenzamine (eticlopride). Contrary to short-term morphine treatment, long-term morphine treatment significantly suppressed Na^+, K^+ -ATPase activity. This effect could be significantly inhibited by D_1 -like dopamine receptor antagonist *R*(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrochloride (SCH 23390).

However, both short-term and long-term morphine treatment-induced changes in Na^+, K^+ -ATPase activity could be reversed by opioid receptor antagonist naltrexone. It was further found that cAMP-dependent protein kinase (PKA) was crucially involved in regulating Na^+, K^+ -ATPase activity by morphine. Different regulation of the phosphorylation levels of the $\alpha 3$ subunit of Na^+, K^+ -ATPase by PKA was related to the distinct modulations of Na^+, K^+ -ATPase by short-term and long-term morphine treatment. Short-term morphine treatment inhibited PKA activity and then decreased the phosphorylation of Na^+, K^+ -ATPase, leading to increase in enzyme activity. These effects were sensitive to eticlopride or naltrexone. Conversely, long-term morphine treatment stimulated PKA activity and then increased the phosphorylation of Na^+, K^+ -ATPase, leading to the reduction of enzyme activity. These effects were sensitive to SCH 23390 or naltrexone. These findings demonstrate that dopamine receptors are involved in regulation of Na^+, K^+ -ATPase activity after activation of opioid receptors by morphine.

Repeated exposure to morphine leads to drug dependence, which is characterized by a somatic abstinence syndrome and by the subjective responses such as euphoria and craving

after morphine withdrawal (Robbins and Everitt, 1999). The mechanisms underlying morphine dependence have not been fully understood yet.

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Morphine dependence is involved in multiple brain regions and neurotransmitter systems. The mesolimbic and nigrostriatal dopamine systems play a vital role in mediating drug reward (Koob, 1992) and addiction-related behaviors (Graybiel et al., 1990). Change in dopaminergic activity in these brain regions has been found to underlie the reinforcing effects and the expression of somatic abstinence of opiates and other drugs of abuse (Koob, 1992; Harris and Aston-Jones, 1994). The mesolimbic and nigrostriatal dopaminergic

ABBREVIATIONS: SCH 23390, *R*(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrochloride; DARPP-32, dopamine- and cAMP-regulated phosphoprotein of 32 kDa; PMSF, phenylmethylsulfonyl fluoride; db-cAMP, *N*⁶,2'-*O*-dibutyryl adenosine 3',5'-cyclic monophosphate sodium salt; H-89, *N*-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline; PAGE, polyacrylamide gel electrophoresis; PKA, cAMP-dependent protein kinase; TBST, Tris-buffered saline/Tween 20; Blotto, bovine lacto transfer technique optimizer; eticlopride, *S*(-)-3-chloro-5-ethyl-*N*-[(1-ethyl-2-pyrrolidinyl)methyl]-6-hydroxy-2-methoxybenzamine hydrochloride; SKF 38393, ($\pm$)-1-phenyl-2,3,4,5-tetrahydro-(1*H*)-3-benzazepine-7,8-diol hydrochloride; quinpirole, *trans*(-)-(4*aR*)-4,4*a*,5,6,7,8,8*a*,9-octahydro-5-propyl-1*H*-pyrazolo[3,4-*g*]quinoline hydrochloride; DTT, dithiothreitol; IP, immunoprecipitation; buffer A, NaCl, KCl, MgCl_2 , EGTA, and Tris-HCl; buffer B, NaCl, KCl, MgCl_2 , EGTA, Tris-HCl, and ouabain.

systems originate in ventral tegmental area and substantia nigra and project to the ventral striatum (nucleus accumbens) and dorsal striatum (caudate putamen), respectively. Dopamine regulates the two major striatal efferent neurons through differential dopamine receptors. It regulates the striatonigral neurons via D₁ dopamine receptors but regulates the striatopallidal neurons via D₂-dopamine receptors (Gerfen et al., 1990; LeMoine and Bloch et al., 1995).

In the striatum, dopaminergic and opiodergic neurons display interactions in regulating the function of efferent striatal neurons. For example, morphine acutely increases dopamine release in caudate putamen and nucleus accumbens (Pothos et al., 1991; Di Chiara and North, 1992; Pontieri et al., 1995) through the disinhibition of dopamine neurons by activation of μ -opioid receptors located on GABA-containing interneurons in the substantia nigra and ventral tegmental area (Johnson and North, 1992; Bontempi and Sharp, 1997). Therefore, the D₁-like dopamine receptor antagonist SCH 23390 can block a short-term morphine-induced increase in *c-fos* expression in the nucleus accumbens and caudate putamen (Bontempi and Sharp, 1997). In addition, it has been shown that the up-regulation of Δ FosB in the nucleus accumbens and caudate putamen upon long-term exposure to morphine is also attenuated by SCH 23390 (Muller and Unterwald, 2005). Furthermore, the down-regulation of D₂ dopamine receptor mRNA levels after long-term morphine treatment has also been reported (Navarro et al., 1992; Georges et al., 1999). Although these studies suggest that in the striatum, dopamine receptors may be essentially required for the cellular and molecular adaptations in response to morphine treatments, the mechanisms underlying the dopaminergic neurons contribution to opiate dependence remain illusive.

Indirect activation of dopamine receptors by opiates such as morphine may act on several effectors such as ion channels, and adenylyl cyclase. Na⁺,K⁺-ATPase is another well-studied downstream target of the action of dopamine in the striatum (Bertorello et al., 1990; Fienberg et al., 1998; Nishi et al., 1999). Na⁺,K⁺-ATPase is a protein responsible for maintaining the cell resting membrane potential by pumping sodium and potassium against the electrochemical gradient across cell membrane for these ions. This ubiquitous protein is particularly abundant in brain areas such as the striatum, in which it plays a fundamental role in maintaining cellular ionic gradients that are required for neural activity, transport of amino acids and glucose, and regulation of cell pH and volume. Given its important role in regulating neural excitability, the impairment of Na⁺,K⁺-ATPase activity might lead to a neural dysfunction. Morphine has been demonstrated to modulate Na⁺,K⁺-ATPase activity in several brain regions including forebrain, cortex, hippocampus, locus ceruleus, and myenteric plexus of the guinea pig (Kong et al., 1997, 2001; Sharma et al., 1998; Wu et al., 2006). Moreover, the impairment of Na⁺,K⁺-ATPase in locus ceruleus and myenteric plexus of the guinea pig after long-term exposure to morphine is linked to opioid tolerance and dependence (Kong et al., 1997, 2001; Taylor and Fleming, 2001). However, the effect of repeated exposure to morphine on striatal Na⁺,K⁺-ATPase activity has never been addressed specifically, although dopamine pathway in the striatum is believed to be a crucial brain region associated with opiate addiction. In this regard, the present study was undertaken to investi-

gate how short-term and long-term morphine treatments modulate Na⁺,K⁺-ATPase activity in the striatum and what role dopamine receptors might play in morphine-mediated changes of striatal Na⁺,K⁺-ATPase activity.

Materials and Methods

Materials. Morphine hydrochloride was purchased from Qinghai Pharmaceutical General Factory (Qinghai, China). SDS and dithiothreitol (DTT) were purchased from AMRESCO (Solon, OH). PMSF and Triton X-100 were obtained from Merck (Darmstadt, Germany). Naltrexone, db-cAMP, H-89, SCH 23390, eticlopride, SKF 38393, quinpirole, 3-isobutyl-1-methylxanthine, and ouabain were supplied by Sigma Chemical Co. (St. Louis, MO).

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 a 12-h light/dark cycle in temperature-controlled environment with free access to food and water. All animals were treated 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 subcutaneous injection of morphine at a dose of 10 mg/kg or across a range of doses from 1 to 20 mg/kg for 1 h, or treated with morphine (10 mg/kg s.c.) for across a range of times from 0.5 to 4 h. Naltrexone, eticlopride, or SCH 23390 was given intraperitoneally 30 min before morphine administration at the doses of 4, 1, or 1 mg/kg, respectively (Taylor et al., 2005; Wu et al., 2006). db-cAMP was administrated i.c.v. 30 min before morphine injection at a dose of 100 ng. In some experiments, the animals were treated with SKF 38393 (3 and 6 mg/kg i.p.), quinpirole (1 and 2 mg/kg i.p.), or saline (10 ml/kg i.p.) for 30 min. For long-term treatment, mice were treated with morphine (10 mg/kg s.c.) twice per day at 12-h intervals for 10 days as described previously (Pu et al., 2002). Naltrexone, eticlopride, or SCH 23390 was given intraperitoneally 30 min before morphine administration at the doses of 4, 1, or 1 mg/kg, respectively. In a control group, mice were treated similarly except that saline was used as a substitute for morphine or antagonists. In some experiments, 1 h after the injection or final injection of morphine, animals were injected i.c.v. with H-89 (1 nmol), and then they were decapitated 30 min later (Pu et al., 2002). After treatment, the animals were sacrificed by decapitation and then the striata were isolated rapidly on ice and then stored at –80°C until use.

Membrane Preparation. Plasma membranes were prepared as described previously (Roth et al., 1981). In brief, striatal tissues from three mice were homogenized on ice in 1 ml of homogenization buffer, pH 7.4, composed of 5 mM HEPES, 1 mM PMSF, 50 μ M CaCl₂, 10% (w/v) sucrose, and 1 mM DTT and centrifuged at 1000g for 10 min at 4°C to remove cellular debris and nuclei. The supernatant was centrifuged at 12,000g for 20 min at 4°C to yield the crude plasma membranes (P₂ pellets). The obtained pellet was washed an additional three times by resuspension and recentrifugation at 14,000g 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, 100 μ l of aliquot containing 40 μ g of protein was preincubated at 37°C for 10 min with 850 μ l of reaction buffer A containing 100 mM NaCl, 20 mM KCl, 2 mM MgCl₂, 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 (reaction buffer B). The reaction was initiated by adding

50 μ l 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 the addition of trichloroacetic acid [0.2 ml, 50% (w/v)]. The tube was put on ice for 15 min, followed by centrifugation at 10,000g at 4°C for 10 min. Then, 80 μ l of supernatant was taken for the assay of liberated inorganic phosphate. In brief, 80 μ l of the supernatant was pipetted into the appropriate well of the 96-well plate, 160 μ l of ammonium molybdate solution color reagent was added to the well, and the absorbance was read at 700 nm with a microplate reader (Molecular Devices, Sunnyvale, CA) after a 5-min incubation at room temperature, using Na₂HPO₄ as standard. Na⁺,K⁺-ATPase activity was obtained by the difference between total ATPase and ouabain-insensitive Mg²⁺-ATPase activity (Esmann, 1988).

In Vitro Assay. The crude striatal synaptosomes (P₂ pellets) from morphine-untreated control mice 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 CaCl₂, 25 mM HEPES, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, and 10 mM glucose, pH 7.4, to give a protein concentration of 1 mg/ml. A 100- μ l sample of aliquot was pipetted into the appropriate well of the 24-well plate, followed by addition of 890 μ l of the Krebs-Ringer-HEPES medium into the well, and then the plate was preincubated for 15 min at 37°C; 10 μ l of different concentrations of morphine was added to the appropriate wells followed by an additional incubation for 10 min at 37°C, or 10 μ l of morphine (1 mM) was added to the appropriate wells followed by an additional incubation for 1 to 15 min at 37°C. In some experiments, naltrexone (10 μ M), eticlopride (100 μ M), or SCH 23390 (100 μ M) was added to the appropriate wells and incubated for 5 min at 37°C before morphine treatments. After incubation, 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 μ l/tube) followed by centrifugation at 14,000g for 20 min at 4°C. The pellets were resuspended in 950 μ l of the assay buffer A or B (see above) and preincubated at 37°C for 10 min. The reaction was initiated by adding 50 μ l of ATP disodium solution (final ATP concentration in the medium was 2.5 mM), followed by incubation for 10 min at 37°C. Na⁺,K⁺-ATPase activity was determined as described above.

PKA Activity Assay. PKA activity was determined as described previously (Pu et al., 2002). The striatum was homogenized on ice in homogenization buffer (25 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM DTT, and 100 μ M leupeptin). The homogenate was centrifuged at 20,000g for 5 min at 4°C. The supernatant was taken for PKA activity assay using PepTag nonradioactive PKA assay kit (Promega, Madison, WI) as described in Promega Technical Bulletin. All reaction components were added on ice in a final volume of 25 μ l of the following mixture: 5 μ l of PepTag PKA reaction buffer, 5 μ l of PepTag A1 Peptide (0.4 μ g/ μ l), 5 μ l of cAMP (5 μ M), 1 μ l of PepTag A1 Peptide protection buffer, 4 μ l of H₂O, and 5 μ l of sample homogenate (0.2 μ g/ μ l). The mixture was incubated for 30 min at room temperature. The reaction was terminated by boiling samples at 100°C for 10 min in water bath, and then the samples were loaded onto the 0.8% agarose gel for electrophoresis. Before loading samples, 1 μ l of 80% glycerol was added to the sample. 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. The phosphorylated species migrated toward the positive electrode, whereas the nonphosphorylated substrate migrated toward the negative electrode.

Immunoblotting Assay. For immunoblotting assay, the crude plasma membranes were prepared as described above 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 μ M 3-isobutyl-1-methylxanthine, 50 mM NaF, 2 μ g/ml aprotinin, and 5 μ g/ml leupeptin. The final pellet was suspended on ice in the same homogenization buffer to give a protein concentration of 3 mg/ml. An equal

volume of 2 $\times$ 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 using the Mini-Protein 3 apparatus (Bio-Rad, Hercules, CA). In brief, equal quantities of protein were separated by SDS-polyacrylamide gel electrophoresis (PAGE) on 8% SDS polyacrylamide gel for approximately 90 min at 120 V and then electroblotted onto nitrocellulose membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK). 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 primary antibody against the α 1 or α 3 subunit of Na⁺,K⁺-ATPase (1:600; Santa Cruz Biotechnology, Santa Cruz, CA) or the β -actin (1:5000; Sigma), and the membrane was incubated overnight at 4°C followed by washing in TBST for 15 min. Wash was repeated twice more. The membrane was then incubated for 120 min at room temperature in the appropriate secondary antibody (1:2000; horseradish peroxidase-conjugated IgG; Calbiochem, Darmstadt, Germany) in Blotto. After three 15-min extensive washes in TBST, the antibody binding was detected using an enhanced chemiluminescence method (GE Healthcare) according to the manufacturer's instructions.

Immunoprecipitating Assay. The crude plasma membranes were prepared as described above. Protein phosphorylation assay was performed as described previously (Wu et al., 2006). 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 Na₄O₇P₂, 1 mM Na₃VO₄, 1 mM PMSF, 10 μ g/ml leupeptin, 4 μ g/ml aprotinin, and 1% Triton X-100) and incubated with rotation at 4°C for 2 h. Insoluble material was removed by centrifugation at 12,000g for 20 min at 4°C, and the concentration of protein in the supernatant was determined as described above. Equal amount of protein from the supernatants were incubated with rotation overnight at 4°C with Na⁺-K⁺-ATPase α 1 or α 3 subunit antibodies. A saturating amount of Protein A/G PLUS-Agarose beads (prewashed with IP buffer for three times) were 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 8000g for 5 min at 4°C. An equal volume of 2 $\times$ sample buffer were added and boiled at 100°C for 10 min. The samples were separated by 8% SDS-PAGE gels transferred to nitrocellulose membranes and probed with anti-pan phosphorylated protein antibody (1:250; Zymed, South San Francisco, CA), or α 1 or α 3 subunit antibody (1:600) as described above (see Immunoblotting Assay).

Statistical Analysis. All data are presented as the mean $\pm$ S.D. from three independent experiments, and the results of each experiment performed in duplicate were from three animals in each group. Unless indicated, statistical analysis was performed by one-way analysis of variance followed by Newman-Keuls test. Differences with a *P* value of less than 0.05 were considered statistically significant.

Results

In Vivo Short-Term Morphine Treatment Increased Na⁺,K⁺-ATPase Activity, and This Effect Was Reversed by Opioid Receptor Antagonist Naltrexone and Significantly Inhibited by D₂-Like Dopamine Receptor Antagonist Eticlopride. Injection of morphine (subcutaneously) produced a dose-dependent increase in ouabain-sensitive Na⁺,K⁺-ATPase activity in mouse striatum, with maximal increase in Na⁺,K⁺-ATPase activity by 40% at the dose of 10 mg/kg (Fig. 1A). When animals were treated with morphine (10 mg/kg) for different times, the maximal stimulation occurred at 1 h and then decreased and returned to the basal level at 4 h (Fig. 1B). The stimulatory effect of morphine on

striatal Na^+, K^+ -ATPase activity is naltrexone-reversible. Naltrexone, given intraperitoneally 30 min before morphine administration at a dose of 4 mg/kg, fully antagonized the stimulatory effect of short-term morphine treatment on striatal Na^+, K^+ -ATPase activity, and an obvious alteration in Na^+, K^+ -ATPase activity was not observed in the animals treated with naltrexone alone (Fig. C). Previous studies have demonstrated that dopamine contributes to the regulation of striatal Na^+, K^+ -ATPase activity (Bertorello et al., 1990; Nishi et al., 1999) and that in vivo administration of morphine leads to the release of dopamine (Di Chiara and North, 1992). To determine whether dopamine receptors were involved in morphine-stimulated Na^+, K^+ -ATPase activity in the striatum, the effect of selective D_1 -like dopamine receptor antagonist SCH 23390 or D_2 -like dopamine receptor antagonist eticlopride was detected. As shown in Fig. 1C, injection of eticlopride but not SCH 23390 (1 mg/kg i.p.) 30 min before morphine administration partially but significantly suppressed short-term morphine-stimulated Na^+, K^+ -ATPase activity. Treatment with eticlopride or SCH 23390 alone did not significantly affect the basal Na^+, K^+ -ATPase activity. The results suggest that dopamine release and activation of D_2 dopamine receptor may be involved in the enhancement of mouse striatal Na^+, K^+ -ATPase activity after short-term morphine treatment.

In Vitro Short-Term Morphine Treatment Also Enhanced Striatal Synaptosomes Na^+, K^+ -ATPase Activity, and This Effect Was Reversed by Naltrexone but Not by Eticlopride or SCH 23390. To prove that in vivo dopamine release and activation of D_2 -dopamine receptor are involved in regulating the striatal Na^+, K^+ -ATPase activity after short-term morphine treatment, the effect of in vitro administration of morphine on striatal Na^+, K^+ -ATPase activity was directly assessed in striatal synaptosomes in which dopamine release induced by morphine might not occur because the striatal circuits that are required for the synapse transmission between opiodergic and dopaminergic neurons are broken. As shown in Fig. 2A, similar to in vivo administration of morphine, incubation of the striatal synaptosomes with morphine also produced a dose-dependent increase in Na^+, K^+ -ATPase activity, with the maximal stimulation at a dose of 10 μM . When striatal synaptosomes were incubated with 10 μM morphine for different times, the maximal enhancement of Na^+, K^+ -ATPase activity occurred at 10 min (Fig. 2B). Next, the effect of eticlopride or SCH 23390 on in vitro morphine-stimulated Na^+, K^+ -ATPase activity in striatal synaptosomes was detected. The striatal synaptosomes were incubated with morphine in the presence of SCH 23390 or eticlopride at a dose of 100 μM . Distinct from in vivo administration, eticlopride failed to inhibit morphine-stimu-

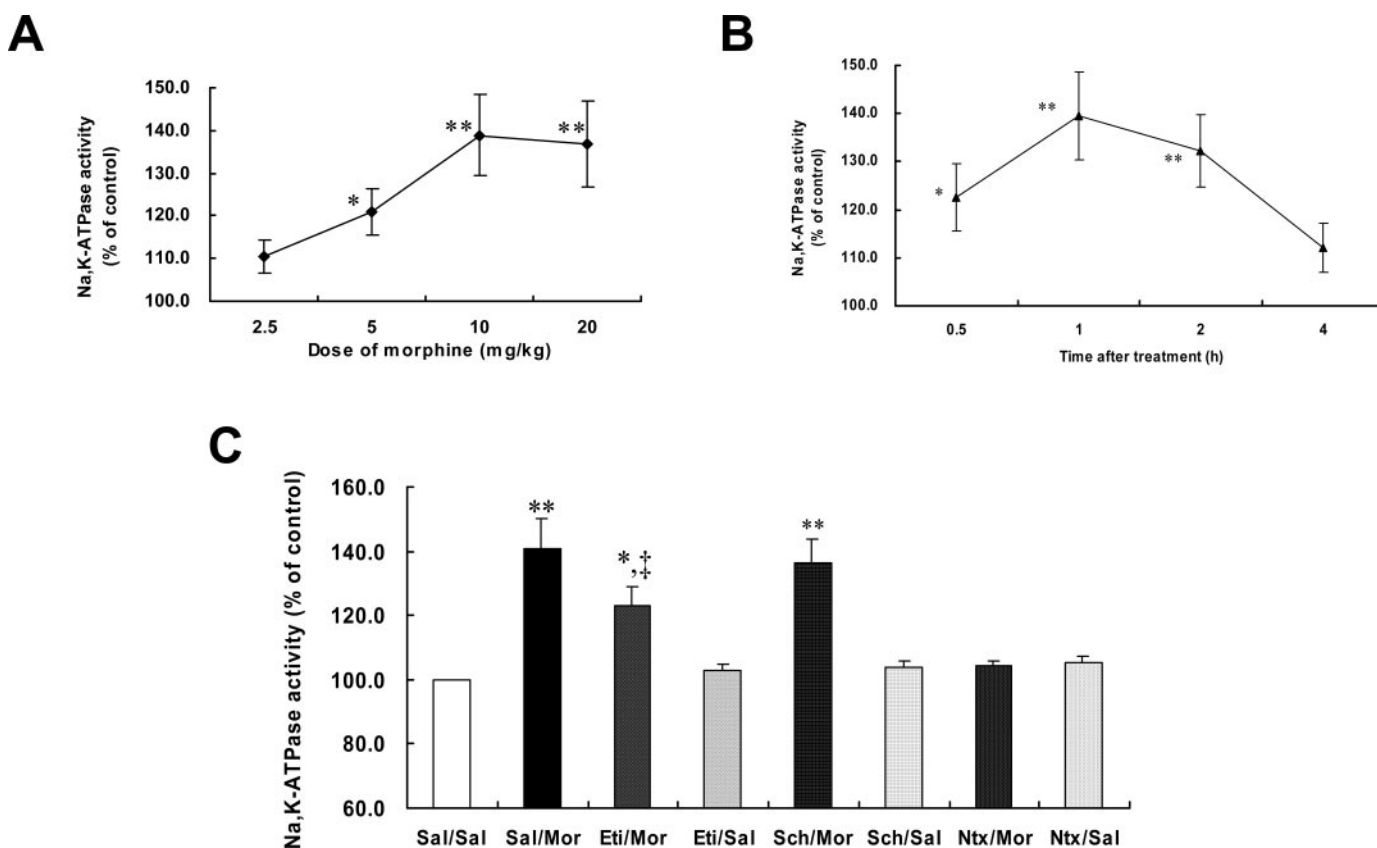


Fig. 1. In vivo short-term morphine treatment stimulated the striatal Na^+, K^+ -ATPase activity in a dose-dependent manner, and this effect was naltrexone reversible and significantly inhibited by eticlopride. A, mice were treated with saline (as control) or increasing doses of morphine for 1 h. B, mice were treated with 10 mg/kg of morphine for 0 (as control), 0.5, 1, 2, and 4 h. C, mice were treated with morphine (10 mg/kg s.c.) or saline (10 ml/kg s.c.) or were concomitantly treated with eticlopride (1 mg/kg i.p.), SCH 23390 (1 mg/kg i.p.), naltrexone (4 mg/kg i.p.), or saline (10 ml/kg i.p.). Eticlopride, SCH 23390, naltrexone, or saline was injected 30 min before morphine or saline administration. After treatment, animals were decapitated, and the striatum was quickly isolated. Na^+, K^+ -ATPase activity was measured as described under *Materials and Methods*. Data represent a percentage of the Na^+, K^+ -ATPase activity obtained from saline-treated or nontreated (0 h, B) controls and expressed as the mean $\pm$ S.D. of three separate experiments performed in duplicate. *, $P < 0.05$; **, $P < 0.01$ compared with control mice; †, $P < 0.01$ compared with mice treated with morphine alone. Sal, saline; Mor, morphine; Eti, eticlopride; Sch, SCH 23390; Ntx, naltrexone.

lated striatal Na⁺,K⁺-ATPase activity (Fig. 2C). Likewise, SCH 23390 was also unable to inhibit morphine-stimulated striatal Na⁺,K⁺-ATPase activity (Fig. 2C). However, as expected, the stimulatory effect of *in vitro* morphine administration on striatal Na⁺,K⁺-ATPase activity could be reversed by naltrexone (Fig. 2C). By themselves, eticlopride, SCH 23390, and naltrexone had no effect on the basal Na⁺,K⁺-ATPase activity (Fig. 2C). The results indicate that dopamine release and dopamine receptor activation are not involved in the enhancement of the Na⁺,K⁺-ATPase activity of striatal synaptosomes after morphine treatment.

Decrease in PKA Activity Contributed to Increase in Striatal Na⁺,K⁺-ATPase Activity after Short-Term Morphine Treatment, and This Effect Was Naltrexone-Reversible and Significantly Suppressed by Eticlopride. PKA plays a role in regulating Na⁺,K⁺-ATPase activity in the striatum (Pinto Ferreira et al., 1998). Opioid- and D₂-dopamine receptors both couple to inhibitory G protein (G_{i/o}). Activation of opioid and dopamine receptors by their agonist results in the inhibition of adenylyl cyclase and attenuation of PKA activation. To determine whether the enhancement of Na⁺,K⁺-ATPase activity upon *in vivo* short-term morphine administration was attributed to the attenuation of PKA activity, the effect of *in vivo* administra-

tion of morphine on the striatal PKA activity was assessed. As shown in Fig. 3A, treatment of mice for 1 h with morphine (10 mg/kg, *i.p.*) yielded a significant reduction of the striatal PKA activity, and this effect was reversed by pretreatment of mice with naltrexone (4 mg/kg, *i.p.*). Eticlopride (1 mg/kg *i.p.*), given before morphine administration, partially but significantly suppressed short-term morphine treatment-induced decrease in PKA activity in the striatum (Fig. 3B), suggesting that D₂-dopamine receptors involve morphine-mediated reduction of PKA activity. To determine whether the reduction of PKA activity is associated with the enhancement of striatal Na⁺,K⁺-ATPase activity after short-term morphine treatment, the effect of db-cAMP, a PKA activator, on short-term morphine treatment-induced enhancement of Na⁺,K⁺-ATPase activity was examined. Primary study showed that db-cAMP exhibited a significant inhibition of basal Na⁺,K⁺-ATPase activity in a dose-dependent manner, with maximal inhibition at the dose of 100 ng (data not shown). Concomitant administration with morphine, db-cAMP (100 ng, *i.c.v.*) significantly suppressed morphine-stimulated striatal Na⁺,K⁺-ATPase activity (Fig. 3C). Moreover, the inhibition of striatal Na⁺,K⁺-ATPase activity by db-cAMP was fully reversed by H-89 (1 nmol *i.c.v.*), a specific PKA inhibitor (Fig. 3D). The results support that the reduc-

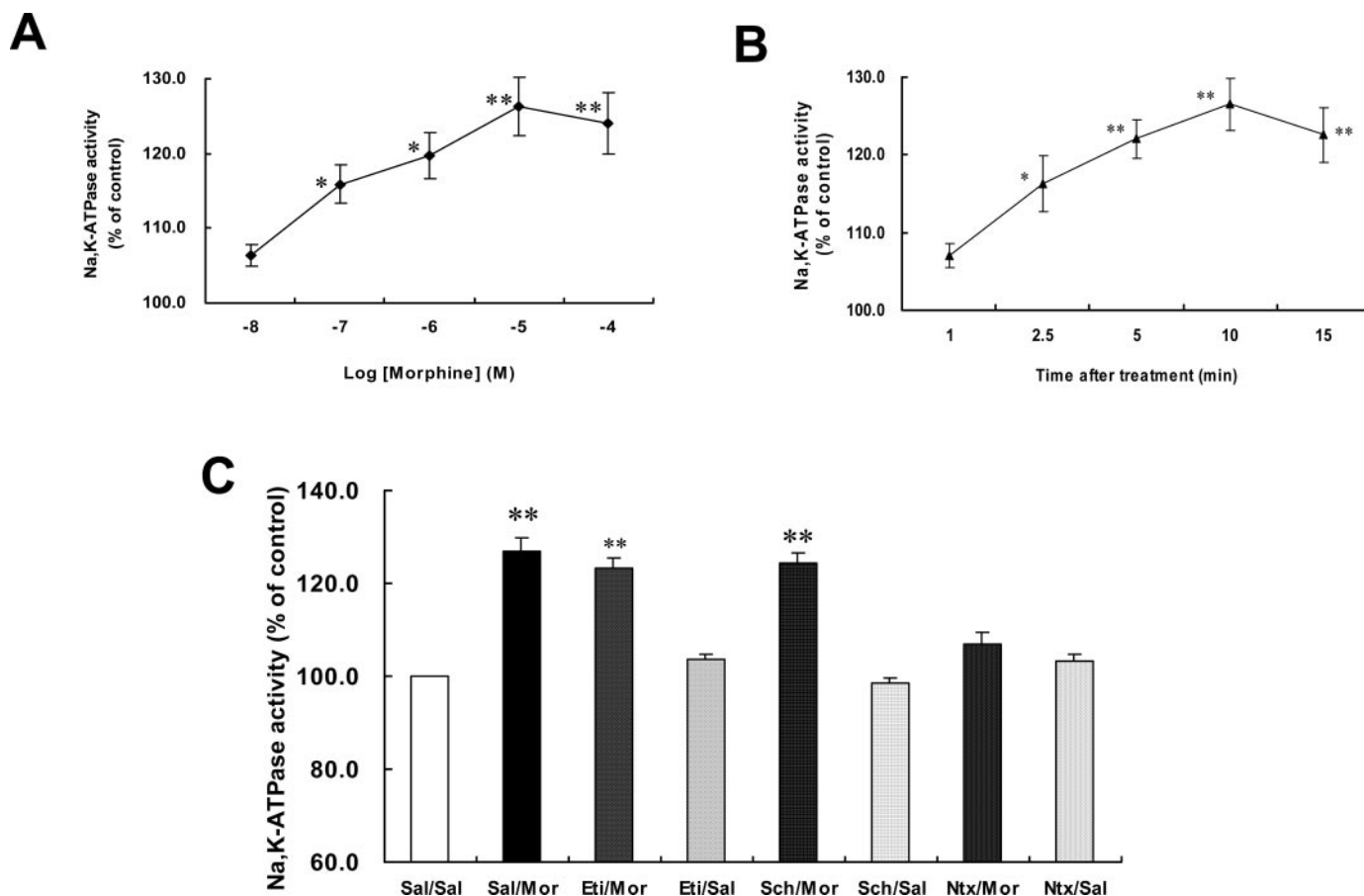


Fig. 2. *In vitro* short-term morphine treatment resulted in a dose-dependent enhancement of striatal Na⁺,K⁺-ATPase activity, and this effect was naltrexone- but not eticlopride- or SCH 23390-reversible. The crude synaptosomes (P₂ pellets) prepared from the striatum of naive mice were treated with saline (as control) or different concentrations of morphine (10⁻⁸–10⁻⁴ M) for 10 min at 37°C (A), or with 10⁻⁵ M of morphine for 0 (as control), 2.5, 5, 10, and 15 min at 37°C (B). Striatal crude synaptosomes were pretreated with eticlopride (100 μM), SCH 23390 (100 μM), naltrexone (10 μM), or saline for 5 min at 37°C and then treated with 10⁻⁵ M concentration of morphine or saline for 10 min at 37°C (C). Data represent a percentage of the Na⁺,K⁺-ATPase activity obtained from saline-treated or nontreated (0 hour, B) controls and expressed as the mean ± S.D. of three separate experiments performed in duplicate. *, *P* < 0.05, **, *P* < 0.01 compared with control crude synaptosomes. Sal, saline; Mor, morphine; Eti, eticlopride; Sch, SCH 23390; Ntx, naltrexone.

tion of PKA activity contributes to the enhancement of Na^+, K^+ -ATPase activity and suggest that PKA activity is inversely correlated with Na^+, K^+ -ATPase activity.

In Vivo Long-Term Morphine Treatment Decreased Striatal Na^+, K^+ -ATPase Activity through Enhancement of PKA Activity, and This Effect Was Naltrexone- and SCH 23390-Reversible. It is well established that short-term and long-term morphine treatments differentially mediate intracellular cAMP concentrations (Liu and Anand, 2001). Contrary to the inhibition of cAMP/PKA signal pathway by short-term morphine treatment, long-term morphine treatment leads to the up-regulation of cAMP/PKA signal pathway. To determine whether dopamine receptors are also involved in the alteration of striatal Na^+, K^+ -ATPase activity after long-term morphine treatment, the effects of dopamine receptor antagonists on long-term morphine treatment-induced change in the striatal Na^+, K^+ -ATPase activity were detected. First, the effect of long-term morphine treatment on mouse striatal Na^+, K^+ -ATPase activity was detected. Treatment of mice with morphine (10 mg/kg i.p.) twice per day for 10 consecutive days markedly suppressed mouse striatal Na^+, K^+ -ATPase activity,

and this effect was reversed by concomitant administration of naltrexone (4 mg/kg, i.p.) (Fig. 4A). Next, the effect of eticlopride or SCH 23390 on long-term morphine treatment-induced reduction of striatal Na^+, K^+ -ATPase activity was assessed. As shown in Fig. 4A, pretreatment of mice with SCH 23390 but not eticlopride (1 mg/kg i.p.) significantly inhibited the reduction of striatal Na^+, K^+ -ATPase activity by long-term morphine treatment, suggesting that D_1 -dopamine receptors are involved in the reduction of striatal Na^+, K^+ -ATPase activity after long-term morphine treatment.

The role of PKA in long-term morphine treatment-mediated decrease in striatal Na^+, K^+ -ATPase activity, then, was determined. As shown in Fig. 4B, treatment of mice with morphine for 10 consecutive days significantly increased striatal PKA activity compared with the saline control group, and this effect was abolished by concomitant administration of naltrexone (4 mg/kg i.p.). Likewise, SCH 23390 (1 mg/kg i.p.), given before morphine treatment, also abolished the effect of long-term morphine treatment on striatal PKA activity (Fig. 4C), suggesting that D_1 dopamine receptors are involved in the enhancement of striatal PKA activity upon

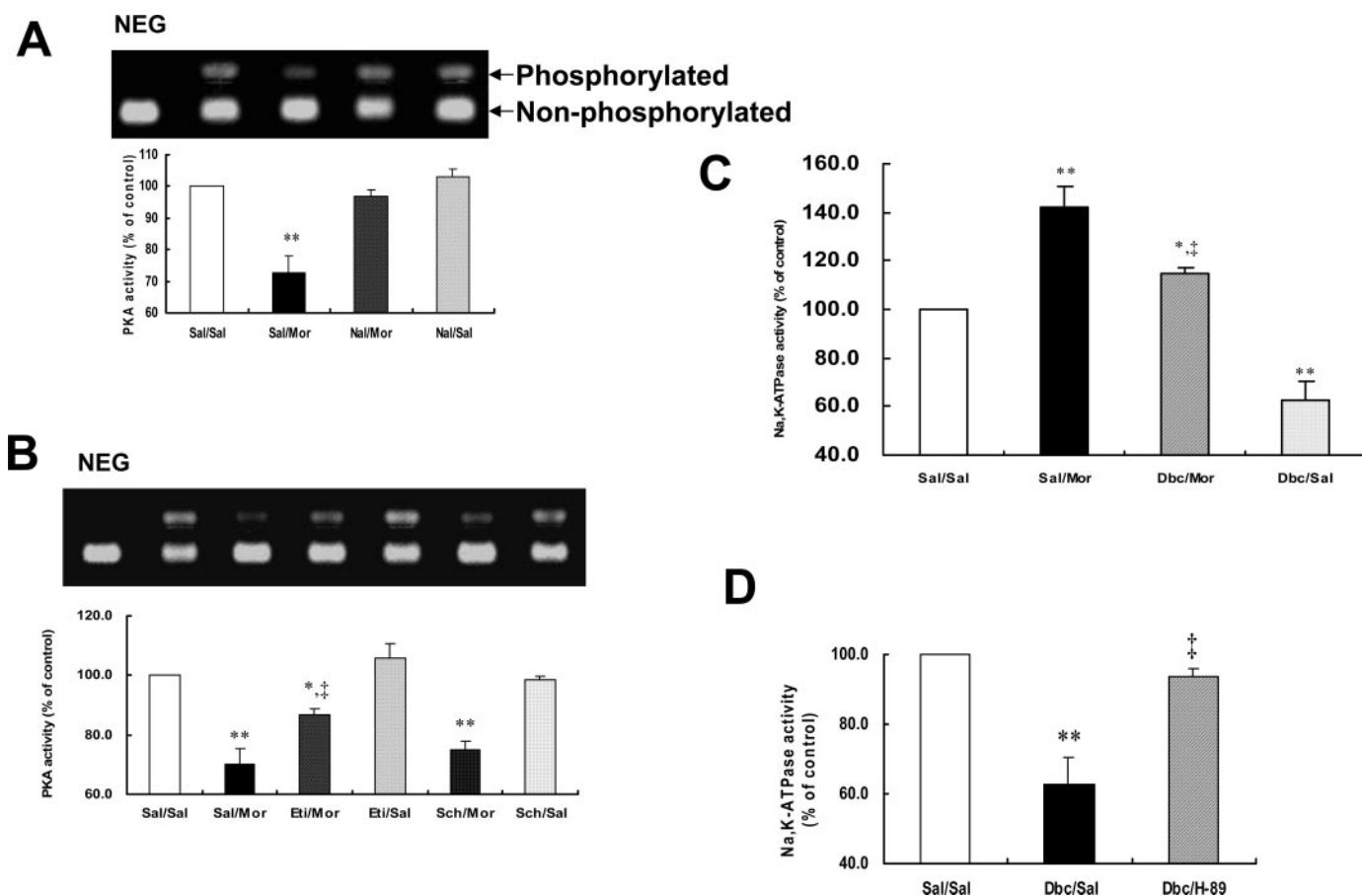


Fig. 3. Decrease in PKA activity contributed to increment of striatal Na^+, K^+ -ATPase activity after short-term morphine treatment, and this effect was naltrexone- and eticlopride-reversible. A and B, mice were treated with morphine (10 mg/kg s.c.) or saline (10 ml/kg s.c.) or were concomitantly treated with eticlopride (1 mg/kg i.p.), SCH 23390 (1 mg/kg i.p.), naltrexone (4 mg/kg i.p.), or saline (10 ml/kg i.p.). Eticlopride, SCH 23390, naltrexone, or saline was injected 30 min before morphine or saline administration. C, animals were treated with morphine (10 mg/kg s.c.) or saline (10 ml/kg s.c.) or were concomitantly treated with db-cAMP (100 ng i.c.v.) or saline (5 μl i.c.v.). db-cAMP and saline were injected 30 min before morphine administration. D, the mice were pretreated with db-cAMP (100 ng i.c.v.) or saline (5 μl i.c.v.) for 30 min and then treated with H-89 (1 nmol i.c.v.) or saline (5 μl i.c.v.) for 30 min. PKA activity and Na^+, K^+ -ATPase activity were measured as described under *Materials and Methods*. A and B, top, representative gel electrophoresis of PKA activity assays; bottom, quantitative determination of the PKA activity by spectrophotometry. Data are expressed as the mean $\pm$ S.D. of three separate experiments performed in duplicate. *, $P < 0.05$, **, $P < 0.01$ compared with saline-treated control mice; †, $P < 0.01$ compared with mice treated with morphine alone. Sal, saline; Mor, morphine; Eti, eticlopride; Sch, SCH 23390; Dbc, db-cAMP; Ntx, naltrexone; NEG, negative control.

long-term morphine treatment. To further confirm the involvement of change in PKA activity in long-term morphine-mediated decrement of striatal Na⁺,K⁺-ATPase activity, the effect of H-89 on long-term morphine-mediated reduction of striatal Na⁺,K⁺-ATPase activity was tested. Administration of H-89 (1 nmol i.c.v.) to suppress long-term morphine-mediated up-regulation of PKA activity significantly reversed the inhibition of striatal Na⁺,K⁺-ATPase activity by morphine (Fig. 4D), suggesting that enhancement of PKA activity is related to the reduction of striatal Na⁺,K⁺-ATPase activity after long-term morphine treatment.

In Vivo Administration of Quinpirole Increased Striatal Na⁺,K⁺-ATPase Activity through Attenuation of PKA Activity, whereas in Vivo Administration of SKF 38393 Decreased Striatal Na⁺,K⁺-ATPase Activity via Augmentation of PKA Activity. The results shown above indicated that activation of D₂ dopamine receptors was involved in short-term morphine treatment-mediated enhancement of striatal Na⁺,K⁺-ATPase activity via the reduction of PKA activity, whereas activation of D₁-dopamine receptor was implicated in long-term morphine treatment-mediated decrease in striatal Na⁺,K⁺-ATPase activity through increase in PKA activity. To prove that activation of D₁- or

D₂-dopamine receptors is able to differentially regulate PKA and Na⁺,K⁺-ATPase activity in response to different morphine treatments, the effects of D₁-like agonist SKF 38393 and D₂-like agonist quinpirole on striatal PKA and Na⁺,K⁺-ATPase activity were determined. Quinpirole at a dose of 1 or 2 mg/kg i.p. (for 30 min) significantly inhibited PKA activity but stimulated Na⁺,K⁺-ATPase activity in the striatum (Fig. 5, A and C). As expected, SKF 38393 at a dose of 3 or 6 mg/kg i.p. (for 30 min) significantly stimulated PKA activity but inhibited Na⁺,K⁺-ATPase activity in the striatum (Fig. 5, B and D). Quinpirole or SKF 38393 mimicked the effect of short-term or long-term morphine treatment on striatal Na⁺,K⁺-ATPase and PKA, indicating that dopamine release induced by morphine is capable of regulating striatal Na⁺,K⁺-ATPase activity.

Alteration of the Phosphorylation Levels but Not Protein Expression Abundance of Na⁺,K⁺-ATPase Involved the Modulation of Striatal Na⁺,K⁺-ATPase Activity by Morphine. Phosphorylation of the subunits of Na⁺,K⁺-ATPase is a primary regulatory mechanism for activity of Na⁺,K⁺-ATPase (Bertorello et al., 1991; Cheng et al., 1997; Nishi et al., 1999). The results shown above suggest that PKA-regulated phosphorylation may involve in the mod-

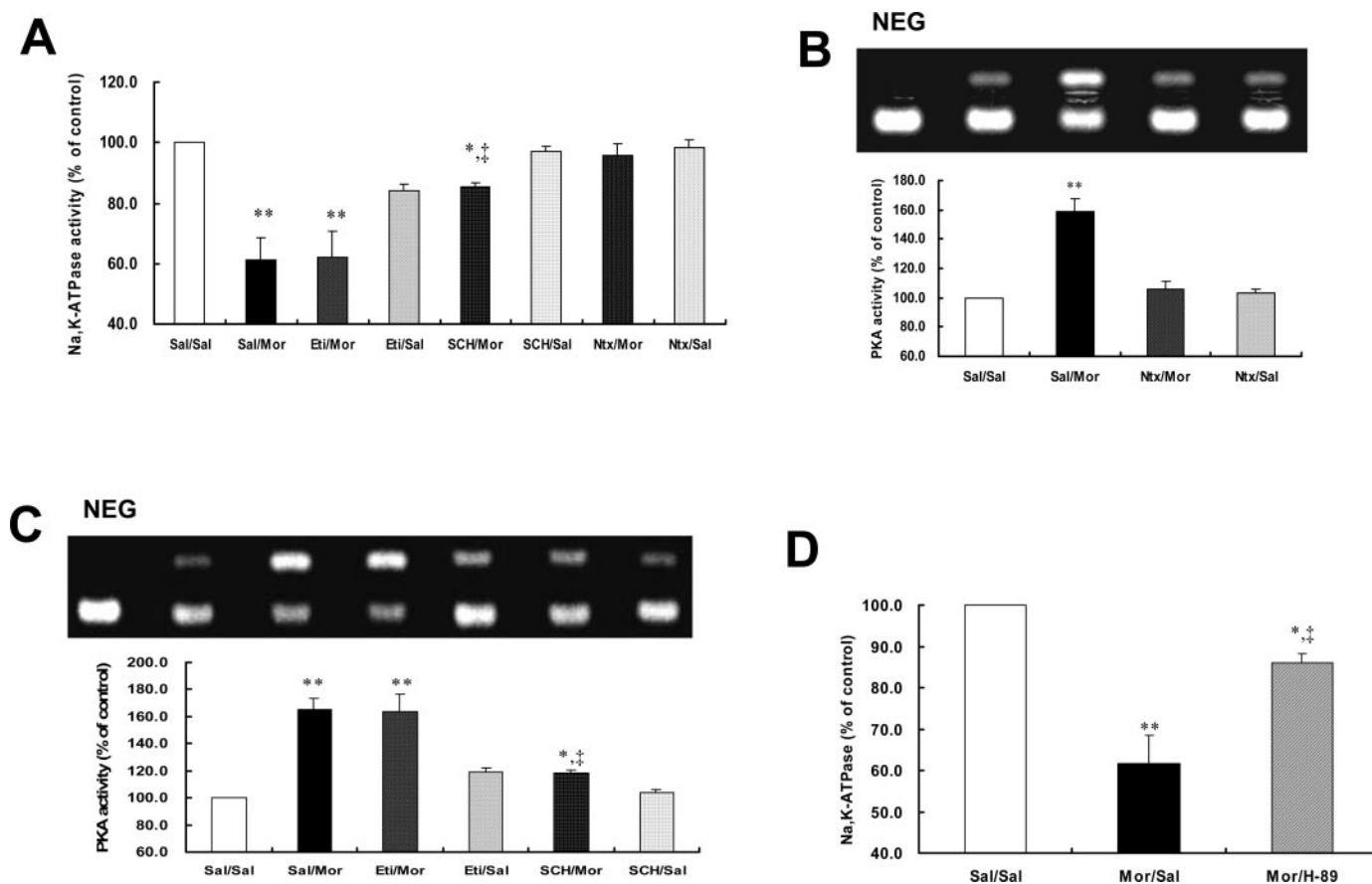


Fig. 4. Increase in PKA activity was involved in decrement of striatal Na⁺,K⁺-ATPase activity after long-term morphine treatment, and this effect was naltrexone- and SCH 23390-reversible. A to C, mice were continually treated with morphine (10 mg/kg s.c.) or saline (10 ml/kg s.c.) or with a combination of morphine with eticlopride (1 mg/kg i.p.), SCH 23390 (1 mg/kg i.p.), naltrexone (4 mg/kg i.p.), or saline (10 ml/kg i.p.) for 10 consecutive days. Mice were sacrificed 1 h after the final morphine or saline administration. D, mice were continually treated as described above. Animals were treated with H-89 (1 nmol in 5 μ l i.c.v.) or saline (5 μ l i.c.v.) 1 h after the final morphine administration. 30 min later, mice were decapitated, and the striatum was quickly isolated. Na⁺,K⁺-ATPase activity (A and D) and PKA activity (B and C) were determined as described under *Materials and Methods*. B and C, top, representative gel electrophoresis of PKA activity assays; bottom, quantitative determination of the PKA activity by spectrophotometry. Data are expressed as the mean $\pm$ S.D. of three independent experiments performed in duplicate. *, $P < 0.05$, **, $P < 0.01$ compared with saline-treated control mice; †, $P < 0.01$ compared with mice treated with morphine alone. Sal, saline; Mor, morphine; Eti, eticlopride; Sch, SCH 23390; naltrexone, Ntx; NEG, negative control.

ulation of Na^+, K^+ -ATPase activity in the striatum by short-term and long-term morphine treatment. It has been reported that the $\alpha 3$ subunit seems to be expressed predominantly in brain and plays a key role in the function of Na^+, K^+ -ATPase (McGrail et al., 1991). To further determine the mechanisms by which morphine modulates striatal Na^+, K^+ -ATPase activity, the role of PKA in short-term and long-term morphine treatment-induced alteration of the phosphorylation levels of the $\alpha 3$ subunit of Na^+, K^+ -ATPase was examined using the antibody against the $\alpha 3$ subunit of Na^+, K^+ -ATPase, and the antibody (anti-pan) recognizing serine-, threonine-, and tyrosine-phosphorylated proteins. As shown in Fig. 6A, short-term morphine treatment (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 striatum. Pretreatment with eticlopride (1 mg/kg i.p.) or db-

cAMP (100 ng i.c.v.) could significantly reverse the reduction of the phosphorylation levels of the $\alpha 3$ subunit of the enzyme induced by short-term morphine treatment (Fig. 6, A and B). Contrary 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 striatum (Fig. 6C). Concurrent treatment with SCH 23390 (1 mg/kg i.p.) remarkably inhibited the increase of the phosphorylation levels of $\alpha 3$ subunit of the enzyme induced by long-term morphine treatment. Likewise, H-89 (1 nmol i.c.v.) also significantly suppressed the increase of the phosphorylation levels of $\alpha 3$ subunit of the enzyme induced by long-term morphine treatment (Fig. 6C). Opioid receptor antagonist naltrexone fully reversed both short-term and long-term morphine treatment-induced alterations in the phosphorylation levels of the $\alpha 3$ subunits of Na^+, K^+ -ATPase (data not

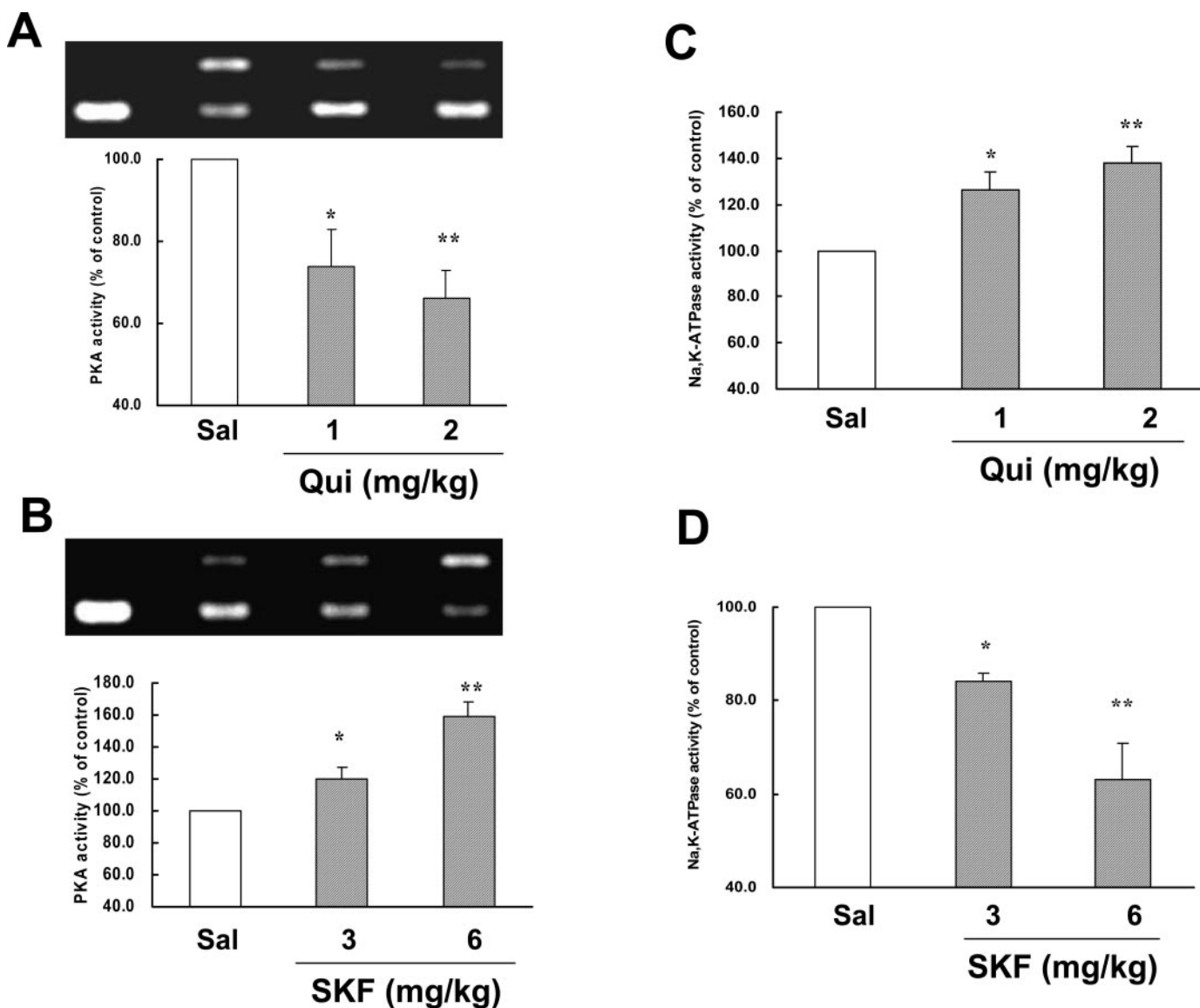


Fig. 5. In vivo administration of quinpirole increased striatal Na^+, K^+ -ATPase activity via attenuation of PKA activity and in vivo administration of SKF 38393 decreased striatal Na^+, K^+ -ATPase activity through augmentation of PKA Activity. A and C, mice were treated with quinpirole (1 and 2 mg/kg i.p.) or saline (10 ml/kg i.p.) for 30 min. B and D, mice were treated with SKF 38393 (3 and 6 mg/kg i.p.) or saline (10 ml/kg i.p.) for 30 min. After treatment, the animal was sacrificed, and the striatum was quickly isolated. Striatal PKA activity (A and B) and Na^+, K^+ -ATPase activity (C and D) were determined as described under *Materials and Methods*. Data are expressed as the mean $\pm$ S.D. of three independent experiments performed in duplicate. *, $P < 0.05$, **, $P < 0.01$ compared with saline-treated control mice. Sal, saline; Qui, quinpirole; SKF, SKF 38393.

shown). Besides determination of the $\alpha 3$ subunit of Na⁺,K⁺-ATPase, the effects of short- and long-term morphine treatments on the phosphorylation levels of the $\alpha 1$ subunit were also detected. As shown in Fig. 6D, neither short- nor long-term morphine treatment could significantly modulate the basal phosphorylation levels of $\alpha 1$ subunit. These results suggest that activation of dopamine receptors mediates, at least in part, the alteration in the phosphorylation levels of the $\alpha 3$ subunit of Na⁺,K⁺-ATPase induced 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., 2000). To determine whether changes in the expression abundance of

Na⁺,K⁺-ATPase after morphine treatment is also involved in alteration of N Na⁺,K⁺-ATPase activity, two subunits ($\alpha 1$ and $\alpha 3$) of Na⁺,K⁺-ATPase were measured by Western blot analyses. An apparent change in the abundance of the two subunits of Na⁺,K⁺-ATPase in the striatum was not observed by either short-term or long-term morphine treatment (Fig. 7), and this was supported by previous study (Cheng and Aperia, 1998).

Discussion

Adaptive alteration of brain neurochemistry in response to prolonged exposure to morphine is a probable mechanism of

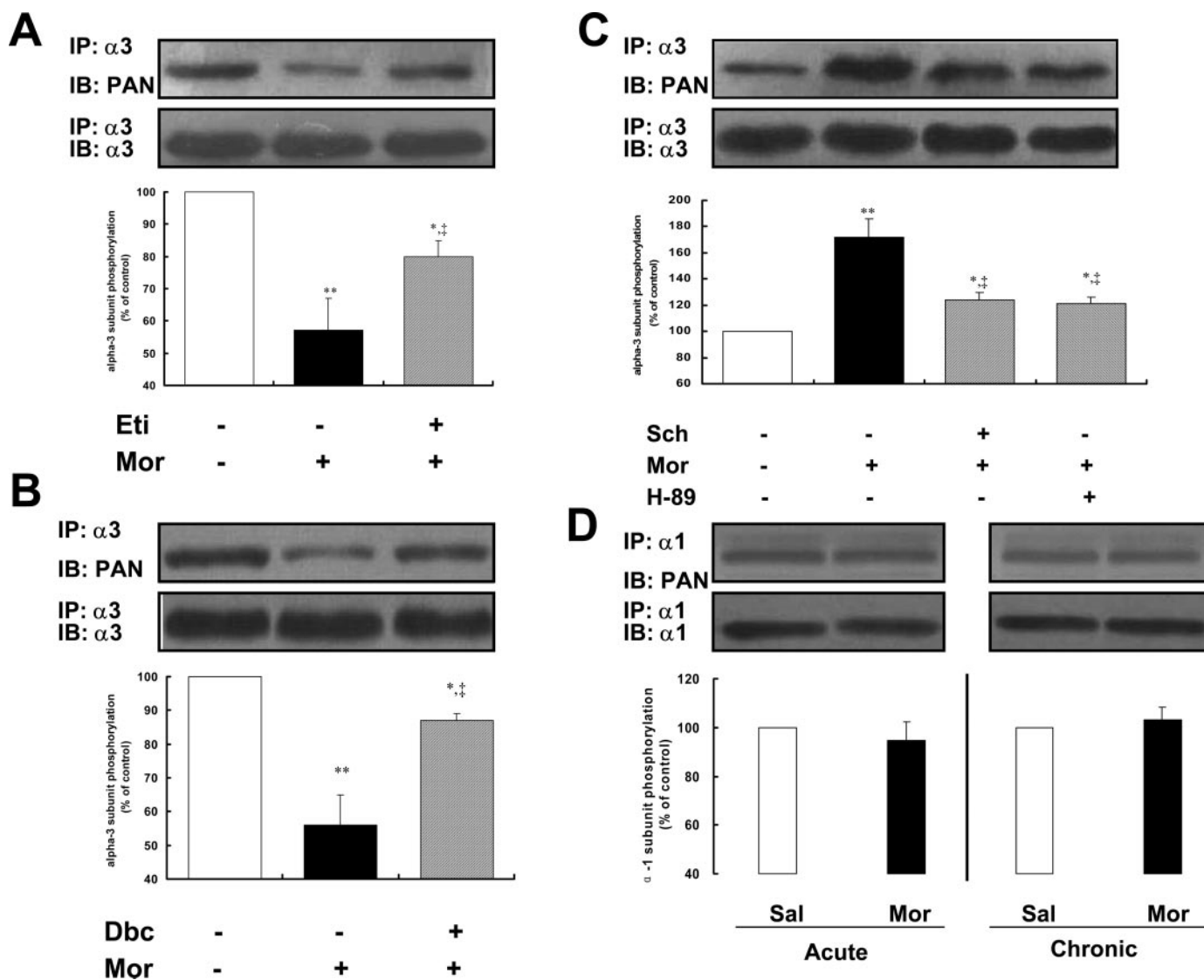


Fig. 6. Alteration of the phosphorylation levels of $\alpha 3$ but not $\alpha 1$ subunit of Na⁺,K⁺-ATPase involved the modulation of striatal Na⁺,K⁺-ATPase activity by morphine treatment. A and B, mice were treated with morphine (10 mg/kg s.c.) or saline (10 ml/kg s.c.), or concomitantly treated with eticlopride (A, 1 mg/kg i.p.) or db-cAMP (B, 100 ng i.c.v.) or the vehicle saline for 1 h. Eticlopride or db-cAMP was administered 30 min before morphine treatment. Mice were decapitated 1 h after morphine administration, and the striatum was isolated. C, mice were consecutively treated with morphine (10 mg/kg s.c.) or saline (10 ml/kg s.c.), or concomitantly treated with SCH 23390 (1 mg/kg i.p.) or saline (10 ml/kg i.p.) for 10 days. SCH 23390 and saline were administered 30 min before morphine treatment. H-89 (1 nmol in 5 μ l i.c.v.) or saline (5 μ l i.c.v.) was injected 1 h after the final morphine or saline treatment. Thirty minutes later, mice were decapitated, and the striatum was isolated. D, animals were treated with morphine for 1 h or 10 days as described above. Phosphorylation assay was performed as described under *Materials and Methods*. Top, representative immunoblots for the phosphorylated $\alpha 3$ or $\alpha 1$ subunit of Na⁺,K⁺-ATPase; bottom, quantitative estimation by scanning densitometry of the phosphorylation levels of $\alpha 3$ or $\alpha 1$ subunit of the enzyme. Data were expressed as a percentage of the control, and values represent the mean $\pm$ 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. Sal, saline; Mor, morphine; Eti, eticlopride; Sch, SCH 23390; IP, immunoprecipitation; IB, immunoblotting.

drug addiction. Previous studies of our laboratory and others have demonstrated that long-term morphine treatment induces impairment of Na^+, K^+ -ATPase function in several brain regions associated with morphine dependence, including forebrain, hippocampus, and locus ceruleus (Sharma et al., 1998; Kong et al., 2001; Wu et al., 2006). The striatum is a major brain region that mediates drug reward and addiction-related behaviors. The present study was designed to characterize adaptive change in Na^+, K^+ -ATPase activity in the striatum after exposure to morphine and to examine whether dopamine receptors were involved in this adaptation. Results from this study showed that the short-term and long-term morphine treatment differentially modulated striatal Na^+, K^+ -ATPase activity in a PKA-dependent manner through indirect activation of dopamine receptors.

In vivo short-term morphine treatment stimulated striatal Na^+, K^+ -ATPase activity, and this effect was significantly but not entirely reversed by the D_2 -like dopamine receptor antagonist eticlopride. Contrary to short-term morphine treatment, long-term morphine treatment suppressed striatal Na^+, K^+ -ATPase activity, and this effect was reversed by D_1 -like dopamine receptor antagonist SCH 23390. The possible explanation for the inability of eticlopride to entirely reverse the enhancement of Na^+, K^+ -ATPase activity by short-term morphine treatment is that, besides increase of Na^+, K^+ -ATPase activity via promotion of dopamine release, morphine can directly increase Na^+, K^+ -ATPase activity without indirect activation dopamine receptors, as shown by this study (Fig. 2C) and previous studies (Masocha et al.,

2002; Wu et al., 2006). Opioid and D_2 dopamine receptors both couple to inhibitory G protein ($\text{G}_{i/o}$). Activation of both receptors by their agonists inhibits adenylyl cyclase and decreases PKA activation, leading to an enhancement of Na^+, K^+ -ATPase activity. Eticlopride only reverses D_2 receptor-mediated increase in Na^+, K^+ -ATPase activity. Opioid receptor antagonist naltrexone reversed both short-term and long-term morphine-induced changes of Na^+, K^+ -ATPase activity. These results suggest that D_2 -dopamine receptors are implicated in regulating striatal Na^+, K^+ -ATPase activity after short-term morphine treatment, whereas D_1 -dopamine receptors are involved in regulating striatal Na^+, K^+ -ATPase activity upon long-term morphine treatment. Involvement of dopamine receptors in regulating Na^+, K^+ -ATPase activity in vivo by morphine is further supported by the observation that eticlopride failed to suppress the enhancement of Na^+, K^+ -ATPase activity induced by in vitro direct administration of morphine to isolated striatal synaptosomes (Fig. 2C). In isolated striatal synaptosomes, the afferent input has been cut off because striatal circuits are broken. In this case, morphine is unable to promote dopamine release and activate D_2 dopamine receptors. Our findings are consistent with earlier in vivo studies showing that short-term and long-term exposures to morphine increase dopamine release in the nucleus accumbens and caudate putamen by activating μ -opioid receptors (Pothos et al., 1991; Pontieri et al., 1995) and that morphine indirectly acts on the dopaminergic system (Johnson and North, 1992). Dopamine is a general dopamine receptor agonist and activates both D_1 and D_2 dopamine receptors. Activation of D_2 dopamine receptors stimulates Na^+, K^+ -ATPase activity (Yamaguchi et al., 1996), whereas activation of D_1 dopamine receptors inhibits Na^+, K^+ -ATPase activity (Fienberg et al., 1998). It seems that short-term morphine treatment preferentially influences D_2 dopamine receptors in striatopallidal neurons, whereas long-term morphine treatment mainly affects D_1 receptors in striatonigral neurons. Several possible reasons can be proposed to explain these results. First, the affinity of D_2 dopamine receptors for dopamine has been shown to be higher than that of D_1 dopamine receptors for dopamine (Missale et al., 1998). Thus, the relatively lower level of synaptic or extrasynaptic dopamine after short-term morphine treatment is probably sufficient to only activate D_2 but not D_1 receptors. Second, long-term morphine treatment probably results in greater dopamine release because there is a relatively higher morphine concentration in the striatum because of consecutive treatment with morphine. Indeed, it has been reported that nicotine, another abused drug that is also shown to promote dopamine release in the striatum, activates D_2 dopamine receptors in striatopallidal neurons at low concentrations but activates D_1 dopamine receptors in striatonigral neurons at high concentrations (Hamada et al., 2004). Finally, predominant action on D_1 dopamine receptors upon long-term morphine treatment may be attributed to the down-regulation of D_2 but not D_1 receptors, as reported by previous studies (Navarro et al., 1992; Georges et al., 1999).

The present study further demonstrated that PKA signal pathway was involved in dopamine-mediated change of Na^+, K^+ -ATPase activity after short-term or long-term morphine treatment, because the PKA activator db-cAMP reversed short-term morphine treatment-induced enhancement of Na^+, K^+ -ATPase, and the PKA inhibitor H-89

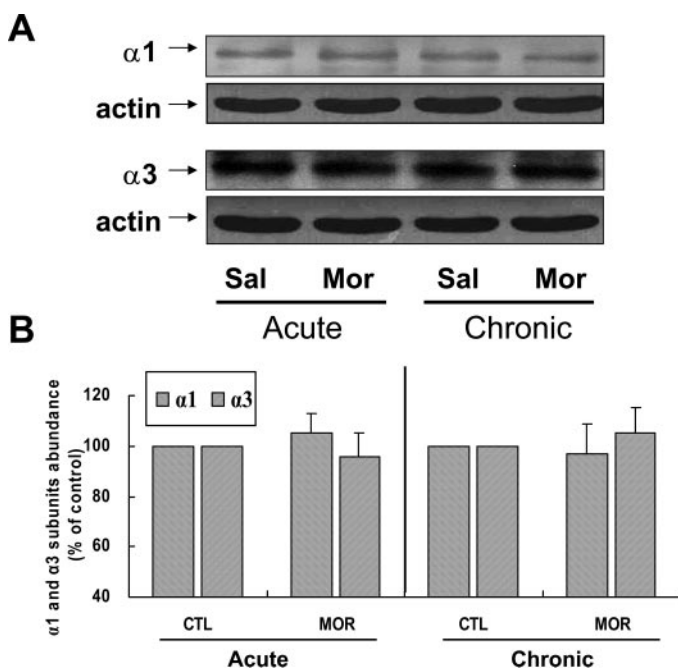


Fig. 7. Effects of short-term and long-term morphine treatment on the expression of $\alpha 1$ and $\alpha 3$ subunits of striatal Na^+, K^+ -ATPase. Equal quantities of protein prepared from short- and long-term morphine-treated mice were separated by SDS-PAGE and then incubated with the primary antibody against the $\alpha 1$ or $\alpha 3$ subunit of mouse Na^+, K^+ -ATPase (1:600) or β -actin (1:5000) overnight at 4°C . The blots were then incubated for 120 min at room temperature with second IgG in Blotto. The antibody binding was detected using an enhanced chemiluminescence method following the manufacturer's instructions. A, representative immunoblots for $\alpha 1$ and $\alpha 3$ subunits of Na^+, K^+ -ATPase and β -actin; B, quantitative estimation (by scanning densitometry) of the expression of the indicated protein. Sal, saline; Mor, morphine.

reversed long-term morphine treatment-induced reduction of Na⁺,K⁺-ATPase activity in the striatum. Different regulation of the phosphorylation levels of the $\alpha 3$ subunit of Na⁺,K⁺-ATPase by PKA may underlie the distinct effects of short-term and long-term morphine treatment on Na⁺,K⁺-ATPase activity. Short-term morphine treatment inhibited striatal PKA activity and therefore decreased the phosphorylation of Na⁺,K⁺-ATPase, leading to increase of Na⁺,K⁺-ATPase activity. Contrary to short-term morphine treatment, long-term morphine treatment stimulated PKA activity and increased the phosphorylation of Na⁺,K⁺-ATPase, leading to decrease of Na⁺,K⁺-ATPase activity. The phosphorylation levels of Na⁺,K⁺-ATPase in inverse correlation with Na⁺,K⁺-ATPase activity are consistent with the results as reported previously (Bertorello et al., 1991; Cheng et al., 1997). The different striatal PKA activity between the short-term and long-term morphine treatment mice may be due to different regulation of cAMP production by short-term and long-term morphine treatment. Although cAMP concentrations in response to different morphine treatment were not assessed in the present study, the phenomenon of the inhibition by short-term morphine treatment and the up-regulation by long-term morphine treatment of cAMP levels have been described in the locus ceruleus (Nestler and Tallman, 1988), nucleus accumbens (Terwilliger et al., 1991), and the hippocampus (Wu et al., 2006) and in primary cultures of striatal neurons (Van Vliet et al., 1991). In addition, a profound increase in D₁ dopamine receptor-stimulated cAMP production was also observed in long-term morphine-treated striatal neurons (Van Vliet et al., 1991).

It seems that the phosphorylation of Na⁺,K⁺-ATPase by morphine is $\alpha 3$ subunit-selective in the striatum because neither short- nor long-term morphine treatment could induce significant change in the phosphorylation levels of the $\alpha 1$ subunit of Na⁺,K⁺-ATPase. The results from the present study support that selective phosphorylation of the $\alpha 3$ subunit of Na⁺,K⁺-ATPase is responsible for regulating the function of this enzyme (Shulman and Fox, 1996; Biser et al., 2000; Wu et al., 2006). The $\alpha 1$ -low and $\alpha 3$ -high expressions in the striatum may explain this specific regulation of the phosphorylation of the $\alpha 3$ subunit of enzyme (McGrail et al., 1991; Fig. 7). 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). It should be noted that although the present study demonstrated the involvement of PKA-mediated phosphorylation of Na⁺,K⁺-ATPase in regulating enzyme activity after morphine treatment, the results did not exclude possible involvement of other protein kinases in regulating the phosphorylation of Na⁺,K⁺-ATPase. In addition, further work is needed to elucidate whether PKA acts on Na⁺,K⁺-ATPase directly or indirectly. Efforts are underway in our laboratory to determine whether DARPP-32 involves the regulation of the phosphorylation of Na⁺,K⁺-ATPase after morphine treatment. DARPP-32 is highly expressed in all striatal projection neurons, and plays an important role in regulating the state of phosphorylation and activity of large number of phosphoproteins (Greengard et al., 1998).

Drug addiction might be attributed to drug-induced aberrant reward-related learning (Wickens et al., 2003). Synaptic plasticity in the striatum induced by addictive drugs is a

potential mechanism for aberrant reward-related learning (Hyman et al., 2006). DARPP-32 may serve as a molecular switch at the nexus of reward pathway plasticity (Gould and Manji, 2005). Na⁺,K⁺-ATPase is an important downstream effector of DARPP-32 and might essentially involve synaptic plasticity and aberrant reward-related learning induced by addictive drugs. The phosphorylation of Thr-34 of DARPP-32 by protein kinases such as PKA converts it into a potent inhibitor of protein phosphatase-1, regulating the state of phosphorylation and activity of large number of phosphoproteins (Greengard et al., 1998). The regulation by DARPP-32/protein phosphatase-1 pathway in striatal neurons of ionic channels, neurotransmitter receptors and electrogenic pumps, Na⁺,K⁺-ATPase definitely plays a vital role in coordinating the effects of various neurotransmitters on the excitability of these cells (Greengard et al., 1998). Therefore, impairment of Na⁺,K⁺-ATPase activity in the striatum after long-term morphine treatment would lead to the dysfunction of neurons, which might contribute to the alteration of synaptic plasticity and produce aberrant reward-related learning. Further work is required to address this issue.

In summary, dopamine has been shown to participate morphine-induced several molecular adaptations in the striatum, including alteration of cAMP response element binding protein phosphorylation and changes in transcription factor Δ FosB and cFos expressions (Bontempi and Sharp, 1997; Chartoff et al., 2003; Muller and Unterwald, 2005). The present study demonstrated a new effect of dopamine in response to activation of opioid receptors: stimulation of Na⁺,K⁺-ATPase activity in response to short-term morphine treatment and inhibition of Na⁺,K⁺-ATPase activity in response to long-term morphine treatment. Further study to confirm the role of impairment of striatal Na⁺,K⁺-ATPase activity in morphine-induced reward-related learning will be beneficial to elucidating the mechanisms of opiate dependence.

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