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

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**Running Title:** PKA mediates morphine regulation of Na<sup>+</sup>,K<sup>+</sup>-ATPase

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**Abbreviations:** db-cAMP, 2'-O-dibutyryl adenosine 3',5'-cyclic monophosphate sodium salt; forskolin, 7β-Acetoxy-8,13-epoxy-1α,6β,9α-trihydroxy labd-14-en-11-one; H-89, (N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide) dihydrochloride; IBMX, 3-isobutyl-1-methylxanthine; icv, intracerebroventricularly; MAPK, mitogen-activated protein kinase; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethyl sulfonyl fluoride; PKA, cAMP-dependent protein kinase; TBST, Tris-buffered saline-Tween 20.

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### ABSTRACT

The depolarization of neurons induced by impairment of  $\text{Na}^+, \text{K}^+$ -ATPase activity after chronic opiate treatment has been shown to involve the development of opioid dependence. However, the mechanisms underlying changes in  $\text{Na}^+, \text{K}^+$ -ATPase activity following opioid treatment are unclear. The best-established molecular adaptation to chronic opioid exposure is up-regulation of the cAMP/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  $\text{Na}^+, \text{K}^+$ -ATPase activity. The results demonstrated that acute morphine treatment dose-dependently stimulated  $\text{Na}^+, \text{K}^+$ -ATPase activity. This action could be significantly suppressed by adenylyl cyclase activator forskolin, or the cAMP analogue db-cAMP. Contrary to acute morphine treatment, chronic morphine treatment significantly inhibited  $\text{Na}^+, \text{K}^+$ -ATPase activity. Moreover, an additional decrease in  $\text{Na}^+, \text{K}^+$ -ATPase activity was observed by naloxone precipitation. The effects of both acute and chronic morphine treatment on  $\text{Na}^+, \text{K}^+$ -ATPase activity were naltrexone-reversible. The regulation of  $\text{Na}^+, \text{K}^+$ -ATPase activity by morphine was inversely correlated with intracellular cAMP accumulation. H-89, a specific PKA inhibitor, mimicked the stimulatory effect of acute morphine but antagonized the inhibitory effect of chronic morphine on  $\text{Na}^+, \text{K}^+$ -ATPase activity. However, okadaic acid, a protein phosphatase inhibitor, suppressed acute morphine stimulation but potentiated chronic morphine inhibition of  $\text{Na}^+, \text{K}^+$ -ATPase activity. The regulation of  $\text{Na}^+, \text{K}^+$ -ATPase activity by morphine treatment appeared to associate with the alteration in phosphorylation level, but not to be relevant to the change in abundance of  $\text{Na}^+, \text{K}^+$ -ATPase. These findings strongly demonstrate that cAMP/PKA signaling pathway involves in regulation of  $\text{Na}^+, \text{K}^+$ -ATPase activity after activation of opioid receptors.

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Opioid analgesics are clearly the most efficacious agents currently available for treatment of moderate to severe pain. However, their use for chronic pain management is often limited due to the development of tolerance and/or dependence upon prolonged administration. The mechanisms underlying these adaptations to repeated or chronic opiate administration are still poorly understood. To date, the best-established molecular adaptations to chronic 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 evidences suggest that morphine and other opiates acutely enhance neural  $\text{Na}^+, \text{K}^+$ -ATPase activity in vivo (Desaiah and Ho, 1977; Sharma et al., 1998) and in vitro (Masocha et al., 2002; Horvath et al., 2003), whereas chronic 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  $\text{Na}^+, \text{K}^+$ -ATPase, which has been suggested to be linked to opioid tolerance and dependence (Kong et al., 1997; 2001). However, the mechanism underlying the modulation of  $\text{Na}^+, \text{K}^+$ -ATPase activity by acute and chronic morphine treatment has not yet been determined and what relationship exists between the up-regulation of cAMP/PKA signaling pathway and changes in  $\text{Na}^+, \text{K}^+$ -ATPase activity upon chronic 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 acute and chronic morphine treatments modulate  $\text{Na}^+, \text{K}^+$ -ATPase activity and whether the up-regulation of cAMP/PKA signaling pathway plays a role in modulation of the mouse hippocampal  $\text{Na}^+, \text{K}^+$ -ATPase activity.

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### Materials and Methods

**Materials.** Morphine hydrochloride was purchased from Qinghai Pharmaceutical General Factory (Qinghai, China). Dimethyl sulphoxide (DMSO), sodium dodecyl sulfate (SDS), and dithiothreitol (DTT) were purchased from Amresco (Solon, Ohio, USA). Phenylmethyl sulfonyl fluoride (PMSF) and Triton-X 100 were obtained from Merck (Darmstadt, Germany). Naltrexone hydrochloride, naloxone hydrochloride, pertussis toxin (PTX), forskolin, 7 $\beta$ -Acetoxy-8,13-epoxy-1 $\alpha$ ,6 $\beta$ ,9 $\alpha$ -trihydroxyabund-14-en-11-one, N<sup>6</sup>, 2'-O-dibutyryladenosine 3', 5'-cyclic monophosphate sodium salt (db-cAMP), okadaic acid sodium salt (OA), H-89 (N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamidedihydrochloride), 3-isobutyl-1-methylxanthine (IBMX) and ouabain hydrate were supplied by Sigma Chemical Co. (St Louis, MO, USA). Protein A/G PLUS-Agarose beads were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The cAMP enzyme immunoassay kit was obtained from Sigma.

**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 animal treatments were strictly in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. For acute treatment, animals were treated with a single subcutaneous (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 intraperitoneally (i.p.) given 30 min prior to morphine administration at a dose of 4 mg/kg. PTX was intracerebroventricularly (i.c.v.) given 5 d before the administration of morphine at a dose of 0.5  $\mu$ g/mouse as previously described (Parenti et al., 1986). Forskolin, db-cAMP or OA was administered (i.c.v.) 30 min before morphine injection at the

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doses of 0.1~100 ng, 1~100 ng and 1 pg~100 pg, respectively. For chronic treatment, mice were treated by morphine (s.c., 10 mg/kg) twice per day at 12 h intervals for 10 days as described (Pu et al., 2002). One group of animals were treated concomitantly with naltrexone and morphine, naltrexone was injected (i.p.) 30 min prior to morphine administration. Control group of mice were treated similarly except that normal saline was used as a substitute for morphine. One h after the final injection of morphine, some animals were decapitated immediately, and the other animals were precipitated with naloxone (i.p., 2 mg/kg) 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 H-89 (1 nmol) or OA (100 pg) and then 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  $-80^{\circ}\text{C}$  until use.

**Membrane Preparation.** Plasma membranes were prepared as previously described (Roth et al., 1981) with some modifications. Briefly, hippocampal tissues from 3 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  $\mu\text{M}$   $\text{CaCl}_2$ , 10% (w/v) sucrose, 1 mM DTT, and centrifuged at 1,000 g for 10 min at  $4^{\circ}\text{C}$  to remove cellular debris and nuclei. The supernatant was centrifuged at 12,000 g for 20 min at  $4^{\circ}\text{C}$  to yield the crude plasma membranes ( $\text{P}_2$  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  $4^{\circ}\text{C}$ . 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  $-20^{\circ}\text{C}$ . To avoid the loss of  $\text{Na}^+, \text{K}^+$ -ATPase activity, the stored plasma membrane pellets was used within 3 days. Membrane protein concentrations were determined

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by a bicinchoninic acid assay (Beyotime Biotechnology, Haimen, China).

**Measurement of Na<sup>+</sup>,K<sup>+</sup>-ATPase Activity.** The Na<sup>+</sup>,K<sup>+</sup>-ATPase activity was measured as described previously (Esmann, 1988) with slight modifications. Briefly, 100  $\mu$ l of aliquot containing 40  $\mu$ g of protein was preincubated at 37 °C for 10 min with 850  $\mu$ l of reaction buffer A containing in mM: 100 NaCl, 20 KCl, 2 MgCl<sub>2</sub>, 0.4 EGTA and 50 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  $\mu$ 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 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. 400  $\mu$ l of supernatant was taken for the assay of liberated inorganic phosphate (Pi) as described previously (Taussky and Shorr, 1953) with some modifications. In brief, 800  $\mu$ l of ammonium molybdate solution color reagent was added to the tube containing 400  $\mu$ l 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<sub>2</sub>HPO<sub>4</sub> as standard. Na<sup>+</sup>,K<sup>+</sup>-ATPase activity was obtained by the difference between total ATPase and ouabain-insensitive Mg<sup>2+</sup>-ATPase activity (Esmann, 1988).

**In Vitro Assay.** The crude hippocampal synaptosomes (P<sub>2</sub> pellets) were prepared as described above. The final pellets were suspended in Krebs'-Ringer's-HEPES (KRH) medium containing the following (in mM): 120 NaCl, 4.7 KCl, 2.2 CaCl<sub>2</sub>, 25 HEPES, 1.2 MgSO<sub>4</sub>, 1.2 KH<sub>2</sub>PO<sub>4</sub>, and 10 glucose, pH 7.4, to give a protein concentration of 1 mg/ml. A 100- $\mu$ l of aliquot was pipetted into the appropriate well of the 24-well plate, 890  $\mu$ l of the KRH medium was added to the well, and then the plate was preincubated for 15 min at 37 °C. Ten  $\mu$ l of different concentrations of morphine were then

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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 prior to 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 2 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<sup>+</sup>,K<sup>+</sup>-ATPase activity determination were performed as described above.

**cAMP Assay.** 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 3 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. Following centrifugation at 3,000 g at 4 °C for 10 min the supernatant were 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 pre-acetylated samples and cAMP standards were 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 by adding



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200  $\mu$ l of wash solution for 3 times. After the final wash the wells were aspirated and the plate was firmly taped to remove any remaining wash buffer. Two hundred  $\mu$ l of the substrate solution was added to every well, and incubated at room temperature for 1 h without shaking. Fifty  $\mu$ l 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 pmol/mg protein.

**Immunoblotting Assay.** For immunoblotting assay the crude plasma membranes were prepared as described for Na<sup>+</sup>,K<sup>+</sup>-ATPase activity assay with the exception of the homogenation buffer containing 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 500  $\mu$ M IBMX, 50 mM NaF, 2  $\mu$ g/ml aprotinin and 5  $\mu$ g/ml leupeptin. The final pellet was suspended on ice in a sufficient amount of this homogenation buffer to give a protein concentration of 3 mg/ml. An equal volume of 2X 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 BioRad Mini-Protean<sup>®</sup> 3 apparatus (BioRad Laboratories, Hercules, CA, USA). In brief, equal quantities of protein (24  $\mu$ g) were separated by SDS-polyacrylamide gel electrophoresis (PAGE) on 8% SDS polyacrylamide gel for about 90 min at 120 V, and then electroblotted at 4 °C onto nitrocellulose membrane (0.45  $\mu$ m pore size, BioRad Laboratories, Hercules, CA, USA). The membrane was rinsed briefly in Tris-buffered saline-Tween 20 (TBST) (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 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  $\alpha$ 1 or  $\alpha$ 3 subunit of mouse Na<sup>+</sup>,K<sup>+</sup>-ATPase (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 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

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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 Biotechnology, Rockford, IL, USA) following the manufacturer's instructions.

**Protein Phosphorylation Assay.** The crude plasma membranes were prepared as described for  $\text{Na}^+, \text{K}^+$ -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  $\text{Na}_4\text{O}_7\text{P}_2$ , 1 mM  $\text{Na}_3\text{VO}_4$ , 1 mM PMSF, 10  $\mu\text{g/ml}$  leupeptin, 4  $\mu\text{g/ml}$  aprotinin, and 1% Triton-X 100), and incubated with rotation at 4 °C for 2 h. Insoluble material was removed by centrifugation at 12,000 g for 20 min at 4 °C, and the concentration of protein in the supernatant was determined as described above. Equal amount of protein (200  $\mu\text{g}$ ) from the supernatants were incubated with rotation overnight at 4 °C with 3  $\mu\text{g}$  of goat polyclonal  $\text{Na}^+ \text{K}^+$ -ATPase  $\alpha 1$  or  $\alpha 3$  subunit antibody. A saturating amount of Protein A/G PLUS-Agarose beads (pre-washed with IP buffer for 3 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 8,000 g for 5 min at 4 °C. An equal volume of 2X sample buffer (100 mM Tris-HCl, pH 6.8, 200 mM DTT, 4% SDS, 20% glycerol, and 0.2% Bromphenol Blue) 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 rabbit polyclonal anti-pan phosphorylated protein antibody at a dilution of 1/250 (Zymed Laboratories, South San Francisco, CA, USA) as described above (see *immunoblotting assay*).

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**p44/42 Mitogen-Activated Protein 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<sub>2</sub>, 1 mM PMSF, 10 µg/ml leupeptin, 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. Equal volume of 2X 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-PAGE 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, Beverly, MA, USA). The membrane was stripped and reprobed with rabbit polyclonal anti-p42 MAPK antibody at a dilution of 1/1000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

**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, pH7.4, 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, USA) as described in Promega Technical Bulletin. 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

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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  $\pm$  SD 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 ANOVA followed by Newman-Keuls test was used. Differences with a *P*-value less than 0.05 were considered statistically significant.

## Results

**Acute Morphine Treatment Increased Na<sup>+</sup>,K<sup>+</sup>-ATPase Activity, and This Effect Was Naltrexone and PTX-Reversible.** Injection of morphine (s.c.) produced a dose-dependent increase in ouabain-sensitive Na<sup>+</sup>,K<sup>+</sup>-ATPase activities in the mouse hippocampus, with maximal effect at dose of 10 mg/kg (Fig.1A). To determine that observed morphine effects are direct or indirect, the stimulation of hippocampal synaptosomal Na<sup>+</sup>,K<sup>+</sup>-ATPase activity by morphine was detected. As shown in Fig. 1B, in vitro morphine treatment also increased the hippocampal synaptosomal Na<sup>+</sup>,K<sup>+</sup>-ATPase activity in a dose-dependent manner. The maximal stimulation occurred at the concentration of 10  $\mu$ M. The

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stimulatory effect of acute morphine on the hippocampal  $\text{Na}^+, \text{K}^+$ -ATPase activity was opioid receptor antagonist naltrexone- and  $\text{G}_{i/o}$  protein blocker PTX-reversible. Naltrexone, given (i.p.) 30 min before morphine administration at dose of 4 mg/kg, or in vitro given to hippocampal synaptosome at dose of 10  $\mu\text{M}$ , fully antagonized the stimulatory effect of acute morphine on  $\text{Na}^+, \text{K}^+$ -ATPase activity in the hippocampus, and obvious alteration in  $\text{Na}^+, \text{K}^+$ -ATPase activity was not observed in the animals treated with naltrexone alone (Fig. 2A, B). Similarly, PTX (0.5  $\mu\text{g}$ , i.c.v.), also significantly inhibited the morphine-induced increase in  $\text{Na}^+, \text{K}^+$ -ATPase activity, when injected 5 d before acute morphine administration (Fig. 2C). In addition, a slight but significant decrease in  $\text{Na}^+, \text{K}^+$ -ATPase activity was 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 a review, see Law et al., 2000). To validate that injection of PTX (i.c.v.) 5 d prior to 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 d prior to morphine completely abolished morphine-activated p42/p44 MAPK phosphorylation in the hippocampus (Fig. 2D).

**Decrease in Hippocampal cAMP Concentration in Acute Morphine-Treated Mice May Be Related to Increase in  $\text{Na}^+, \text{K}^+$ -ATPase Activity.** It is widely documented that acute activation of opioid receptors by opiates inhibits the cAMP accumulation and the PKA activation. To determine the relationship between alteration of intracellular cAMP concentration and  $\text{Na}^+, \text{K}^+$ -ATPase activity, hippocampal cAMP concentrations in mice acutely treated with morphine were measured. As was expected, acute treatment of mice with morphine (10 mg/kg, s.c.) significantly decreased cAMP concentrations in the hippocampus as compared with saline-treated control group, and pretreatment of

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naltrexone could abolish this decrease of cAMP concentration (Fig.3A). To further determine the role of alteration of cAMP concentration in changes in  $\text{Na}^+, \text{K}^+$ -ATPase activities, the effects of forskolin, an activator of adenylyl cyclase, and db-cAMP, an analogue of cAMP, on acute morphine treatment-induced enhancement of  $\text{Na}^+, \text{K}^+$ -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  $\text{Na}^+, \text{K}^+$ -ATPase activity were measured. Both forskolin and db-cAMP exhibited a significant inhibition of basal  $\text{Na}^+, \text{K}^+$ -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. 3D, E). The results indicate that reduction of cAMP concentration may contribute to enhancement of  $\text{Na}^+, \text{K}^+$ -ATPase activity upon acute activation of opioid receptors, suggesting that cAMP concentration is inversely correlated with  $\text{Na}^+, \text{K}^+$ -ATPase activity.

**H-89, a Selective Inhibitor of PKA, Mimicked but OA, a Protein Phosphatase Inhibitor, Reversed the Stimulatory Effect of Acute Morphine on  $\text{Na}^+, \text{K}^+$ -ATPase Activity.** To determine whether alteration of PKA activity involved morphine-induced increase in  $\text{Na}^+, \text{K}^+$ -ATPase activity, the effect of H-89, a specific PKA inhibitor, on basal  $\text{Na}^+, \text{K}^+$ -ATPase activity was investigated. Indeed, a single injection of H-89 (1 nmol, i.c.v.), mimicked the action of acute morphine stimulation of

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Na<sup>+</sup>,K<sup>+</sup>-ATPase activity, displaying a significant increase in Na<sup>+</sup>,K<sup>+</sup>-ATPase activity of the hippocampus relative to saline-treated control group (Fig. 4A), indicative of reduction of PKA activity involvement of enhancement of Na<sup>+</sup>,K<sup>+</sup>-ATPase activity. To investigate whether alteration of phosphorylation level of Na<sup>+</sup>,K<sup>+</sup>-ATPase was involved in enhancement of Na<sup>+</sup>,K<sup>+</sup>-ATPase activity following acute morphine treatment, the effect of OA, a protein phosphatase inhibitor, on morphine-mediated changes in Na<sup>+</sup>,K<sup>+</sup>-ATPase activity was assessed. OA can increase the phosphorylation levels of Na<sup>+</sup>,K<sup>+</sup>-ATPase via the blockade of dephosphorylation of rat renal Na<sup>+</sup>,K<sup>+</sup>-ATPase (Li et al, 1998). Preliminary study showed that OA (1~100 pg) dose-dependently inhibited the hippocampal Na<sup>+</sup>,K<sup>+</sup>-ATPase activity (data not shown). Therefore, a dose of 100 pg was used to assess the effect of OA on acute morphine-mediated changes in Na<sup>+</sup>,K<sup>+</sup>-ATPase activity. OA, given (i.c.v.) 30 min prior to morphine administration at dose of 100 pg, markedly attenuated acute morphine-stimulated Na<sup>+</sup>,K<sup>+</sup>-ATPase activity in the hippocampus as compared with acute morphine treatment alone group (Fig. 4B), suggesting that reduction of phosphorylation levels of Na<sup>+</sup>,K<sup>+</sup>-ATPase may be implicated in enhancement of Na<sup>+</sup>,K<sup>+</sup>-ATPase activity induced by acute morphine treatment.

**Chronic Morphine Treatment Decreased Na<sup>+</sup>,K<sup>+</sup>-ATPase Activity but Increased cAMP Concentration.** It has been well established that chronic exposure to opiates leads to a marked increase in cAMP concentration (Liu and Anand, 2001). To further confirm the modulation of Na<sup>+</sup>,K<sup>+</sup>-ATPase activity by cAMP, the effects of chronic morphine treatment and naloxone-precipitated morphine withdrawal on Na<sup>+</sup>,K<sup>+</sup>-ATPase activity and intracellular cAMP concentration were tested. One group of animals was administered with 10 mg/kg of morphine twice per day for 10 consecutive days. The other group of animals was treated identically except that

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animals were precipitated with naloxone after chronic morphine treatment and withdrawal syndromes were observed. The mice receiving chronic 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, chronic treatment of mice with morphine for ten days markedly enhanced hippocampal cAMP concentration (Fig. 5B). In contrast to the acute single morphine treatment, chronic morphine treatment significantly decreased Na<sup>+</sup>,K<sup>+</sup>-ATPase activity in the hippocampus as compared with saline control group (Fig. 5A). An additional reduction of Na<sup>+</sup>,K<sup>+</sup>-ATPase activity and enhancement of cAMP concentration was found upon naloxone-precipitated withdrawal in chronic morphine-treated mice relative to non naloxone-precipitated group (Fig. 5A, B). The attenuation of Na<sup>+</sup>,K<sup>+</sup>-ATPase activity and enhancement of intracellular cAMP concentration by chronic morphine were prevented by concomitant administration of naltrexone (Fig. 5A, B). Naltrexone, co-administrated with morphine, also significantly suppressed chronic morphine treatment-induced withdrawal behaviors (data not shown). These results suggest that increase in cAMP concentration in mouse hippocampus may be related to the reduction of Na<sup>+</sup>,K<sup>+</sup>-ATPase activity following chronic morphine treatment, and that changes in Na<sup>+</sup>,K<sup>+</sup>-ATPase activity may be one of the potential mechanisms underlying development of morphine dependence.

**H-89 Antagonized but OA Enhanced the Inhibitory Effect of Chronic Morphine Treatment on Na<sup>+</sup>,K<sup>+</sup>-ATPase Activity.** To determine whether the alteration of PKA activity and phosphorylation level of Na<sup>+</sup>,K<sup>+</sup>-ATPase was also associated with modulation of Na<sup>+</sup>,K<sup>+</sup>-ATPase activity by chronic morphine, the effects of H-89 or OA treatment on chronic morphine-induced reduction of Na<sup>+</sup>,K<sup>+</sup>-ATPase activity were detected. Application of H-89 (1 nmol, i.c.v.) to reduce the chronic



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morphine-upregulated PKA activity significantly reversed the inhibitory effect of chronic morphine on  $\text{Na}^+, \text{K}^+$ -ATPase activity (Fig. 6A), suggesting that enhancement of PKA activity was involved in reduction of  $\text{Na}^+, \text{K}^+$ -ATPase activity by chronic morphine treatment. However, administration of OA (100 pg, i.c.v.) enhanced the inhibitory effect of chronic morphine on  $\text{Na}^+, \text{K}^+$ -ATPase activity as compared with morphine-treated alone group (Fig. 6B), suggesting that enhancement or maintenance of phosphorylation process of  $\text{Na}^+, \text{K}^+$ -ATPase in hippocampus was associated with reduction of  $\text{Na}^+, \text{K}^+$ -ATPase activity by chronic morphine treatment. Taken together, the findings suggested that reduction of  $\text{Na}^+, \text{K}^+$ -ATPase activity following chronic morphine treatment may attributed to up-regulation of cAMP/PKA signaling pathway.

### **Alteration of the Phosphorylation Levels but not Protein Expression Abundance of $\text{Na}^+, \text{K}^+$ -ATPase Involved the Modulation of $\text{Na}^+, \text{K}^+$ -ATPase Activity by Morphine.**

Phosphorylation is a primary regulatory mechanism for activity of  $\text{Na}^+, \text{K}^+$ -ATPase (Bertorello et al., 1991; Cheng et al., 1997). The results showed above suggested that the PKA-mediated phosphorylation may involve in the regulation of  $\text{Na}^+, \text{K}^+$ -ATPase activity in the hippocampus by acute and chronic morphine administration. Since it has been reported that the  $\alpha 3$  subunit appears to be expressed predominantly in brain and plays a key role in the function of  $\text{Na}^+, \text{K}^+$ -ATPase (McGrail et al., 1991; Wang et al., 2003), next, the effect of acute and chronic morphine treatment on the phosphorylation of  $\alpha 3$  subunit of  $\text{Na}^+, \text{K}^+$ -ATPase was firstly 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, acute morphine administration (10 mg/kg, s.c.) resulted in a significant reduction of the total phosphorylation levels of the  $\alpha 3$  subunit of  $\text{Na}^+, \text{K}^+$ -ATPase in the hippocampus. Pretreatment of naltrexone (4 mg/kg, i.p.) could abolish the decrease in the basal total

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phosphorylation of the  $\alpha 3$  subunit of the enzyme. Contrary to acute morphine treatment, chronic 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 chronic morphine treatment. Similarly, H-89 (1 nmol, i.c.v.) also significantly suppressed the increase of phosphorylation of the enzyme by inhibiting chronic morphine-induced up-regulation of PKA activity. The identical results were also obtained by using the antibody (anti-pan) recognizing serine, threonine and tyrosine phosphorylated proteins first, and then using the antibody against the enzyme  $\alpha 3$  subunit (data not shown). Besides determination of phosphorylation of  $\alpha 3$  subunit of  $\text{Na}^+, \text{K}^+$ -ATPase, the effects of acute and chronic morphine treatments on phosphorylation of  $\alpha 1$  subunit were also detected. As shown in Fig 7C, neither acute nor chronic morphine treatment could significantly regulate the basal phosphorylation of  $\alpha 1$  subunit. The results suggest that alteration in phosphorylation level of  $\text{Na}^+, \text{K}^+$ -ATPase  $\alpha 3$  but not  $\alpha 1$  subunit was involved in modulation of  $\text{Na}^+, \text{K}^+$ -ATPase by morphine.

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

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### Discussion

Opioid receptors belong to the superfamily of GPCRs 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. Acute opiate exposure inhibits adenylyl cyclase activity and reduces cAMP production, whereas chronic 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 chronic 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 acute morphine treatment and inhibition of  $Na^+,K^+$ -ATPase activity after activation by chronic morphine treatment in the hippocampus. The stimulation or inhibition of  $Na^+,K^+$ -ATPase activity in response to differential morphine treatments was due to 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 acute morphine treatment and inhibition of  $Na^+,K^+$ -ATPase by chronic morphine treatment were naltrexone-reversible, indicating that the regulation of  $Na^+,K^+$ -ATPase activity by acute or chronic

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morphine treatment was mediated by opioid receptors. Second, the regulation of Na<sup>+</sup>,K<sup>+</sup>-ATPase activity by acute or chronic morphine treatment was inversely correlated with intracellular cAMP accumulation. Acute morphine treatment enhanced Na<sup>+</sup>,K<sup>+</sup>-ATPase activity but reduced cAMP concentration. The direct evidence to support the inverse relationship between cAMP concentration and Na<sup>+</sup>,K<sup>+</sup>-ATPase activity was that increase in cAMP concentration in hippocampus by concomitant administration of forskolin and db-cAMP markedly suppressed acute morphine-induced enhancement of Na<sup>+</sup>,K<sup>+</sup>-ATPase activity. Contrary to acute morphine treatment, chronic morphine-treatment reduced Na<sup>+</sup>,K<sup>+</sup>-ATPase activity but enhanced cAMP concentration. Moreover, in chronic morphine-treated mice, naloxone precipitation induced a further reduction of Na<sup>+</sup>,K<sup>+</sup>-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<sup>+</sup>,K<sup>+</sup>-ATPase activity. Third, the selective PKA inhibitor, H-89 mimicked the stimulatory effect of acute morphine on Na<sup>+</sup>,K<sup>+</sup>-ATPase activity but antagonized the inhibitory effect of chronic morphine on Na<sup>+</sup>,K<sup>+</sup>-ATPase activity. Importantly, H-89 significantly inhibited chronic morphine-induced the phosphorylation of Na<sup>+</sup>,K<sup>+</sup>-ATPase, supporting that alteration of PKA activity was implicated in the regulation of Na<sup>+</sup>,K<sup>+</sup>-ATPase activity upon activation of opioid receptors. A significant increase in PKA activity in rat hippocampus after chronic 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 acute morphine on Na<sup>+</sup>,K<sup>+</sup>-ATPase activity but potentiated the inhibitory effect of chronic morphine on Na<sup>+</sup>,K<sup>+</sup>-ATPase activity, suggesting that the phosphorylation level of Na<sup>+</sup>,K<sup>+</sup>-ATPase is inversely correlated with Na<sup>+</sup>,K<sup>+</sup>-ATPase activity. This is consistent with previous observations that phosphorylation of Na<sup>+</sup>,K<sup>+</sup>-ATPase is associated with inhibition of the enzyme activity (Bertorello et

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al., 1991; Cheng et al., 1997; Li et al., 1998). The direct evidence to support involvement of changes in basal phosphorylation level of Na<sup>+</sup>,K<sup>+</sup>-ATPase in regulation of Na<sup>+</sup>,K<sup>+</sup>-ATPase activity was that acute morphine administration resulted in significant reduction of the total phosphorylation levels of the  $\alpha 3$  subunit of Na<sup>+</sup>,K<sup>+</sup>-ATPase in the hippocampus, while chronic 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<sup>+</sup>,K<sup>+</sup>-ATPase by acute morphine and enhancement of phosphorylation of the  $\alpha 3$  subunit by chronic morphine. Interestingly, it seems that the regulation of phosphorylation of  $\alpha 3$  subunit is selective, since neither acute nor chronic morphine treatment significantly regulated the phosphorylation of the  $\alpha 1$  subunit of Na<sup>+</sup>,K<sup>+</sup>-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<sup>+</sup>,K<sup>+</sup>-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 due to their intrinsic structural difference as proposed (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, receptors, etc.) necessary for mediating second messenger regulation of activity (Nishi et al., 1999). Taken collectively, these results strongly demonstrate that cAMP/PKA signaling pathway is involved in regulation of Na<sup>+</sup>,K<sup>+</sup>-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<sup>+</sup>,K<sup>+</sup>-ATPase in regulation of Na<sup>+</sup>,K<sup>+</sup>-ATPase by morphine, further work is need to elucidate

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whether PKA acts on Na<sup>+</sup>,K<sup>+</sup>-ATPase directly or effects through the ancillary proteins.

Phosphorylation of the catalytic  $\alpha$ -subunit of Na<sup>+</sup>,K<sup>+</sup>-ATPase by protein kinases has been reported to be associated with triggering for endocytosis of Na<sup>+</sup>,K<sup>+</sup>-ATPase in other tissue (Chibalin et al., 1998a; 1998b). Although the present study demonstrated that neither acute nor chronic morphine treatment could significantly change the abundance of  $\alpha$ 1 or  $\alpha$ 3 subunit of Na<sup>+</sup>,K<sup>+</sup>-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) was applied in this study.

There are two well-established adaptations to chronic opioid exposure, which are linked to opioid tolerance and dependence (for review, see Taylor and Fleming, 2001). One 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 is the depolarization of neuronal cells, as a result of reduction in Na<sup>+</sup>,K<sup>+</sup>-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 if two adaptations were independent mechanisms of tolerance and dependence induction or were 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<sup>+</sup>,K<sup>+</sup>-ATPase, leading to diminution of Na<sup>+</sup>,K<sup>+</sup>-ATPase activity, which may contribute to the depolarization of neuron, thereby resulting in opioid tolerance and

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dependence. Up-regulation of cAMP/PKA signaling pathway upon chronic 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 (CREB), a transcription factor that regulates the expression of numerous genes (for review, see Liu and Anand, 2001; Nestler and Aghajanian, 1997), the present results demonstrated a new adaptation: the phosphorylation of Na<sup>+</sup>,K<sup>+</sup>-ATPase. This may give an insight into the mechanisms underlying opioid tolerance and dependence.

Na<sup>+</sup>,K<sup>+</sup>-ATPase (pump) is ubiquitous in neurons and widely distributed in the mammalian brain (Hauger et al., 1985). It maintains Na<sup>+</sup> and K<sup>+</sup> gradient across cell membrane by returning Na<sup>+</sup> and K<sup>+</sup> 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<sup>+</sup>-Ca<sup>2+</sup> exchanger (Blaustein 1999). Impairment of the Na<sup>+</sup>, K<sup>+</sup>-pump reduces intracellular K<sup>+</sup> and increases intracellular Na<sup>+</sup>, consequently leading to the membrane depolarization and enhancement of intracellular free Ca<sup>2+</sup> due to activation of voltage-gated Ca<sup>2+</sup> channels and reverses operation of the Na<sup>+</sup>-Ca<sup>2+</sup> exchanger (Blaustein 1999). It has been reported that Na<sup>+</sup>,K<sup>+</sup>-ATPase is involved in synaptic plasticity (Glushchenko et al., 1997; Reich et al., 2004; Ross and Soltesz, 2001) and plays a role in the process of learning and memory (Christain et al., 2004; Wyse et al., 2004). Impairment of Na<sup>+</sup>,K<sup>+</sup>-ATPase was proposed to be responsible for memory loss and other cognitive disturbance caused by brain injury (Reich et al., 2004; Ross and Soltesz, 2000). Inhibition of Na<sup>+</sup>,K<sup>+</sup>-ATPase with dihydroouabain (DHO), a ouabain analogue, produced a long-lasting suppression of field excitatory postsynaptic potentials (fEPSPs) in rat hippocampal CA1 cells, which leads to depotentiate long-term potentiation (LTP) (Reich et al., 2004), LTP is thought to be a possible

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neuronal mechanism underlying learning and memory (Bliss and Collingridge, 1993). Recently, drug dependence has been proposed to be an aberrant form of learning mediated by maladaptive recruitment of certain memory systems in brain (Robbins and Everitt, 1999). It has been reported that chronic use of opiates could attenuate hippocampal LTP (Pu et al., 2002). Diminution of  $\text{Na}^+, \text{K}^+$ -ATPase activity induced by up-regulation of cAMP/PKA signaling pathway following chronic exposure to opioids may be a possible 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 acute and chronic morphine treatment could modulate  $\text{Na}^+, \text{K}^+$ -ATPase activity in the mouse hippocampus by cAMP/PKA signaling pathway. The regulation of  $\text{Na}^+, \text{K}^+$ -ATPase activity by morphine treatment appeared to associate with the alteration in phosphorylation level of  $\text{Na}^+, \text{K}^+$ -ATPase. The results suggest that diminution of  $\text{Na}^+, \text{K}^+$ -ATPase mediated by up-regulated cAMP/PKA cascade may be a potential mechanism by which chronic exposure to morphine results in opioid dependence.

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### Figure Legends

**Fig.1** In vivo and in vitro acute morphine stimulation of ouabain-sensitive  $\text{Na}^+, \text{K}^+$ -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, the crude synaptosomes (P2 pellets) prepared from the hippocampus of naïve mice were treated with different concentrations of morphine ( $10^{-9}$ ~ $10^{-4}$  M) for 10 min at 37 °C.  $\text{Na}^+, \text{K}^+$ -ATPase activity was measured as described in *Materials and Methods*. Values of each point are presented as the mean  $\pm$  S.D. of three separate experiments performed in duplicate. \* $P < 0.05$ , \*\* $P < 0.01$  as compared with vehicle-treated control animals or non morphine-treated crude synaptosomes.

**Fig.2** Reversal of the stimulatory effect of acute morphine on hippocampal  $\text{Na}^+, \text{K}^+$ -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 prior to morphine administration. B, hippocampal crude synaptosomes were incubated with  $10^{-5}$  M of naltrexone for 5 min at 37 °C, and then treated with  $10^{-5}$  M of 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  $\mu\text{g}$ , i.c.v.) or vehicle (50% (v/v) glycerol, 50 mM Tris, pH 7.5, 10 mM glycine, 0.5 M NaCl; 5  $\mu\text{l}$ , i.c.v.). PTX or vehicle was administrated 5 days before morphine treatment.  $\text{Na}^+, \text{K}^+$ -ATPase activity (A, B and C) and p44/p42 MAPK phosphorylation (D) were measured as described in *Materials and Methods*. D, *upper panel*: representative immunoblots for phosphorylated p44/p42 MAPK (*top*) and total p42 MAPK (*bottom*); *lower panel*: quantitative estimation by scanning densitometry of ratio of phosphorylated p44/p42 MAPK to total p42 MAPK.

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Data represent the mean  $\pm$  S.D. of there separate experiments performed in duplicate.  $**P<0.01$  as compared with vehicle-treated control mice;  $^{\ddagger}P<0.01$  as compared with mice treated with morphine alone. CTL, control; MOR, morphine; NTX, naltrexone; PTX, pertussis toxin.

**Fig.3** Enhancement of hippocampal cAMP level and PKA activity and inhibition of acute morphine-induced enhancement of  $\text{Na}^+, \text{K}^+$ -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 vehicles. Naltrexone, forskolin and db-cAMP were injected 30 min prior to morphine administration. B and C, mice were treated with forskolin (10 ng in 5 $\mu$ l, i.c.v.) or db-cAMP (100 ng in 5  $\mu$ l, i.c.v.) or vehicles (0.2% DMSO (v/v) in saline, 5 $\mu$ l, i.c.v.) for 30 min. Animals were sacrificed, and the hippocampi were quickly isolated on ice.  $\text{Na}^+, \text{K}^+$ -ATPase activity (D and E), intracellular cAMP concentration (A and B) and PKA activity (C) were measured as described in *Materials and Methods*. C, *upper panel*: representative gel electrophoresis of PKA activity assays; *lower panel*: quantitative determination of the PKA activity by spectrophotometry. Data are expressed as the mean  $\pm$  S.D. of three separate experiments performed in duplicate.  $*P<0.05$ ,  $**P<0.01$  as compared with vehicle-treated control mice;  $^{\ddagger}P<0.01$  as compared with mice treated with morphine alone. CTL, control; MOR, morphine; NTX, naltrexone; FOR, forskolin; DBC, db-cAMP; NEG, negative control.

**Fig.4** H-89 mimicked the stimulatory effect of acute morphine on  $\text{Na}^+, \text{K}^+$ -ATPase activity, but OA suppressed the stimulatory effect of acute morphine on  $\text{Na}^+, \text{K}^+$ -ATPase activity. A, mice were treated with H-89 (1 nmol in 5  $\mu$ l, i.c.v.) or saline (5  $\mu$ l, i.c.v.) for 30 min. B, mice were acutely treated with morphine (10 mg/kg, s.c.) or saline (10 ml/kg), or concomitantly treated with OA (100 pg in 5 $\mu$ l, i.c.v.) or saline (5  $\mu$ l, i.c.v.). OA was injected 30 min prior to morphine administration. After treatment the



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hippocampus was quickly isolated and membrane was prepared.  $\text{Na}^+, \text{K}^+$ -ATPase activity was measured as described in *Materials and Methods*. Data are expressed as the mean  $\pm$  S.D. of three separate experiments performed in duplicate.  $**P < 0.01$  as compared with vehicle-treated control mice;  $^\ddagger P < 0.01$  as compared with mice treated with morphine alone. CTL, control; MOR, morphine.

**Fig.5** Chronic morphine treatment decreased  $\text{Na}^+, \text{K}^+$ -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  $\text{Na}^+, \text{K}^+$ -ATPase activity and enhancement of cAMP concentration. Mice were chronically 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 h 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 exception the mice were precipitated with naloxone (2 mg/kg, i.p.) before sacrifice.  $\text{Na}^+, \text{K}^+$ -ATPase activity (A) and cAMP levels (B) were determined as described in *Materials and Methods*. Data are expressed as the mean  $\pm$  S.D. of three independent experiments performed in duplicate.  $**P < 0.01$  as compared with vehicle-treated mice;  $^\ddagger P < 0.01$  as compared with mice treated with morphine alone. CTL, control; MOR, morphine; NLX, naloxone; NTX, naltrexone.

**Fig.6** Antagonism by H-89 and potentiation by OA of the inhibitory effect of chronic morphine on  $\text{Na}^+, \text{K}^+$ -ATPase activity. A, reversal by H-89 of inhibition of  $\text{Na}^+, \text{K}^+$ -ATPase activity induced by chronic morphine treatment. B, potentiation by OA of inhibition of  $\text{Na}^+, \text{K}^+$ -ATPase activity mediated by chronic morphine treatment. Mice were chronically treated with morphine (10 mg/kg, s.c.) or saline (10 ml/kg, s.c.) for 10 days. One h after the termination of chronic morphine treatment, animals were given with H-89 (1 nmol in 5  $\mu\text{l}$ , i.c.v.) or OA (100 pg in 5  $\mu\text{l}$ , i.c.v.), or saline (5  $\mu\text{l}$ , i.c.v.). 30 min

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later, mice were decapitated, and the hippocampi were quickly isolated and membrane was prepared.  $\text{Na}^+, \text{K}^+$ -ATPase activity was measured as described in *Materials and Methods*. Data are expressed as the mean  $\pm$  S.D. of three independent experiments performed in duplicate. \* $P < 0.05$ , \*\* $P < 0.01$  as compared with vehicle-treated control mice; † $P < 0.05$ , ‡ $P < 0.01$  as compared with morphine-treated mice. CTL, control; MOR, morphine.

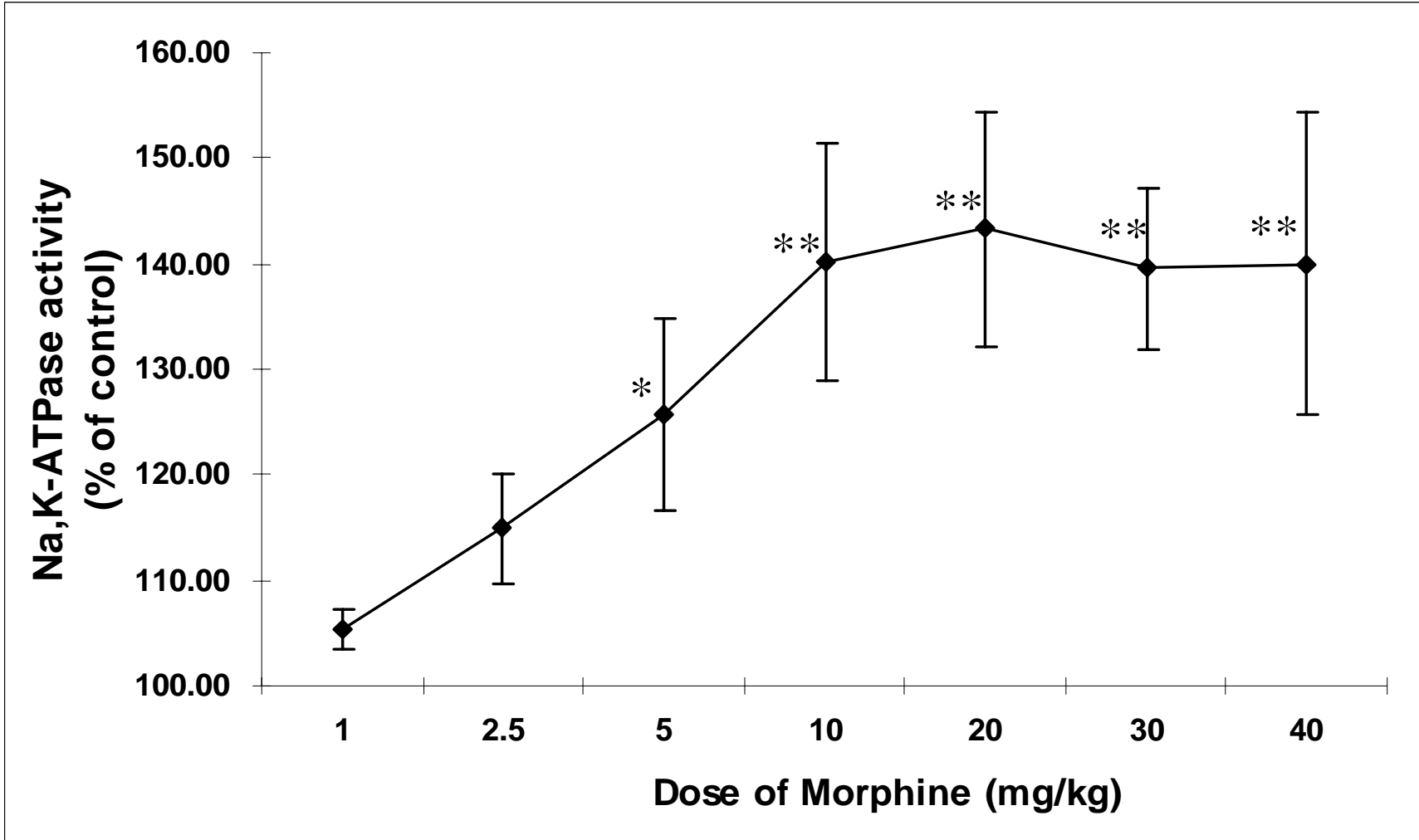
**Fig.7** Reduction by acute morphine treatment and enhancement by chronic morphine treatment of the phosphorylation levels of  $\alpha 3$  but not  $\alpha 1$  subunit of  $\text{Na}^+, \text{K}^+$ -ATPase in the hippocampus. A, mice were acutely treated with morphine (10 mg/kg, s. c.) or saline (10 ml/kg, s.c.), or concomitantly treated with naltrexone (4 mg/kg, i.p.) or saline (10 ml/kg, i.p.). Naltrexone and saline were administered 30 min before morphine treatment. One h after morphine treatment, mice were decapitated and the hippocampi were isolated on ice. B, mice were chronically 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 h after the final morphine or saline treatment, mice were treated with H-89 (1 nmol in 5  $\mu\text{l}$ , i.c.v.) or saline (5  $\mu\text{l}$ , i.c.v.) for 30 min, and then the hippocampi were isolated on ice. C, mice were acutely or chronically treated with morphine or saline as described in A and B. Phosphorylation assay was performed as described in the *Materials and Methods*. The  $\alpha 3$  or  $\alpha 1$  subunit of the enzyme was immunoprecipitated by the anti- $\alpha 3$  or anti- $\alpha 1$  antibody, respectively, and then was probed with the anti-pan antibody. A, B and C, *upper panels*: representative immunoblots for the phosphorylated  $\alpha 3$  and  $\alpha 1$  subunits of  $\text{Na}^+, \text{K}^+$ -ATPase; *lower panels*: quantitative estimation by scanning densitometry of the total phosphorylation levels of the  $\alpha 3$  and  $\alpha 1$  subunits of  $\text{Na}^+, \text{K}^+$ -ATPase. Data were expressed as a percent of the control, and values represent the mean  $\pm$  S.D. of three independent experiments. \*\* $P < 0.01$  as compared with vehicle-treated control mice; † $P < 0.01$  as compared with morphine-treated mice. CTL, control; MOR, morphine; NTX, naltrexone; IP, immunoprecipitation; IB, immunoblotting.

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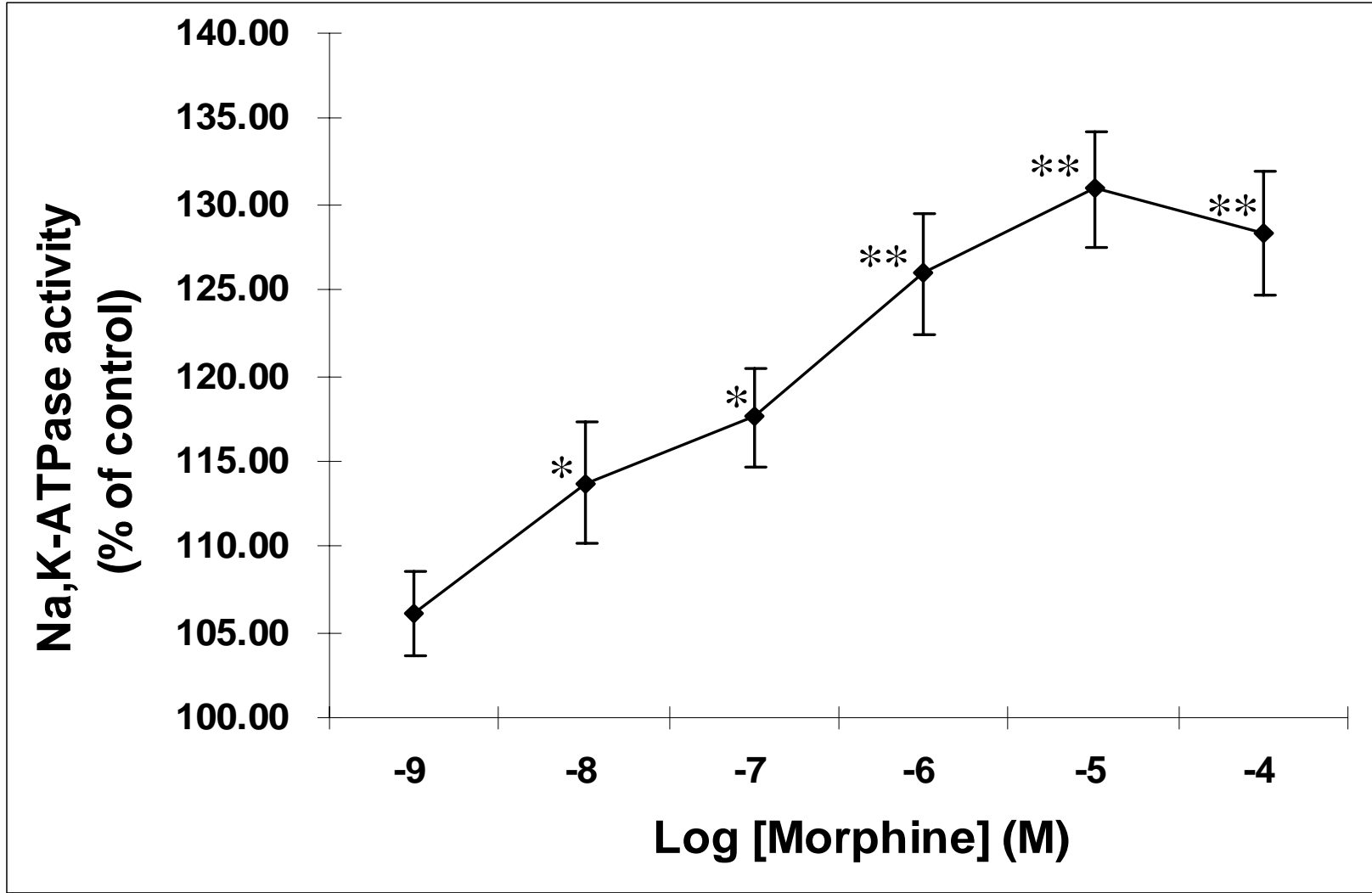
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**Fig.8** Effect of acute and chronic morphine-treatment on the expression of  $\alpha 1$  and  $\alpha 3$  subunits of the mouse hippocampal  $\text{Na}^+, \text{K}^+$ -ATPase. Equal quantities of protein prepared from acute and chronic morphine-treated mice were separated by SDS-PAGE and then incubated with the primary antibody against the  $\alpha 1$  or  $\alpha 3$  subunit of mouse  $\text{Na}^+, \text{K}^+$ -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. *Upper* panels, representative immunoblots for the  $\alpha 1$  and  $\alpha 3$  subunits of  $\text{Na}^+, \text{K}^+$ -ATPase; *Lower* panel, quantitative estimation (by scanning densitometry) of the expression of  $\alpha 1$  and  $\alpha 3$  subunits of  $\text{Na}^+, \text{K}^+$ -ATPase. CTL, control; MOR, morphine.

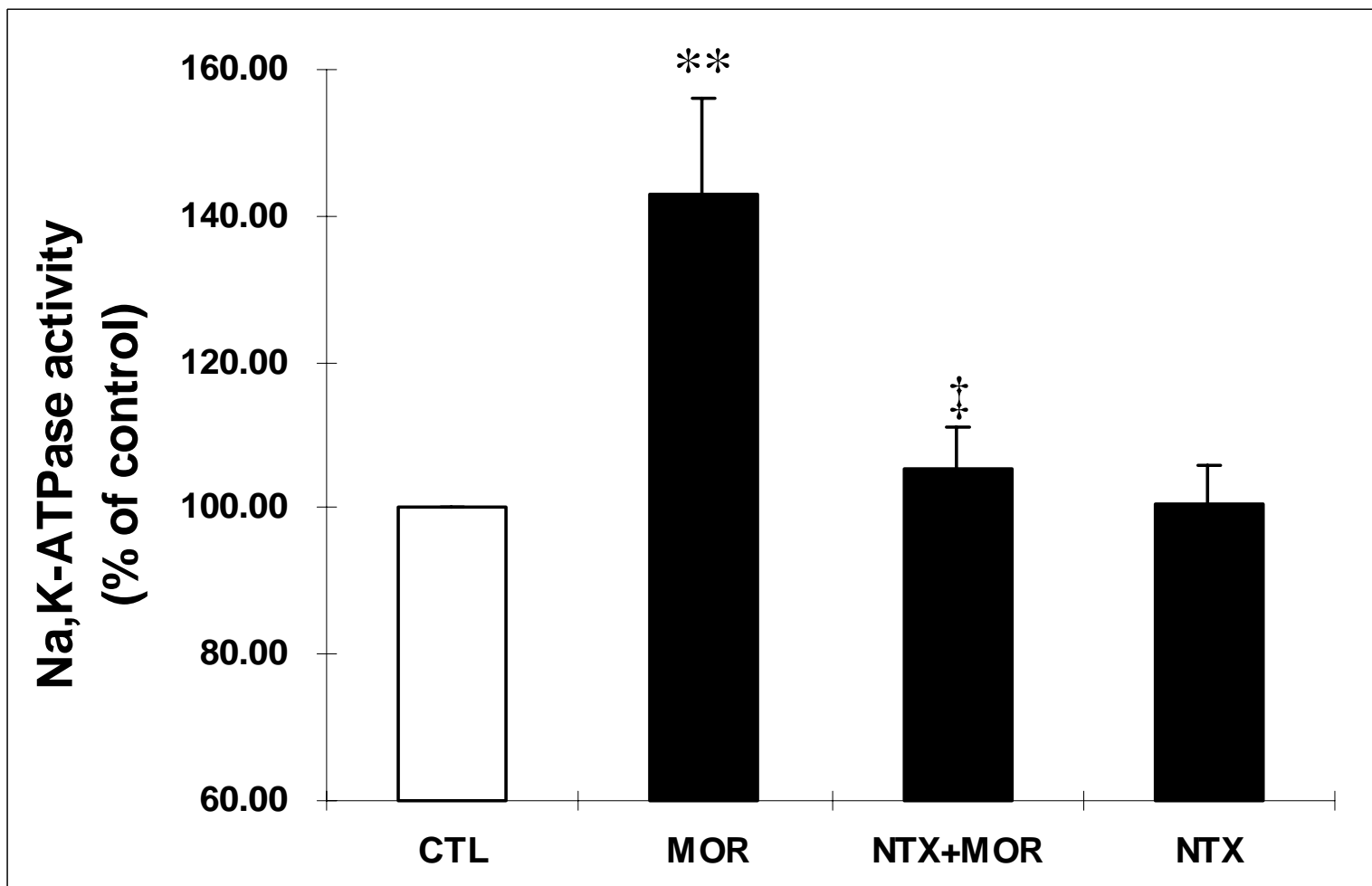
**Fig. 1A**



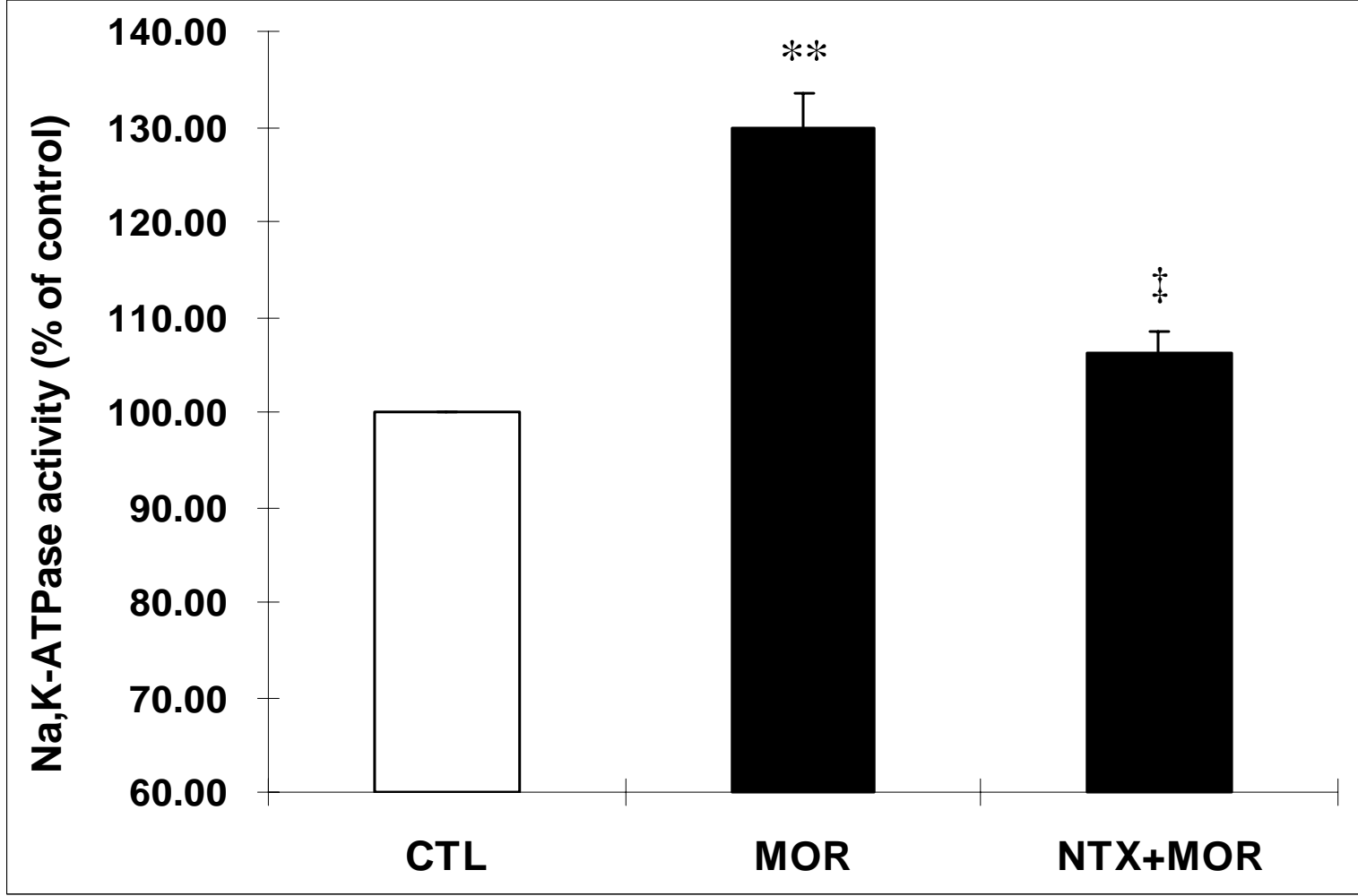
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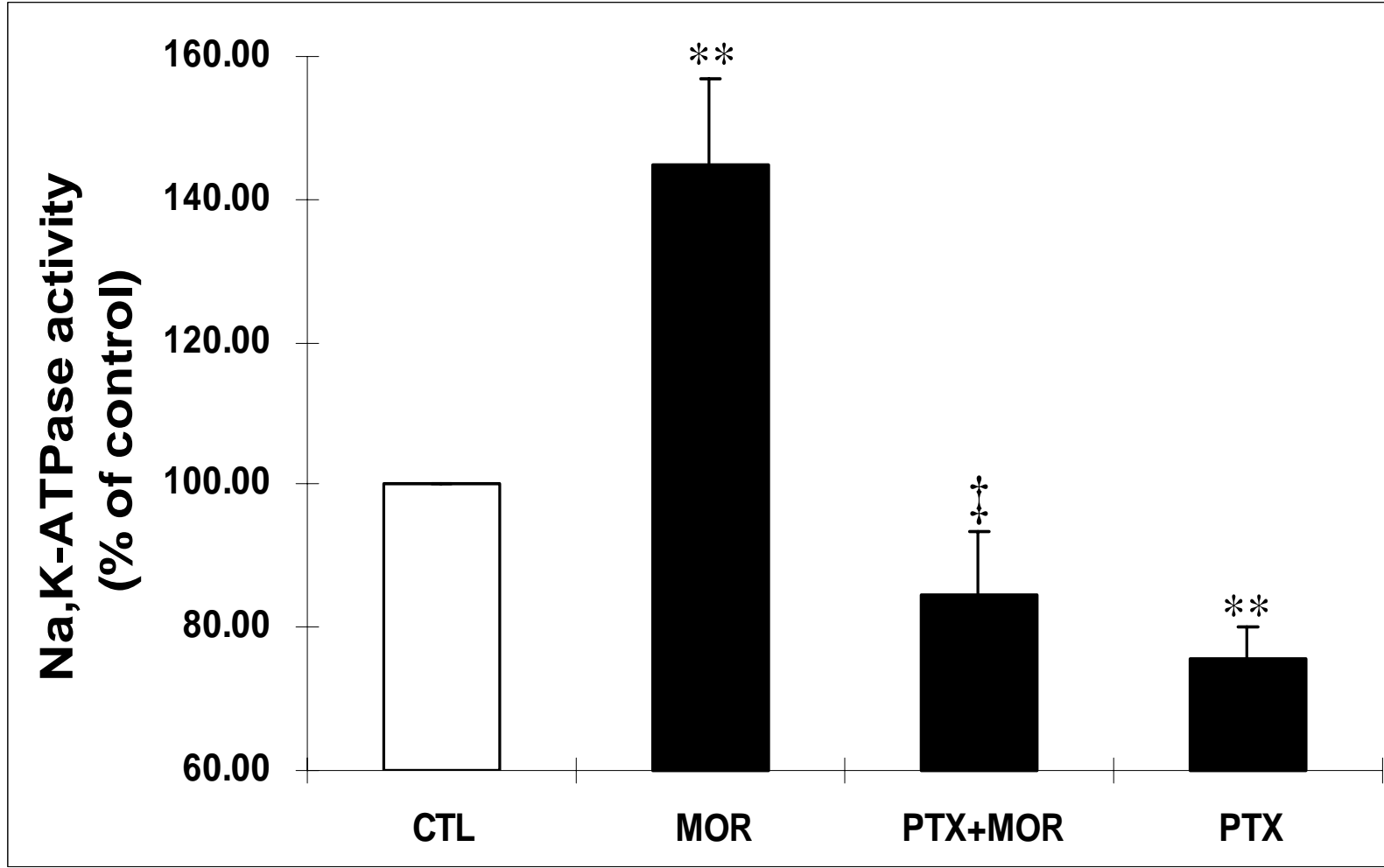
**Fig. 2A**



**Fig. 2B**

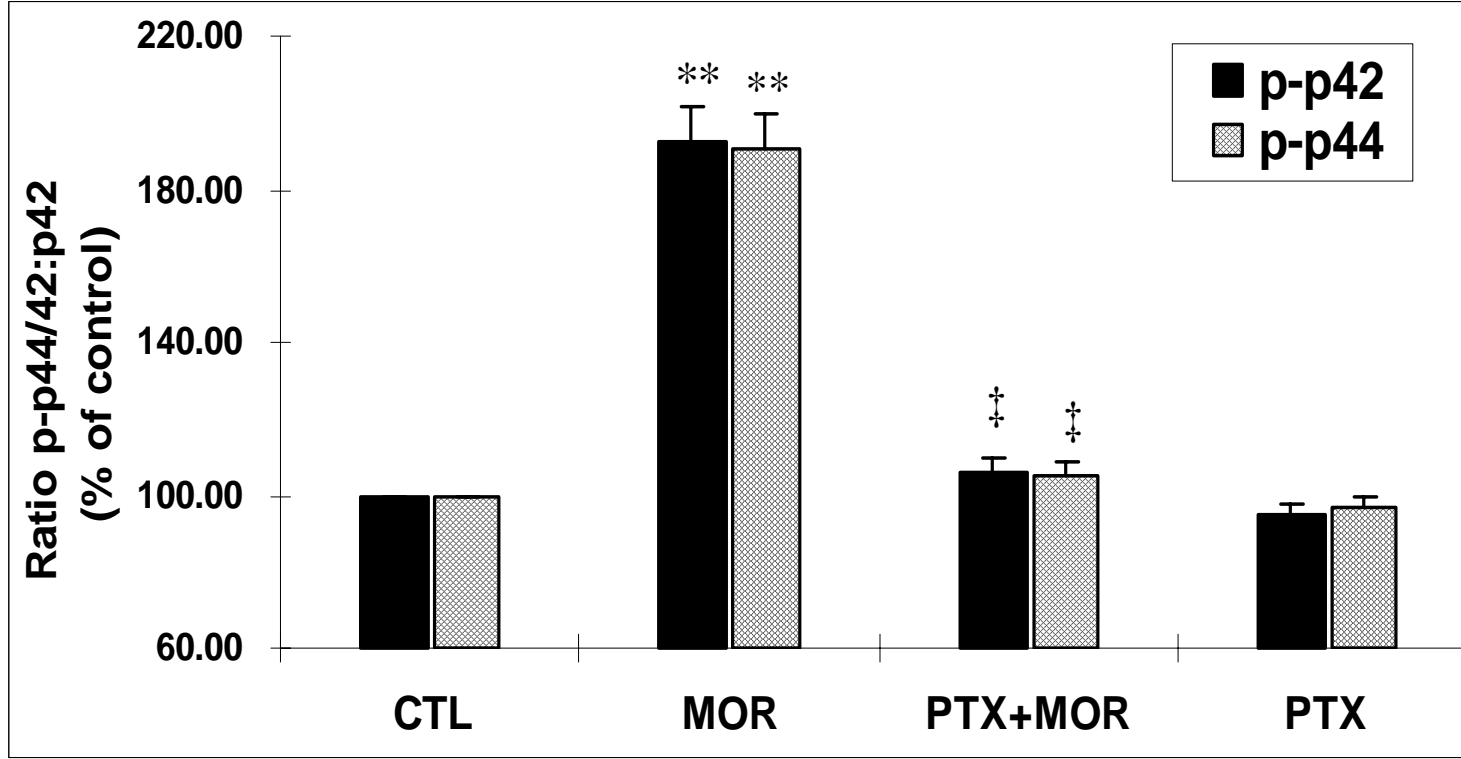
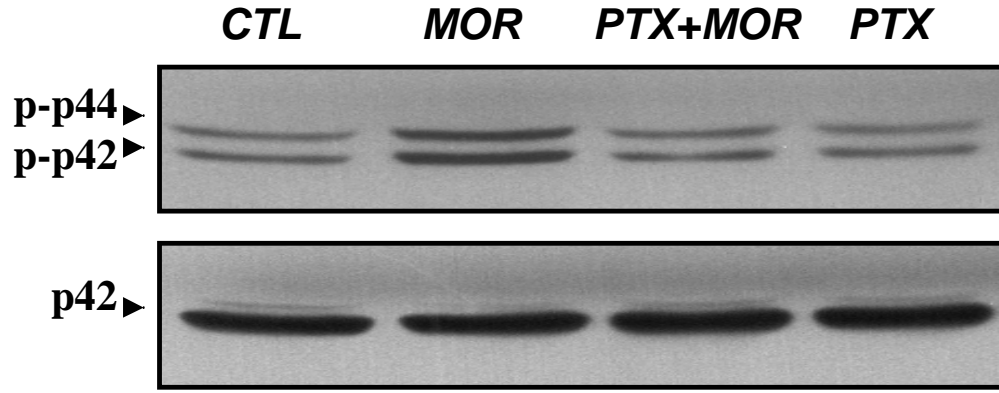


**Fig. 2C**

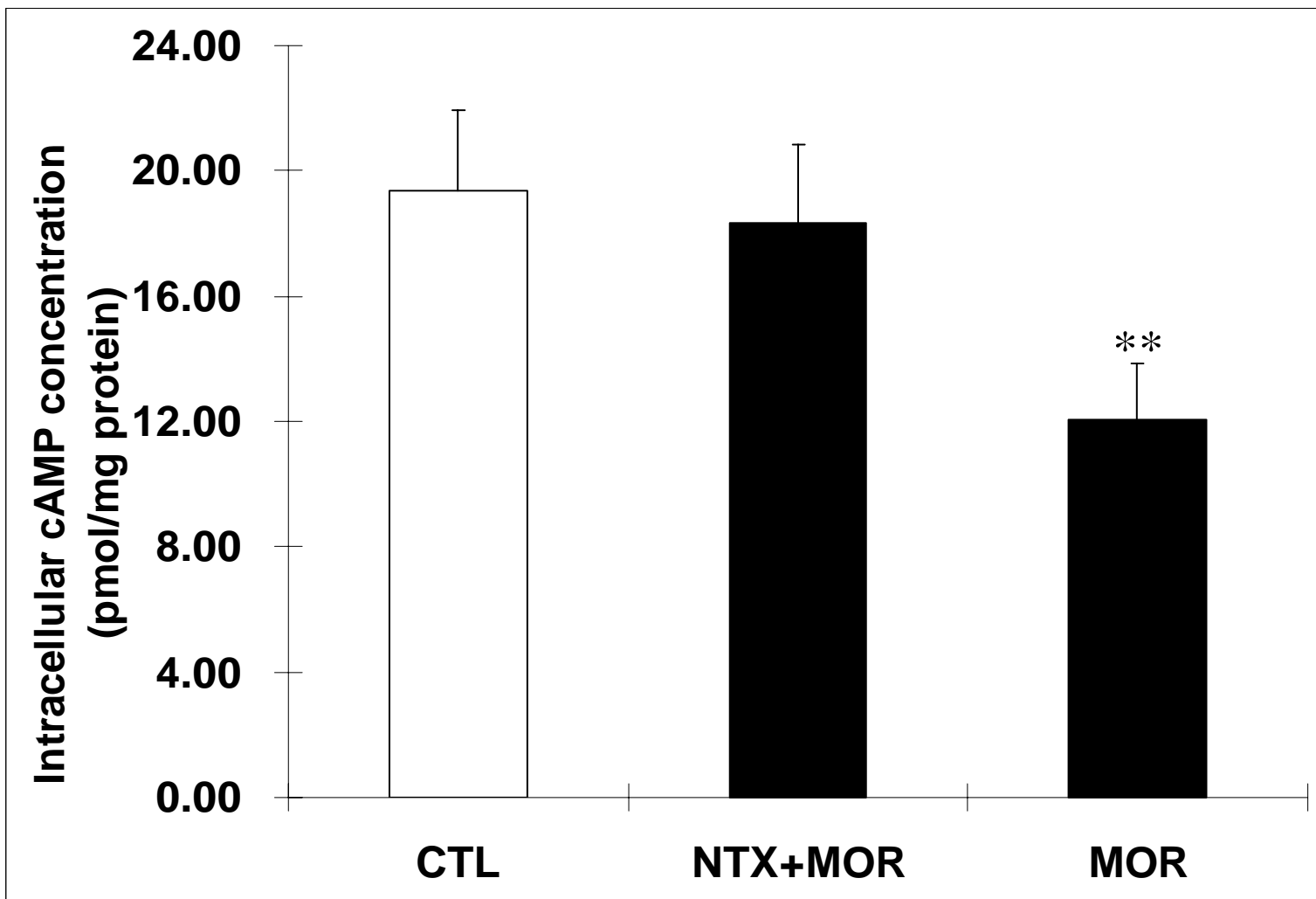




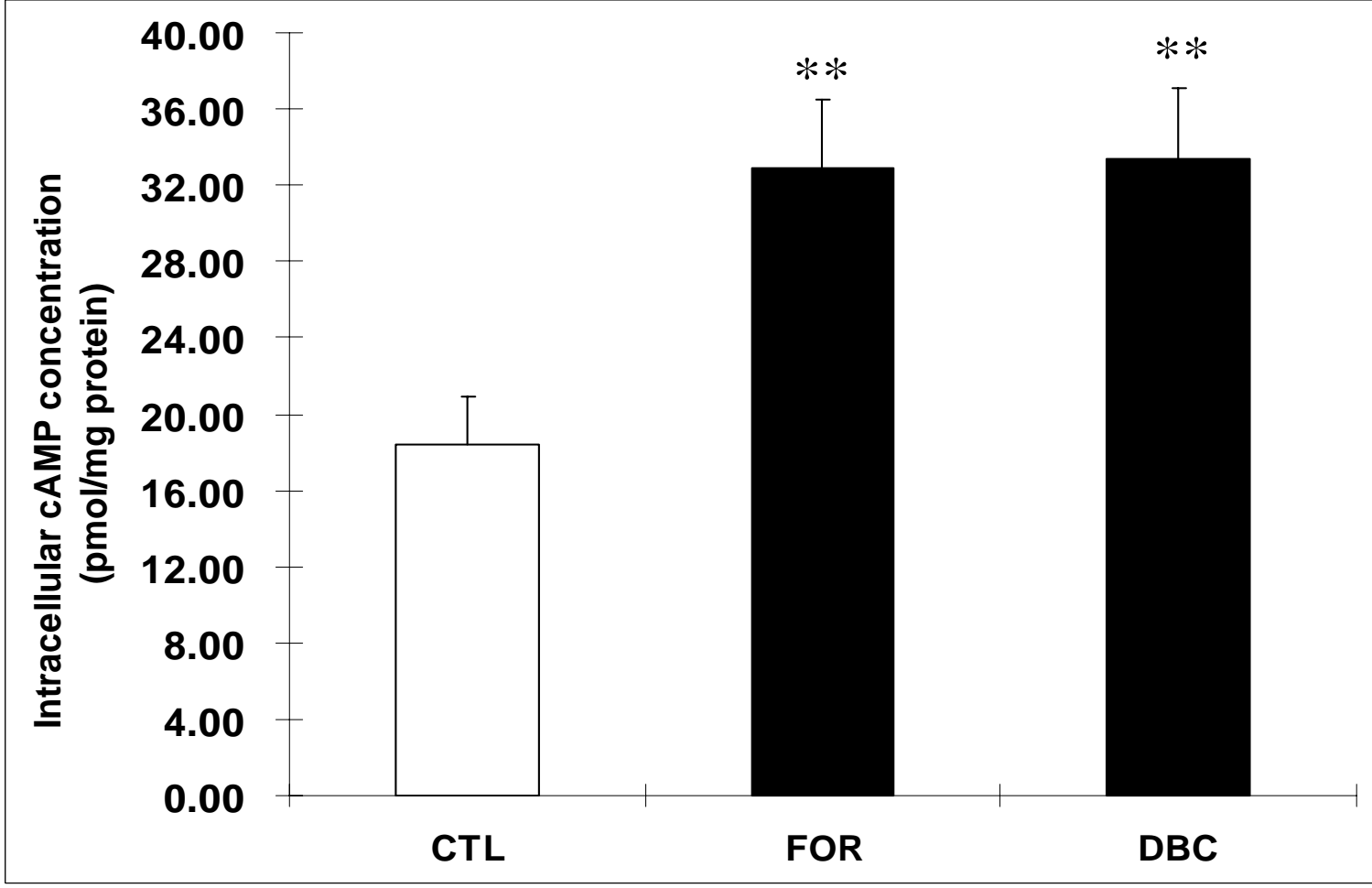
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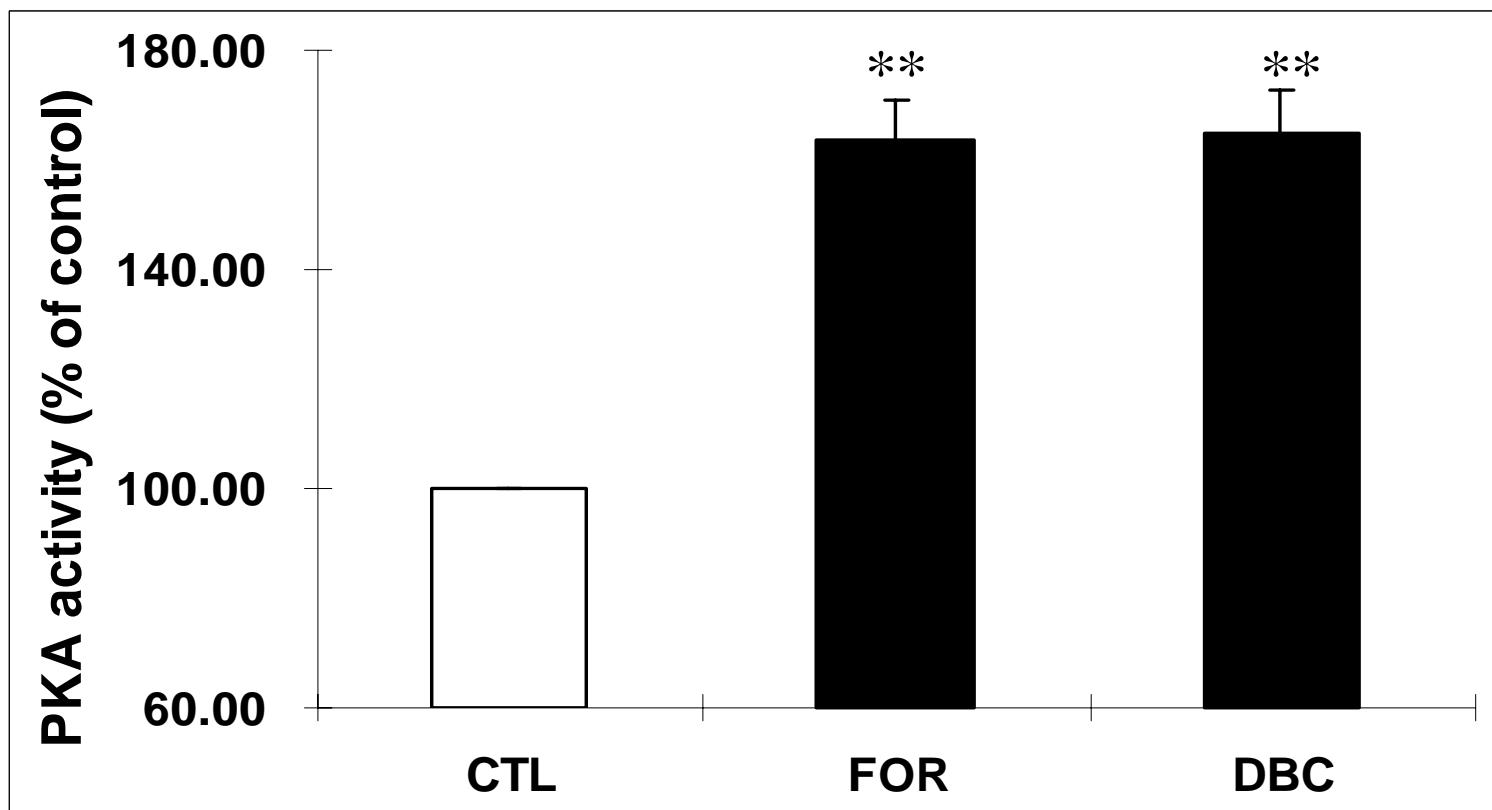
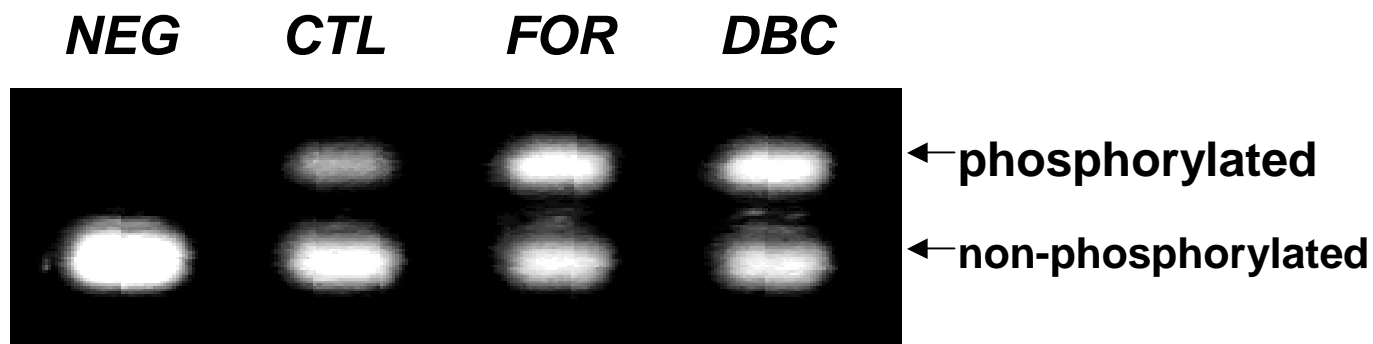
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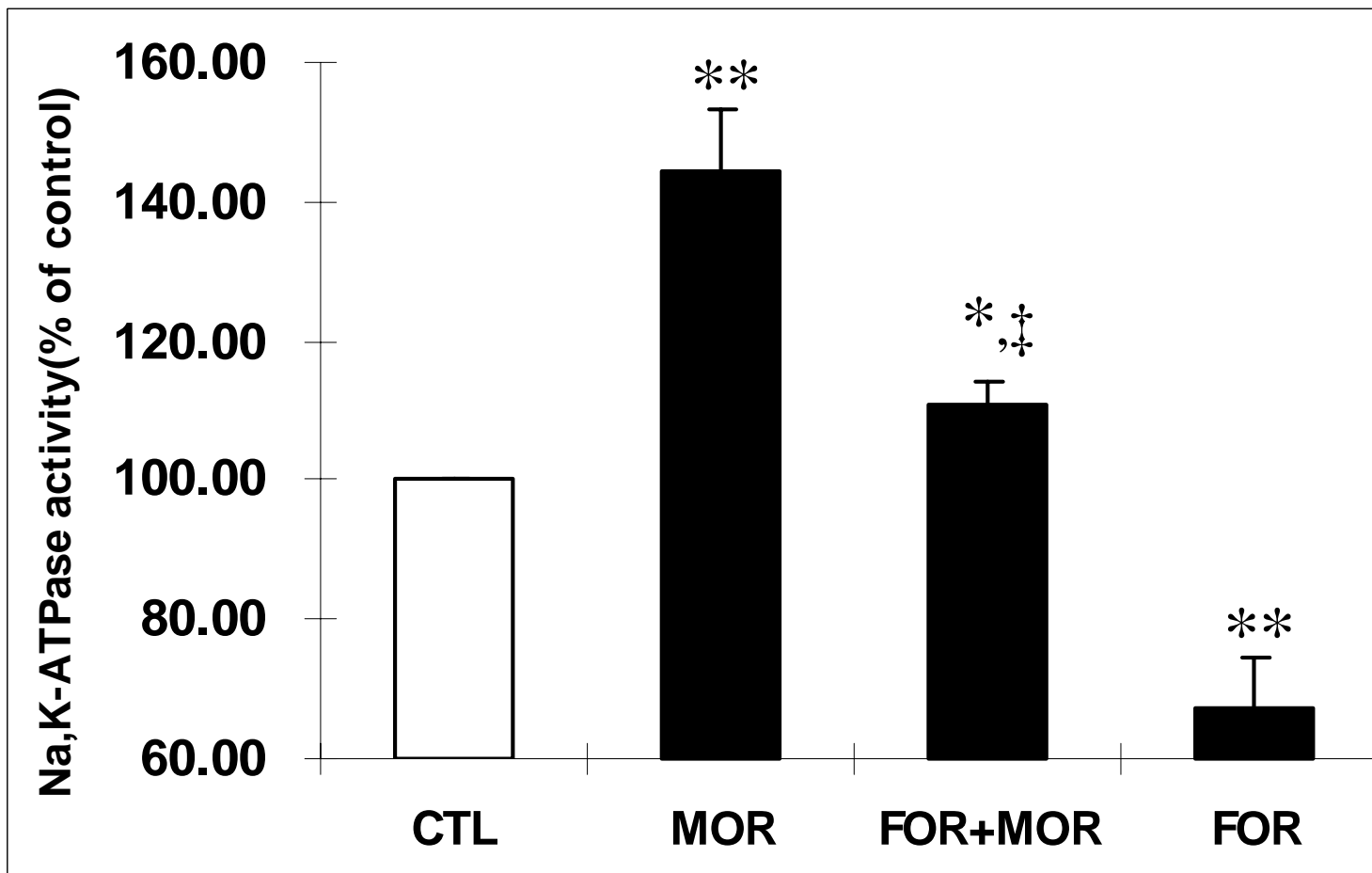
**Fig. 3B**



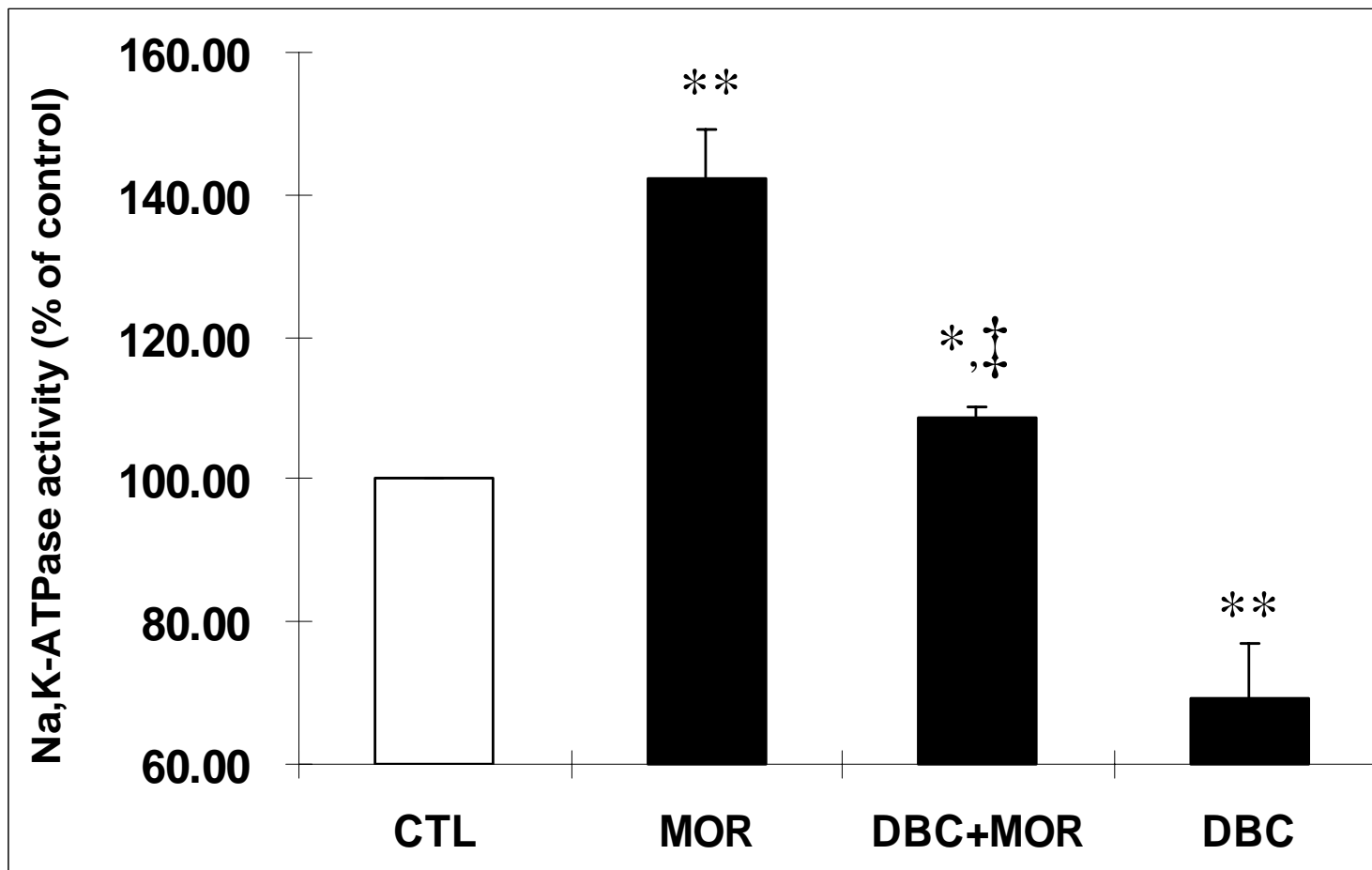
**Fig. 3C**



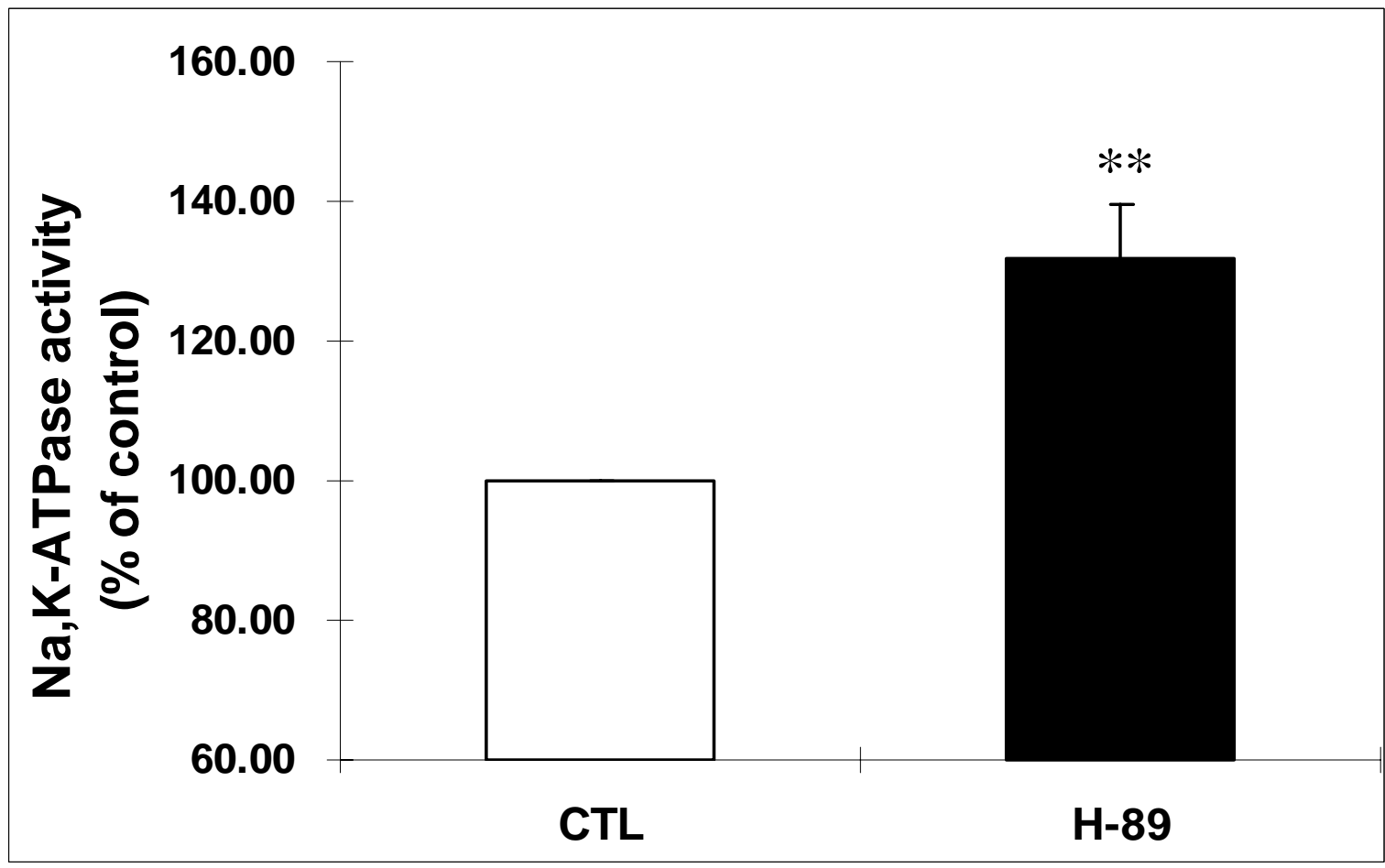
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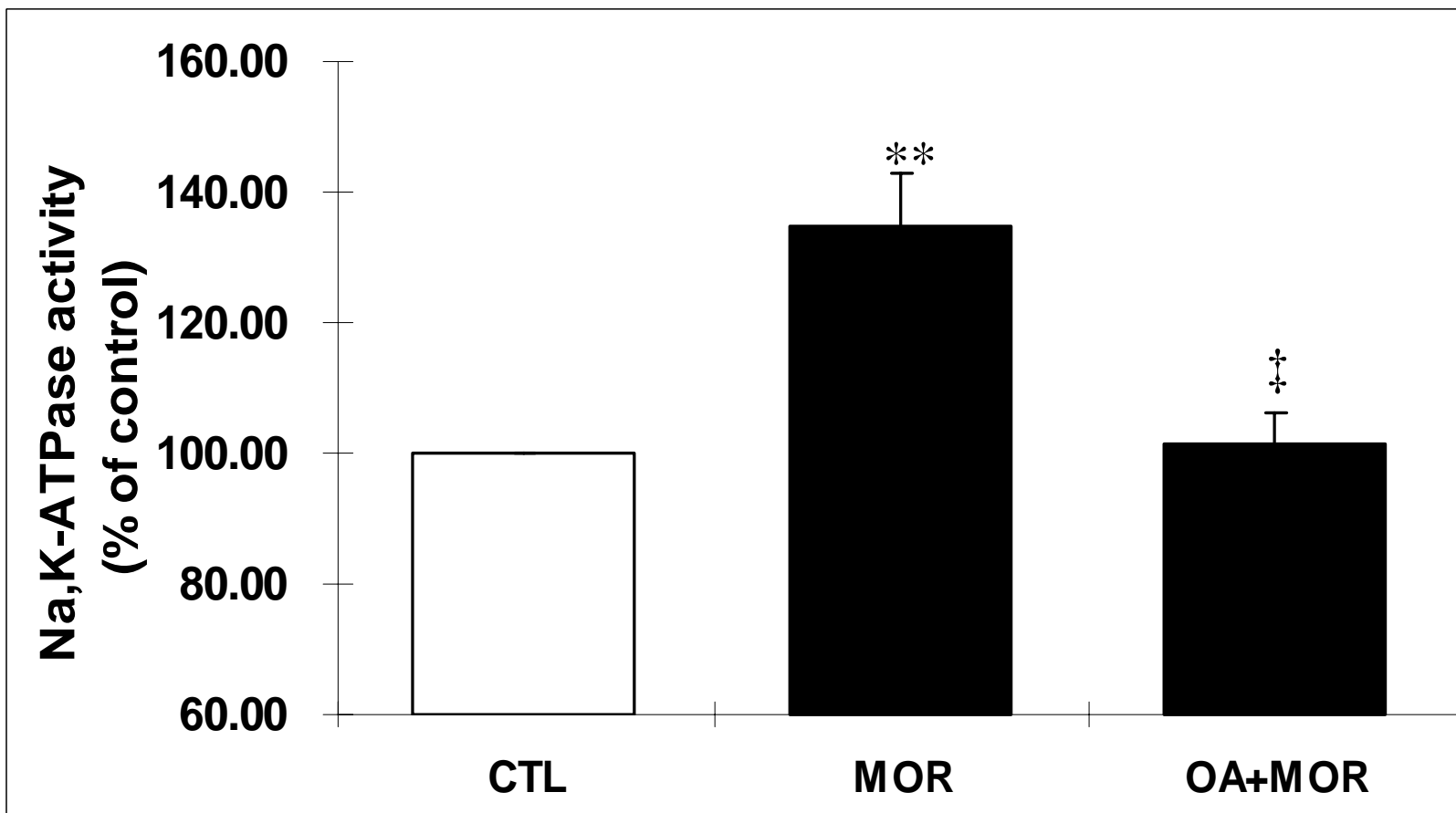
**Fig. 3E**



**Fig. 4A**

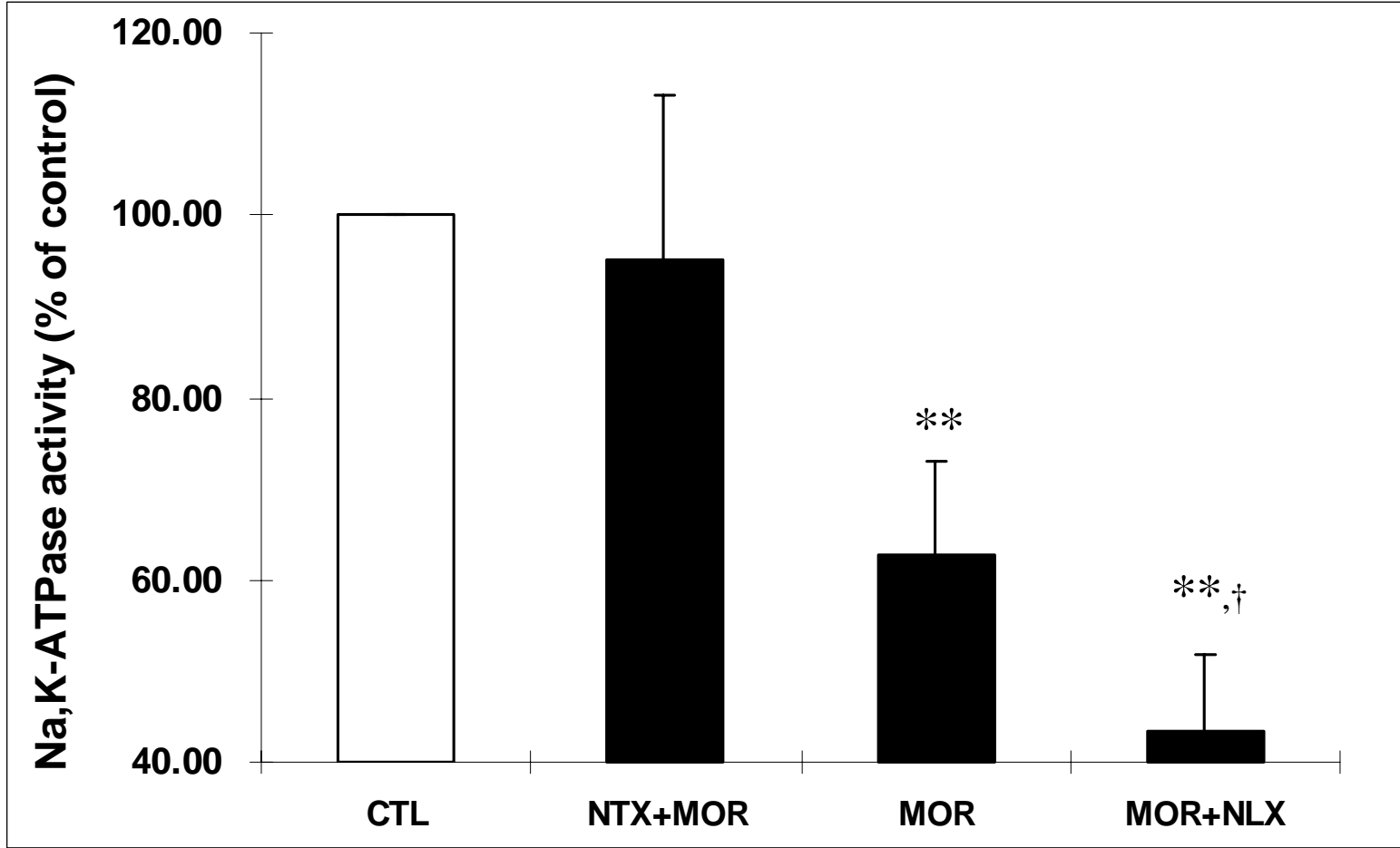


**Fig. 4B**

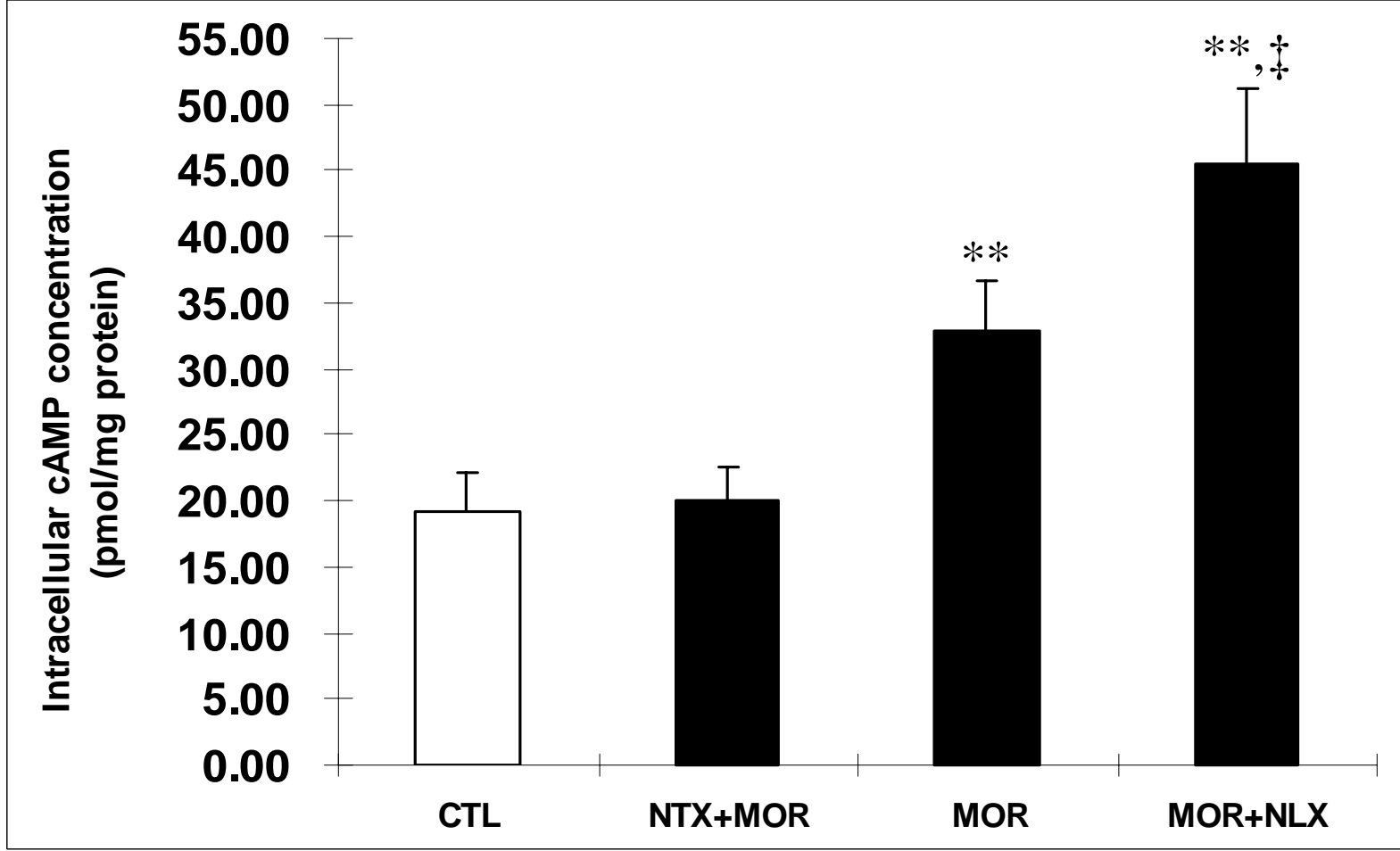




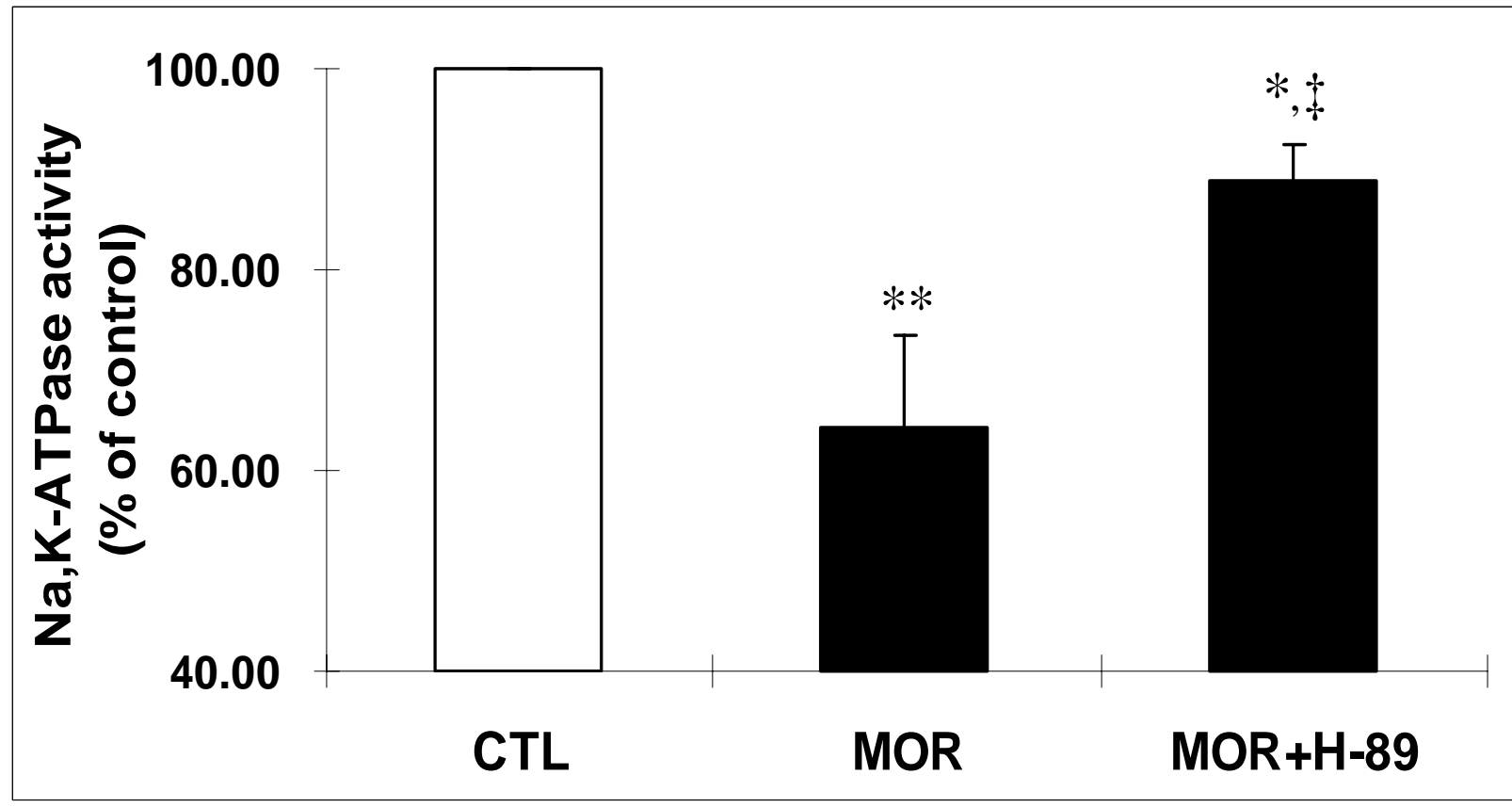
**Fig. 5A**



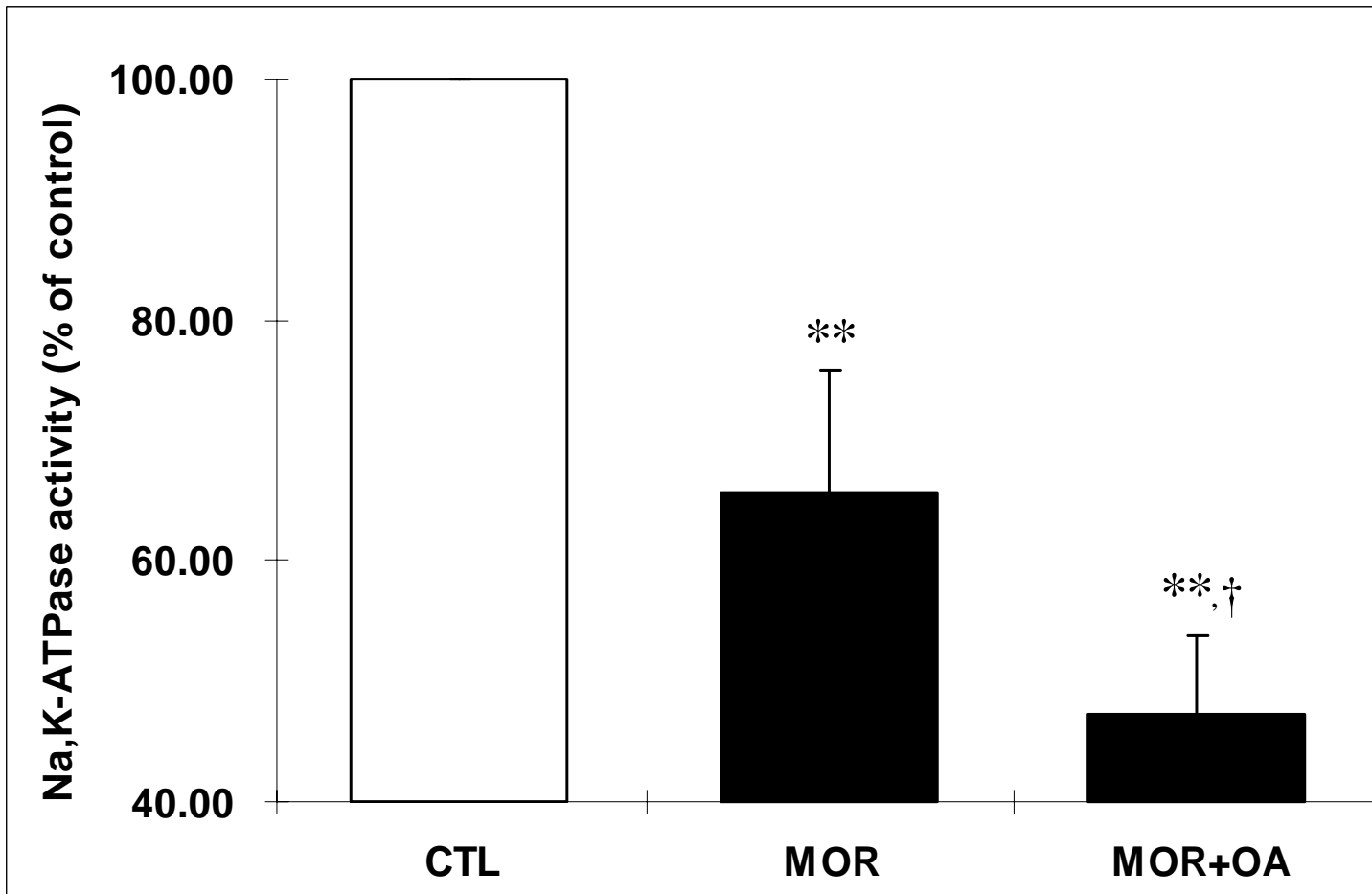
**Fig. 5B**



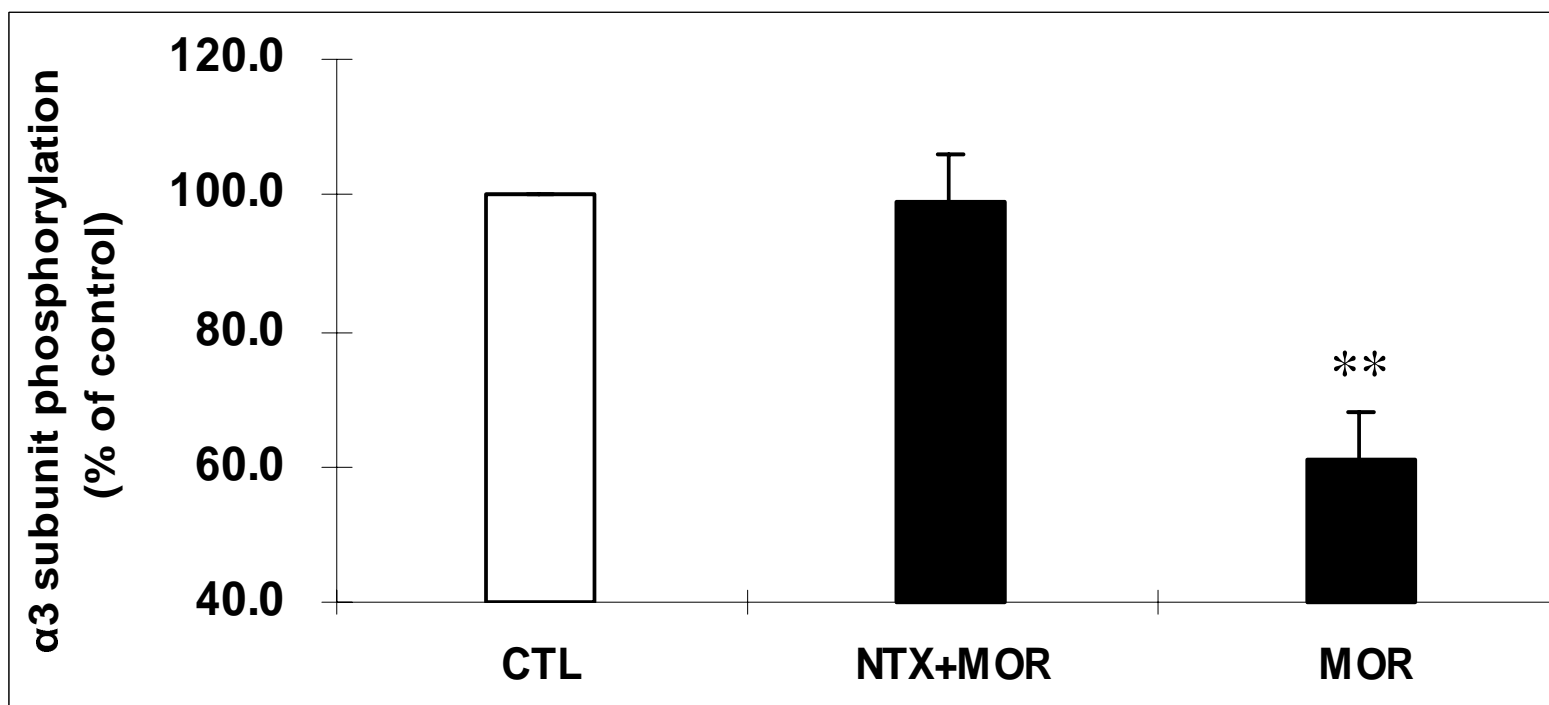
**Fig. 6A**



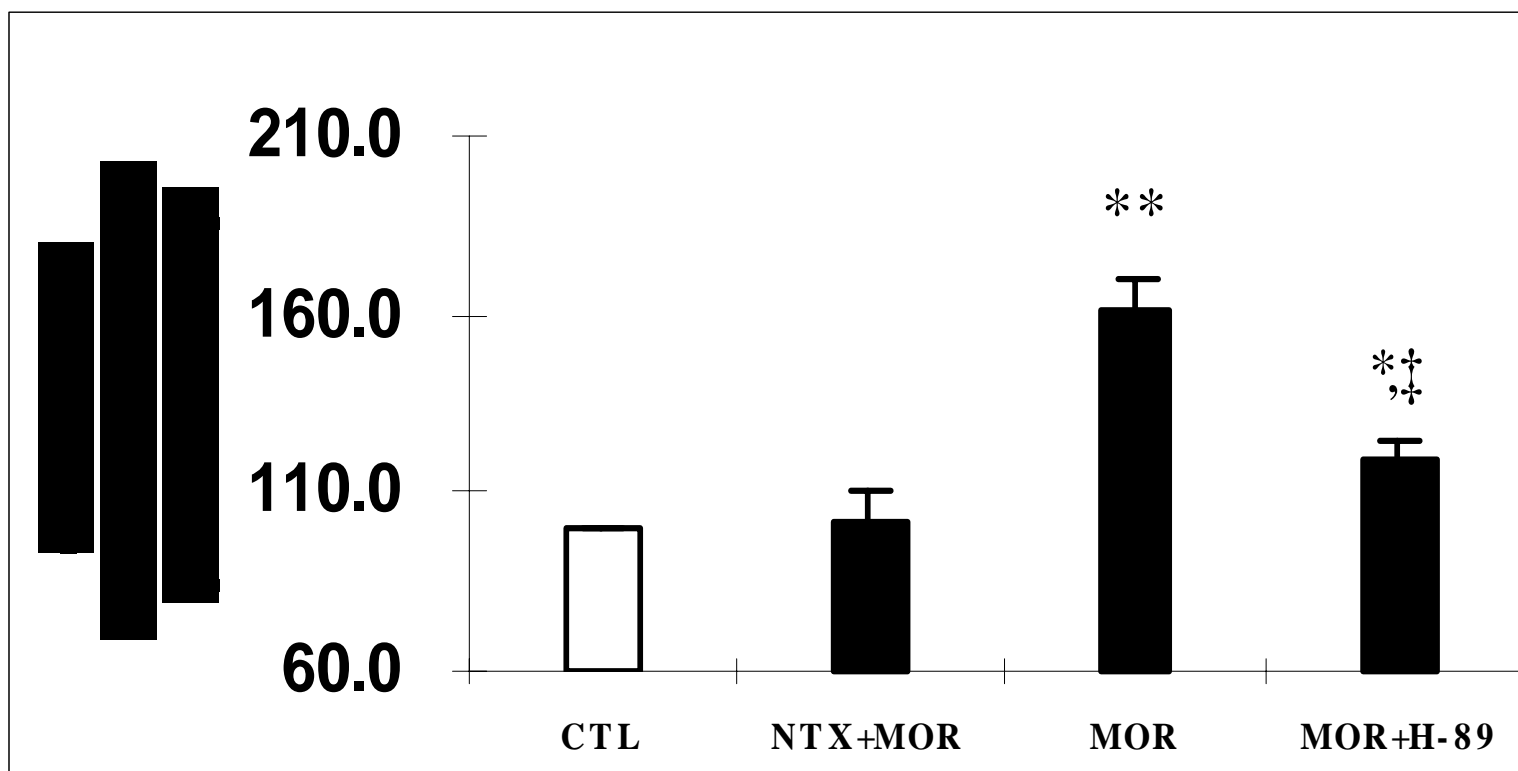
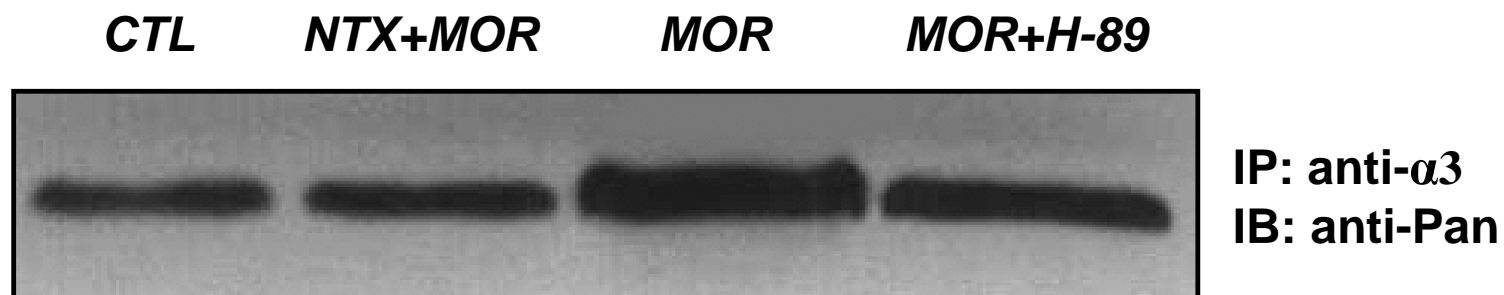
**Fig. 6B**



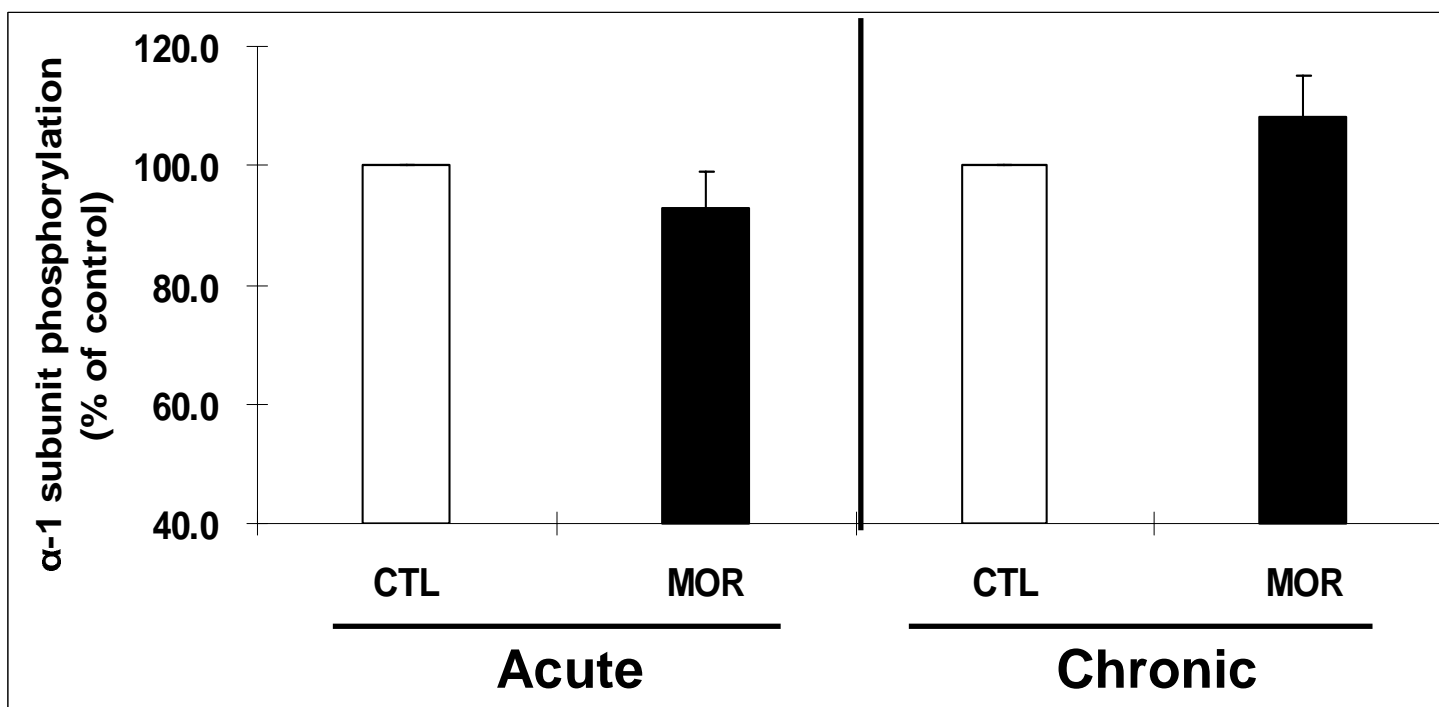
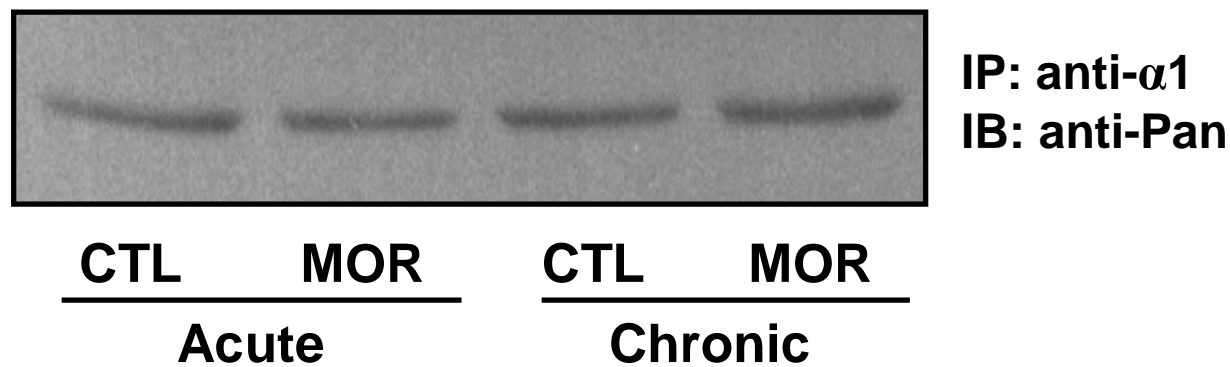
**Fig. 7A**



**Fig. 7B**



**Fig. 7C**



**Fig. 8**

