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Nicotinic regulation of calcium/calmodulin-dependent protein kinase II activation in the spinal cord

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ABBREVIATIONS : nAChR, Acetylcholine nicotinic receptor; CNS, Central nervous system; s.c., subcutaneous injection; i.t., Intrathecal; i.p., Intraperitioneal; α-BGTX, α-bungarotoxin; MLA, Methyllycaconitine ; LCC, L-type calcium channels; DHBE, dihydro-β-erythroidine; %MPE, % Maximum Possible Effect.

Abstract – Recent studies have implicated the involvement of Ca^{2+}-dependent mechanisms, in particular calcium/calmodulin-protein kinase II in nicotine-induced antinociception using the tail-flick test. The spinal cord was suggested as a possible site of this involvement. The present study was undertaken to investigate the hypothesis that the β_2 nicotinic receptor subunit plays a central role in nicotine-induced spinal antinociception via calcium/dependent calmodulin protein kinase II activation. The antinociceptive effects of intrathecal nicotine in the tail-flick test did not significantly differ in wild-type and α_7 knockout animals but were lost in β_2 knockout mice. When calcium/dependent calmodulin protein kinase II activity in the lumbar spinal cord after acute intrathecal administration of nicotine was investigated in wild type and β_2 and α_7 knock out mice, the increase in calcium/dependent calmodulin protein kinase II activity was not significant reduced in α_7 KO mice but was eliminated in the β_2 KO mice. In addition, L-type calcium channel blockers nimodipine and verapamil but not NMDA antagonist MK-801 blocked the increase in the kinase activity induced by nicotine. Taken together, these results are consistent with the hypothesis that increases in intracellular calcium result in activation of calcium-mediated second messengers in the spinal cord that play an important role in nicotine-induced antinociception as measured in the tail-flick test. Furthermore, our findings indicate that nicotinic stimulation of β_2-containing nAChRs in the spinal cord can activate calcium/dependent calmodulin protein kinase II and produce nicotinic analgesia, which may require L-type calcium voltage, gated channels but not the intervention of glutamatergic transmission.

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Introduction

Activation of cholinergic pathways by nicotine elicits antinociceptive effects in a variety of acute and chronic pain models (Aceto et al., 1986; Mattila et al., 1968). There is strong evidence that the antinociceptive effect of nicotine can occur via activation of nAChRs expressed in a variety of brain loci and spinal cord (Iwamoto and Marion, 1993; Mattila et al., 1968; Iwamato, 1991; Scott Bitner et al., 1998; Aceto et al., 1986; Damaj et al., 1998; Khan et al, 1997). Furthermore, nicotinic agonists have well documented antinociceptive properties when administered intrathecally in mice (Damaj et al., 1998). Studies with knockout mice deficient in the α_4 and β_2 nicotinic acetylcholine receptor subunits suggest an important role of $\alpha_4\beta_2$ neuronal subtypes in nicotine-induced antinociception in acute pain models such as the hot-plate and tail-flick tests (Marubio et al., 1999). More recently, Cordero-Erausquin et al (2004), using electrophysiological and molecular approaches, identified spinal $\alpha_4\beta_2$* and $\alpha_3\beta_2$* neuronal subtypes as possible targets for nicotinic analgesia. However, the molecular characterization of this nicotinic effect at the spinal cord is still largely unknown. Activation of calcium-dependent signaling mechanisms seems a plausible target. Studies with peripheral and central preparations showed that significant amounts of Ca^{2+} enter the cell following activation of certain nAChRs such as β_2 and α_7 subtypes, causing a rise in $[Ca^{2+}]_i$ concentration (Fluhler et al., 1992; Mulle et al., 1992; Vijayaraghavan et al., 1992; Barrantes et al., 1994). We recently reported that nicotine increases $[Ca^{2+}]_i$ levels in spinal synaptosomes and elevates calcium-dependent calmodulin protein Kinase II activity in the spinal cord membrane after acute systemic injection in mice (Damaj et al, 2000). In addition, calcium/dependent calmodulin protein kinase II inhibitors blocked nicotine's effects in the tail-flick test in a dose-related manner. Collectively, these studies provide strong evidence that nicotinic receptor-mediated antinociception at the spinal level involves a calcium-dependent protein kinase, namely calcium/dependent calmodulin protein kinase II.

The present study was undertaken to examine and characterize the effect of acute nicotine exposure on the activity of calcium/dependent calmodulin protein kinase II in the spinal cord and investigate whether the regulation of calcium/dependent calmodulin protein kinase II by nicotine occurs directly through nAChRs or indirectly through NMDA receptors or L-type calcium

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channels. We first investigated the effects of intrathecal injection of acute nicotine on calcium/dependent calmodulin protein kinase II activity in the lumbar spinal cord, a region that contains the dorsal horn, which plays an important role in modulating nociceptive transmission. We then determined the time-course of changes in calcium/dependent calmodulin protein kinase II activity after nicotine administration. Investigation of the role of major nicotinic subtypes (β_2 and α_7 nAChRs subtypes) in nicotine-induced changes in calcium/dependent calmodulin protein kinase II followed using two approaches for this characterization: classical pharmacological methods (use of nicotinic antagonists) and genetically modified mice (β_2 and α_7 knock out mice). After characterizing the changes in calcium/dependent calmodulin protein kinase II in the spinal cord, we finally investigated the role of L-type calcium channels and NMDA receptors in nicotine-induced calcium/dependent calmodulin protein kinase II activation.

Materials and Methods

Animals. Mice lacking the α_7 nicotinic receptor (C57BL/6 background), and wild type littermates were purchased from The Jackson Laboratories (B6.129S7-charna7tm1bay, number 003232- Bar Harbor, ME). Breeding pairs of mice lacking the β_2 nicotinic receptor (C57BL/6 background), and wild type littermates were shipped from Institut Pasteur (Paris, France). Homozygous α_7 and β_2 mutant and wild-type controls were obtained from crossing heterozygote mice. Male mice about 8-12 weeks old (together with age- and sex-matched littermates wild-type controls) were used for the kinase experiments. Animals were housed in an AALAC approved facility in groups of three and had free access to food and water. Animals were housed in an AALAC approved facility and the study was approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

Drugs. MK-801, verapamil hydrochloride, α-bungarotoxin (α-BGTX), Methyllycaconitine citrate (MLA) and dihydro-β-erythroidine were purchased from RBI (Natick, MA). Nimodipine was a gift from Miles, Inc. (West Haven, CT) and mecamylamine hydrochloride was a gift from Merck, Sharp and Dohme & Co. (West Point, PA). MLA citrate, dihydro-β-erythroidine mecamylamine HCl and verapamil hydrochloride were dissolved in physiological saline (0.9% sodium chloride). Nimodipine, was prepared in DMSO and its solutions were refrigerated in foil-lined containers. (-)-Nicotine was obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI) and converted to the ditartrate salt as described by Aceto et al. (1979). All doses are expressed as the free base of the drug.

Intrathecal injections. Intrathecal injections (i.t.) were performed free-hand between the L5 and L6 lumbar space in unanesthetized male mice according to the method of Hylden and Wilcox (1980). The injection was performed using a 30-gauge needle attached to a glass microsyringe. The injection volume in all cases was 5 µl. The accurate placement of the needle was evidenced by a quick “flick” of the mouse’s tail. In protocols where two sequential injections were required in an animal, the flicking motion of the tail could be elicited with the subsequent injection.

Antinociceptive Assay. Antinociception was assessed by the tail-flick method of D'Amour and Smith (1941). Briefly, mice were lightly restrained while a radiant heat source was shone onto the upper portion of the tail. Latency to remove the tail from the heat source was recorded for each animal. A control response (2-4 sec) was determined for each mouse before treatment, and test latency was determined after drug administration. In order to minimize tissue damage, a maximum latency of 10 sec was imposed. Antinociceptive response was calculated as percent maximum possible effect (% MPE), where %MPE = [(test-control)/(10-control)] x 100. The mice were tested 5 min after i.t. injection of nicotine. The mice were tested 5 min after i.t. injection of nicotine. Antagonism studies were carried out by pretreating the mice with either i.t. saline or nicotinic antagonists 5 min before nicotine (20 μg/mouse). The animals were tested 5 min after administration of nicotine. Nimodipine (1 μg/mouse), mecamylamine (20 μg/mouse), α-BGTX (2 μg/mouse) and DHβE (20 μg/mouse) doses were based on previously published intrathecally administered doses of these antagonists (Damaj, 2000; Damaj et al. 1995). Because of recent reports questioning the receptor selectivity of MLA, the investigation of the role of spinal α_7 nicotinic receptors in our behavioral and biochemical studies was carried out using MLA and α-BGTX, a selective α_7 antagonist with poor blood brain barrier penetration. The MLA dose (7.5 mg/kg, s.c.) was within the range reported to block α_7 nicotinic receptors after systemic injection (Turek et al. 1995). The MK-801 (2.5 μg/mouse) dose was based on published studies that determined the most effective intrathecal doses for blocking NMDA nociceptive effects (Suh HW, et al., 1995; Suh H, 2000). Groups of six to eight animals were used for each dose and for each treatment.

Calcium/dependent calmodulin protein Kinase II phoshorylation assays.

Calcium/dependent calmodulin protein kinase II activity was measured using a modified assay kit (Upstate Biotechnology, Lake Placid, NY). Briefly, at different times following experimental treatment, mice were killed by cervical dislocation and the spinal column was isolated and divided in thoracic, cervical and lumbar regions. The lumbar segment of spinal cord was removed from the spinal column by gentle flushing with ice cold, isotonic saline. Lumbar spinal cord tissues were homogenized using a microcentrifuge pestle in a calcium-free buffer that

contains 20 mM HEPES (pH = 7.4), 2.6 mM EGTA, 80 mM beta-glycerolphosphate, 20 mM magnesium acetate, 0.1 µM okadiac acid, 0.1 µM calyculin, 0.1 mM DTT, 50 mM sodium-floride, 1 mM sodium-orthovanadate and 0.01 mg/ml CLAPS (0.1 mg/ml each of Pepstatin A, Chymostatin, Aprotinin, Leupeptin, Trypsin-Chymotrypsin Inhibitor). Homogenates were normalized for protein concentration. Samples were centrifuged in order to separate the membrane and the cytosol containing-kinase. The pellet was re-suspended in homogenization buffer plus 1% NP-40 (IGEPAL) and allowed to incubate on ice 1 hour. The tubes are spun again and supernatant was retained (Membrane fraction). Standard phosphorylation reaction solutions contain 15 µg extract protein, 100 µM calcium/dependent calmodulin protein kinase II -specific substrate peptide (Autocamtide-2), 0.25 µM protein kinase inhibitors (0.25 µM each of PKA & PKC inhibitor peptides), 75 mM Mg acetate, 500 uM ATP, 20 mM HEPES, 25 mM beta-glycerolphosphate, 1 mM Na-orthovanadate, 1 mM DTT, 1 µCi of [^{32}P]ATP, 5 µM $CaCl_2$ and 5 µg calmodulin for the measurement of calcium-dependent activity. In aliquots used for calcium-independent activity, 5 mM EGTA was added while $CaCl_2$ and calmodulin were omitted. Standard reactions were performed in triplicate in a shaking water bath at 30˚C for 10 min along with background controls lacking substrate. Activity was quantified by spotting half the reaction on phosphocellulose paper squares. Squares were washed in 0.75 % phosphoric acid (5 times) followed by a brief acetone rinse before analysis by scintillation counting. Calcium/dependent calmodulin protein kinase II activity was expressed in pmol phosphate/min/µg and determined using the following calculations: [(count-specific binding minus background) x (correcting factor)]/[(specific radioactivity) x time (10 min)].

Statistical analysis. Statistical analysis of all analgesic studies was performed using either t-test or analysis of variance (ANOVA) with Tukey's test post hoc test when appropriate. All differences were considered significant at $p < 0.05$.

Results

Calcium/dependent calmodulin protein kinase II activity in lumbar spinal cord tissues after acute intrathecal injection of nicotine in mice: Time-course and dose-response effects.

The activity of calcium/dependent calmodulin protein kinase II in the lumbar spinal cord after intrathecal acute administration of nicotine in mice was investigated. Spinal cord tissues were dissected and the activity of calmodulin protein kinase II (expressed as the number of pmol ^{32}P incorporated into calmodulin protein kinase II substrate peptide/min/mg of protein in the presence or absence of calcium) in the membrane was measured. The onset of action for nicotine (20 µg/animal) on the calcium-dependent activity of calmodulin protein kinase II in the lumbar spinal cord was rapid with maximum activation occurring between 0 and 5 min (Figure 1A). The effect decreased then within 30 min of nicotine administration in mice. In contrast, nicotine's effect on the calcium-independent activity of the enzyme reached its maximal increases 30 min after injection and was still significant 60 min after drug exposure (Figure 1B). A similar time-course was seen at lower doses of nicotine (data not shown).

A dose-response relationship was then established for nicotine in mice by measuring both calcium-dependent and –independent calmodulin protein kinase II activity at the time of maximal effect. As shown in Fig. 2, a dose-dependent increase in the membrane kinase activity was seen after acute injection of nicotine. The increase in the kinase activity induced by nicotine was blocked by mecamylamine pretreatment (Table 1). These results are consistent with the notion that calmodulin protein kinase II activation after acute nicotine exposure is a receptor-mediated event.

Nicotine-induced antinociception in β_2 and α_7 knock out mice after intrathecal injection

The antinociceptive effects of intrathecal nicotine in the tail-flick test are shown in Figure 3. The control latency response to painful stimuli did not significantly differ in wild-type and knockout animals indicating that the endogenous activation of the β_2 and α_7 nAChR subunit is not essential in the perception of acute thermal nociception (data not shown). Nicotine given at a dose of 20 µg/mouse, showed a 87± 7 % maximum possible effect (%MPE) in wild-type and

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90 ± 5 % MPE in $\alpha_7^{-/-}$ mice. In contrast, $\beta_2^{-/-}$ mice did not exhibit a significant antinociceptive response to nicotine (Fig. 3).

Calcium/dependent calmodulin protein kinase II activity in lumbar spinal cord tissues in β_2 and α_7 knock out mice and after intrathecal injection of nicotine.

The activity of calcium/dependent calmodulin protein kinase II in the spinal cord after acute intrathecal administration of nicotine in mice was investigated in wild type and β_2 and α_7 knock out mice. Animals received either saline or nicotine (20 µg/mouse, intrathecally) and were sacrified 15 min after injection. Spinal cord tissues were dissected and the calcium-dependent and –independent activity of calcium/dependent calmodulin protein kinase II in the membrane was measured. As shown in Fig. 4A and 4B, a significant increase in both dependent and independent kinase activity was seen after acute injection of nicotine in wild-type mice. This increase in calcium/dependent calmodulin protein kinase II activity was not significantly reduced in α_7 KO mice but was eliminated in the β_2 KO mice. The increase in the kinase activity induced by nicotine was blocked by mecamylamine and dihydro-β-erythroidine, but not MLA and α-BGTX pretreatment (Table 1). By themselves, nicotinic antagonists did not cause any significant change in calcium/dependent calmodulin protein kinase II activity at the indicated doses and times (data not shown).

Effects of calcium channel blockers and an NMDA antagonist on the nicotine-induced increase in spinal calcium/dependent calmodulin protein kinase II activity in mice.

To further investigate the regulation of calcium/dependent calmodulin protein kinase II activation by nicotine, various L-type calcium channels inhibitors and an NMDA antagonist were evaluated for their ability to alter the increase in spinal calcium/dependent calmodulin protein kinase II activity induced by nicotine. Nimodipine and verapamil, L-type calcium channels inhibitors, given i.t. inhibited the increase in both dependent and independent calcium/dependent calmodulin protein kinase II activity (Fig. 5). In contrast, the NMDA antagonist MK-801 failed to statistically reduce calcium/dependent calmodulin protein kinase II

activation. By themselves, these antagonists did not cause any significant change in calcium/dependent calmodulin protein kinase II (data not shown).

Effects of calcium channel blockers and an NMDA antagonist on nicotine-induced antinociception in mice.

To further investigate the regulation of calcium/dependent calmodulin protein kinase II activation and its correlation with nicotine-induced antinociception, L-type calcium channels and an NMDA antagonist were evaluated for their ability to alter the antinociceptive effects of spinal nicotine. Similar to their effects on calcium/dependent calmodulin protein kinase II activation, nimodipine and verapamil given intrathecally inhibited the antinociceptive responses of spinally injected nicotine (Table 2). In contrast, MK-801 failed to block nicotine-induced antinociception. By themselves these antagonists did not have a significant effects in the tail-flick test.

Discussion

We previously reported that activation of neuronal nAChRs by systemic nicotine administration leads to an influx of calcium, which activates calcium/dependent calmodulin protein kinase II in the spinal cord (Damaj, 2000). In addition, selective calcium/dependent calmodulin protein kinase II inhibitors in the spinal cord blocked nicotine-induced antinociception in the tail-flick test. These results suggested that spinally located calcium/dependent calmodulin protein kinase II might be involved in the modulation of nicotine-induced antinociception. In the present study, we found that spinally injected nicotine directly activates calcium/dependent calmodulin protein kinase II in the lumbar spinal cord section, a region that contains the dorsal horn, which plays an important role in modulating nociceptive transmission.

The difference in kinetics for the increase in Ca^{2+}-dependent as compared to Ca^{2+}-independent activity shows a delay in the increase in the autonomous activity of the enzyme. This delay may reflect the "switch" from the activated to the autonomous form. It is possible also that nicotine induced a shift of autonomous calmodulin protein kinase II to the membrane. However, no significant decrease in the activity of the enzyme in the cytosolic fraction was observed as well as in the total amount of the enzyme (data not shown).

The increase in calcium/dependent calmodulin protein kinase II activity was blocked by mecamylamine, a non-selective nicotinic antagonist, suggesting the involvement of spinal nAChRs in this effect. It is interesting to note that the increase in calcium/dependent calmodulin protein kinase II activity in the spinal cord was sustained until at least 60 –120 min after a single injection of nicotine (Fig. 1). This sustained activation could potentially have important pharmacological and molecular consequences, since calcium/dependent calmodulin protein kinase II is a key element in neuronal plasticity and in calcium-dependent neurotransmitter release. In addition, this extended time-course is consistent with an initiation of neuronal plasticity in some supraspinal regions of the brain as reported in the VTA after an acute exposure to nicotine (Mansvelder and McGehee, 2002; Mansvelder et al., 2003).

Although the behavioral results in our study confirm a previously reported role for β_2 subunits in nicotine-induced antinociception, our data suggest a calcium-dependent kinase

molecular mechanism mediating the activation of the β_2-containing receptor subtype. We now propose that calcium/dependent calmodulin protein kinase II activation in the spinal cord mediates nicotine's antinociceptive effects through the activation of β_2- but not α_7-containing nAChRs. While calcium/dependent calmodulin protein kinase II has long been reported as a calcium-dependent kinase in memory and learning, more recently this kinase was reported to have important roles in nociceptive signaling (Willis, 2001).

Radioligand binding and molecular studies have revealed the existence and location of multiple neuronal nicotinic receptor subtypes in brain as well as in the spinal cord. Molecular biology studies confirmed the existence of multiple nAChRs in the spinal cord. Indeed, Wada et al. (1989) conducted a very extensive analysis of α_2, α_3, α_4, and β_2 mRNA localization in the brain. Although a small portion of this study was directed to the spinal cord, transcripts for these subunits were detected in the spinal cord. In another study, no signal was detected for β_4 mRNA in the spinal cord (Dineley-Miller and Patrick, 1992). However, it has been difficult to determine which subtypes of nAChRs mediate the various effects of nicotine in the spinal cord. Recently, Cordero-Erausquin et al. (2004) identified a β_2-containing nAChRs, $\alpha_4\alpha_6\beta_2$* subtype, as major contributors for nicotine's effects on spinal inhibitory neurons. In the same study, the authors reported that the spinal excitatory neurons expressed an α_7-containing nAChRs. On the other hand, it has been suggested that α_7 nicotinic receptor subtypes may mediate a pronociceptive action at the level of the spinal cord (Khan et al. 1997). This nociceptive effect was blocked by MK-801, suggesting the involvement of spinal NMDA receptors. Interestingly, MK-801 failed to block nicotine's antinociceptive effects and calcium/dependent calmodulin protein kinase II activation as reported in our studies. Taken together, these results are consistent with the involvement of spinal calcium/dependent calmodulin protein kinase II in nicotine-induced antinociception via β_2-containing nAChRs.

It is known that transduction changes can be set in motion by calcium influx through activation of ligand-gated ion channels such NMDA receptors together with contributions from LCC and release from internal calcium stores. Indeed, sustained L-type calcium channels activation triggers calcium release from internal stores. In a recent report neither L- nor N-type channels were shown to be required for nicotinic regulation of CREB phosphorylation (p CREB)

in ciliary ganglion neurons (Chang et al., 2001). Instead, calcium influx through nAChRs themselves, together with calcium-induced calcium release from internal stores, sustained pCREB levels in the ganglionic neurons. In contrast, stimulation of α_7 nAChRs subtypes in rat cultured hippocampal neurons was reported to activate CREB through both glutamatergic and nonglutamatergic components, and does not, however, require activation of LCC (Hu et al., 2002). Our findings in this present case show that nicotinic stimulation of β_2-containing nAChRs in the spinal cord can activate calcium/dependent calmodulin protein kinase II and produce nicotinic analgesia, which may require L-type calcium voltage-gated channels but not the intervention of glutamatergic transmission. It also possible that the LCC antagonists inhibit directly β_2-containing nAChR since earlier reports suggest that these antagonists could block neuronal nAChR present on chromaffin cells (Donnelly-Roberts et al., 1995).

The fact that LCC blockers inhibited the nicotinic effects further suggests that calcium influx through β_2-containing nAChRs in the spinal cord is not sufficient to trigger enough calcium/dependent calmodulin protein kinase II activation for behavioral expression as measured in the tail-flick, an acute pain model. It is possible that these signaling cascades may play a critical role in mediating nicotine-induced long-lasting changes in brain neurochemistry and, therefore, may be involved in the development of nicotine tolerance and/or dependence.

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1 Time course of increase in spinal calmodulin protein kinase II calcium-dependent **(A)** and calcium-independent **(B)** activity after acute intrathecal injection of nicotine (20 µg/animal). Animals were sacrificed at different times after nicotine or saline injection. Each point represents the mean ± S.E. of 6-8 mice. *Statistically different from saline at $P < 0.05$.

Fig. 2 Dose-response curve of nicotine-induced increase in calmodulin protein Kinase II dependent and independent spinal membrane activity after acute intrathecal injection of nicotine at different doses. Animals were sacrificed 5 min after nicotine or saline injection for the dependent activity and 30 min for the independent activity. Each point represents the mean ± S.E. of 6-8 mice. *Statistically different from saline at $P < 0.05$.

Fig. 3 Antinociceptive response to saline nicotine in wild-type, α_7 KO and β_2 KO mice. Mice were treated i.t. with saline or nicotine (20 µg/mouse) and tested 5 min after injection in the tail-flick test. Each point represents the mean %MPE ± S.E. for 8-10 mice. *Statistically different from saline at $p < 0.05$.

Fig. 4 Dependent **(A)** and independent **(B)** spinal membrane calmodulin protein kinase II activity after acute injection of nicotine (20 µg, i.t.) in wild-type, α_7 KO and β_2 KO mice. Animals were sacrificed 15 min after nicotine or saline injection. Each point represents the mean ± S.E. of 6-8 mice. *Statistically different from saline at $p < 0.05$.

Fig. 5 Effects of nimodipine, verapamil and MK-801 on nicotine-induced increase in spinal calmodulin protein kinase II activity in mice. Mice were pretreated with

the antagonists 5 min before nicotine (20 µg/mouse). Each point represents the mean ± S.E. of 6-8 mice. *Statistically different from saline at $p < 0.05$.

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Table 1. Effects of various nicotinic antagonists on the nicotine-induced increase in spinal calmodulin protein kinase II activity in wild-type mice. Mice were pretreated with nicotinic antagonists 5 min before nicotine (20 µg/mouse). Each number represents the mean ± SE of 6 to 12 mice.

Treatment	Dependent Activity (pmol/min/µg)	Independent Activity (pmol/min/µg)
Veh/Veh	4.7 ± 0.07	0.6 ± 0.02
Veh/Nic (20 µg)	11.9 ± 0.3*	3.37 ± 0.09*
Mecamylamine (20)/Nic (20 µg)	5.5 ± 0.2	0.7 ± 0.05
DHβE (20)/Nic (20 µg)	5.7 ± 0.3	0.5 ± 0.05
α-BGTX (2)/Nic (20 µg)	10.7 ± 0.5*	3.28 ± 0.02*
MLA (7.5)/Nic (20 µg)	11.5 ± 0.4*	3.31 ± 0.03*

*$p<0.05$ from vehicle/vehicle

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Table 2. Effects of various calcium channel blockers and an NMDA antagonist on nicotine-induced antinociception in mice. Mice were pretreated with intrathecal antagonists 5 min before nicotine (20 µg/mouse). Each number represents the mean ± SE of 6 to 8 mice.

Treatment	Antinociception (%MPE)
Veh/Veh	7 ± 3
Veh/Nic (20)	90 ± 5
Nimo (1)/Nic (20)	5 ± 2
Vera (2)/Nic (20)	11 ± 4
MK-801 (2.5)/Nic (20)	85 ± 10

*p<0.05 from vehicle/vehicle

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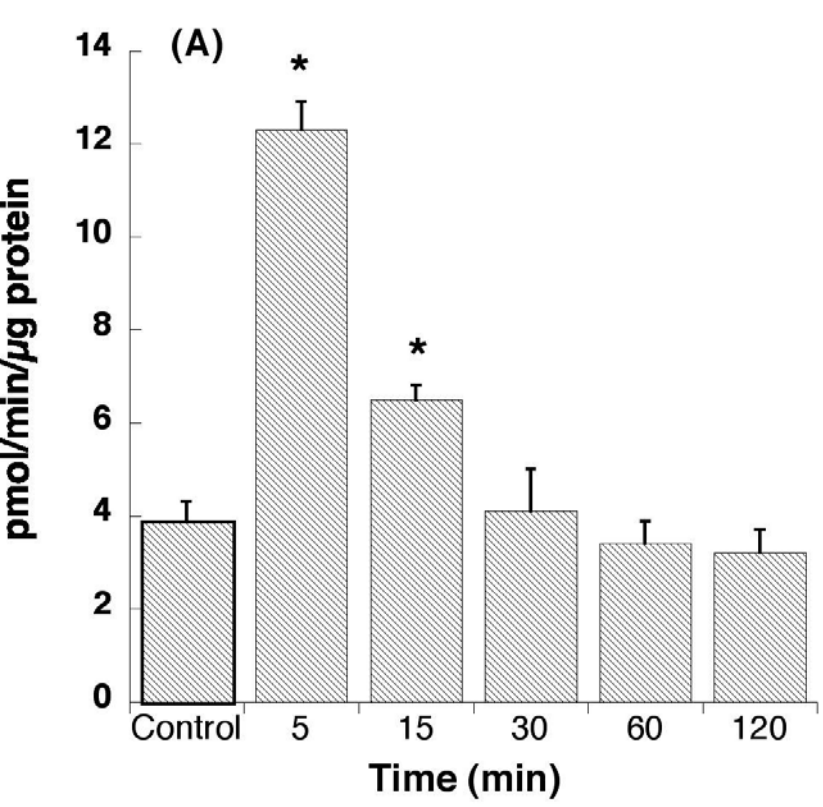

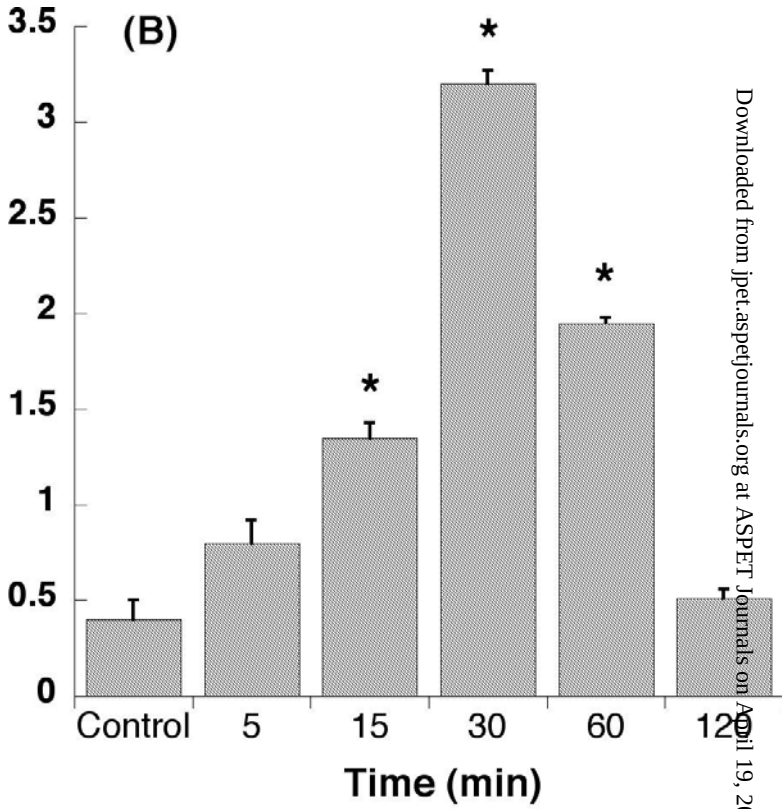

Figure 1

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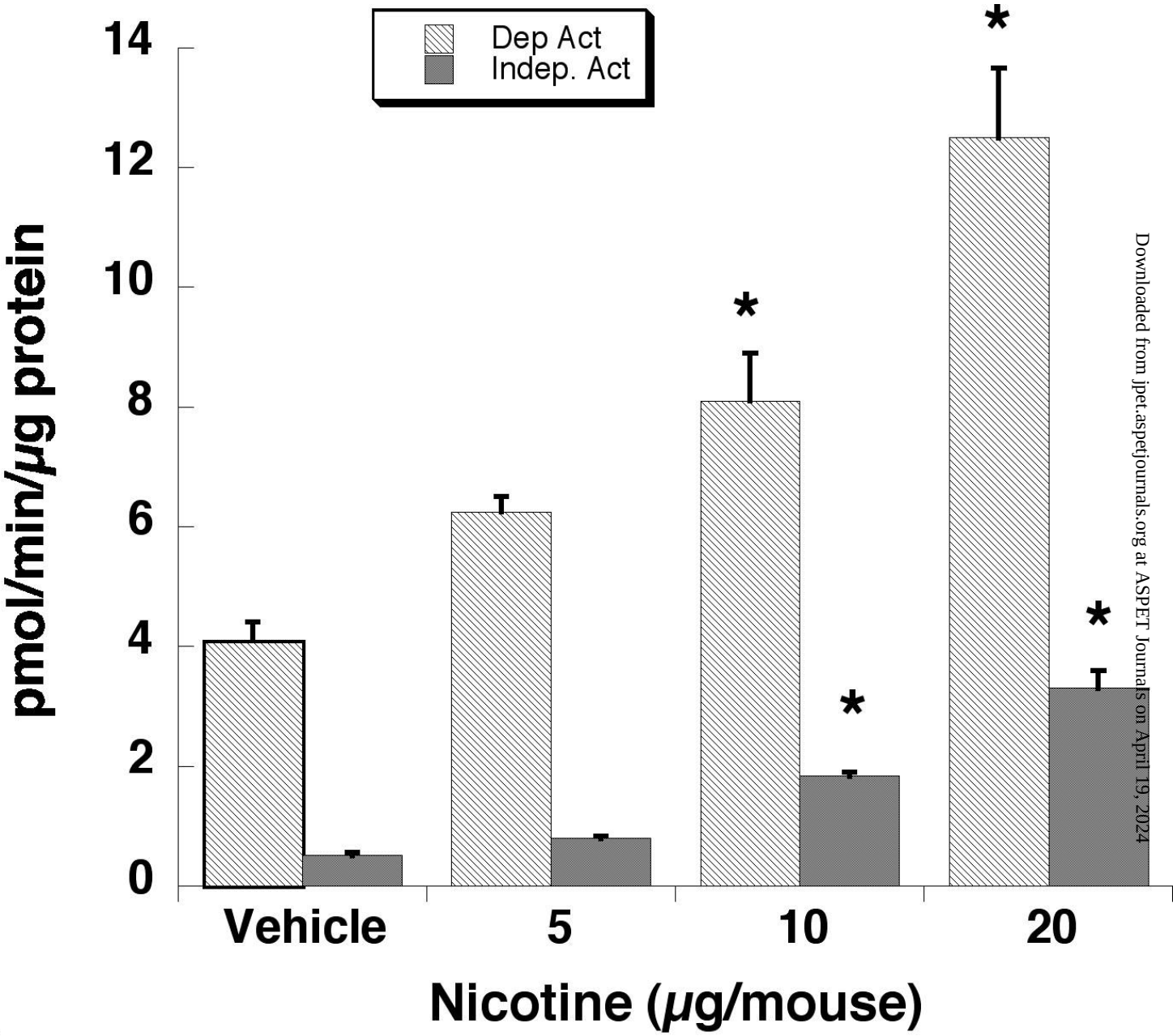

Figure 2

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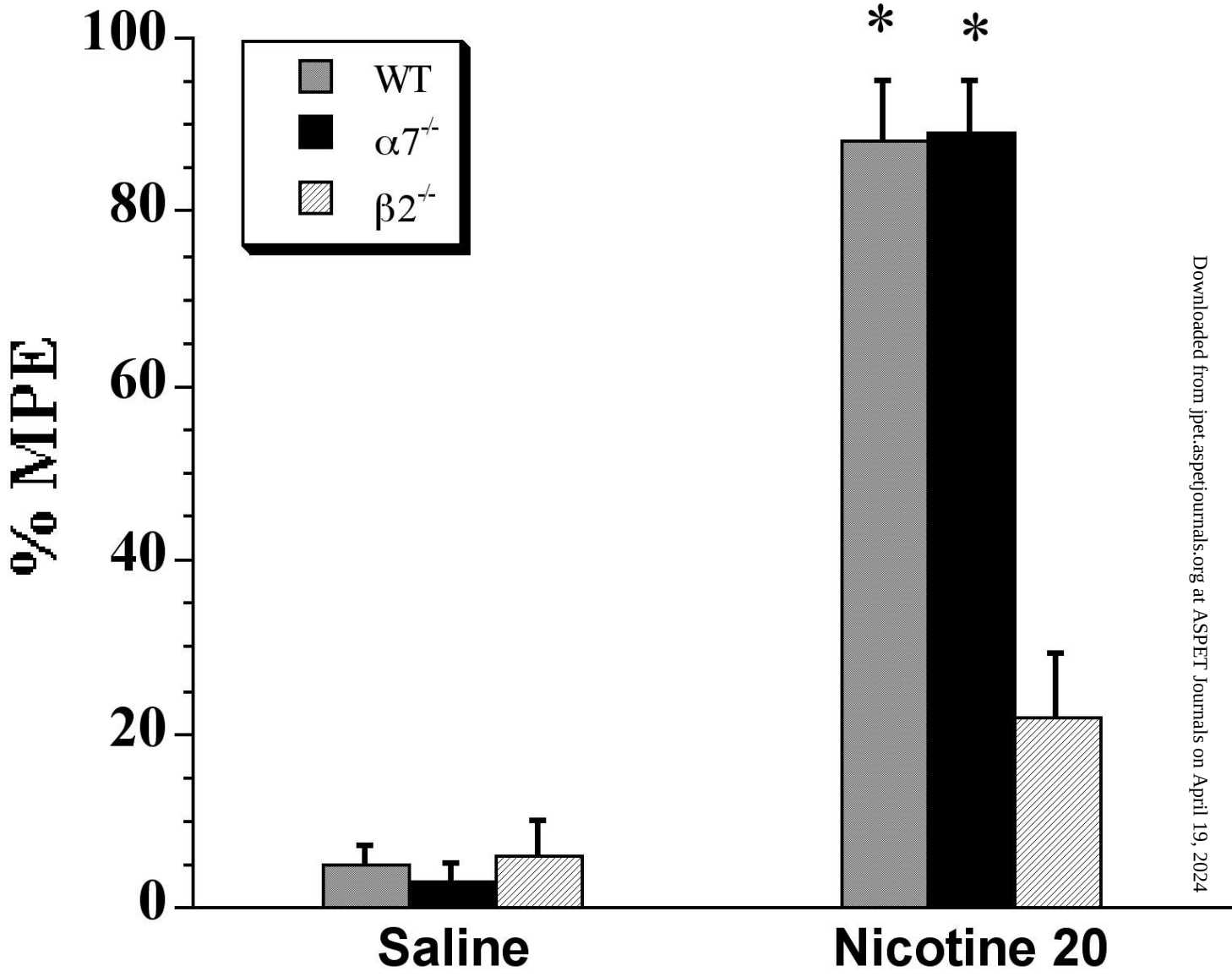

Figure 3

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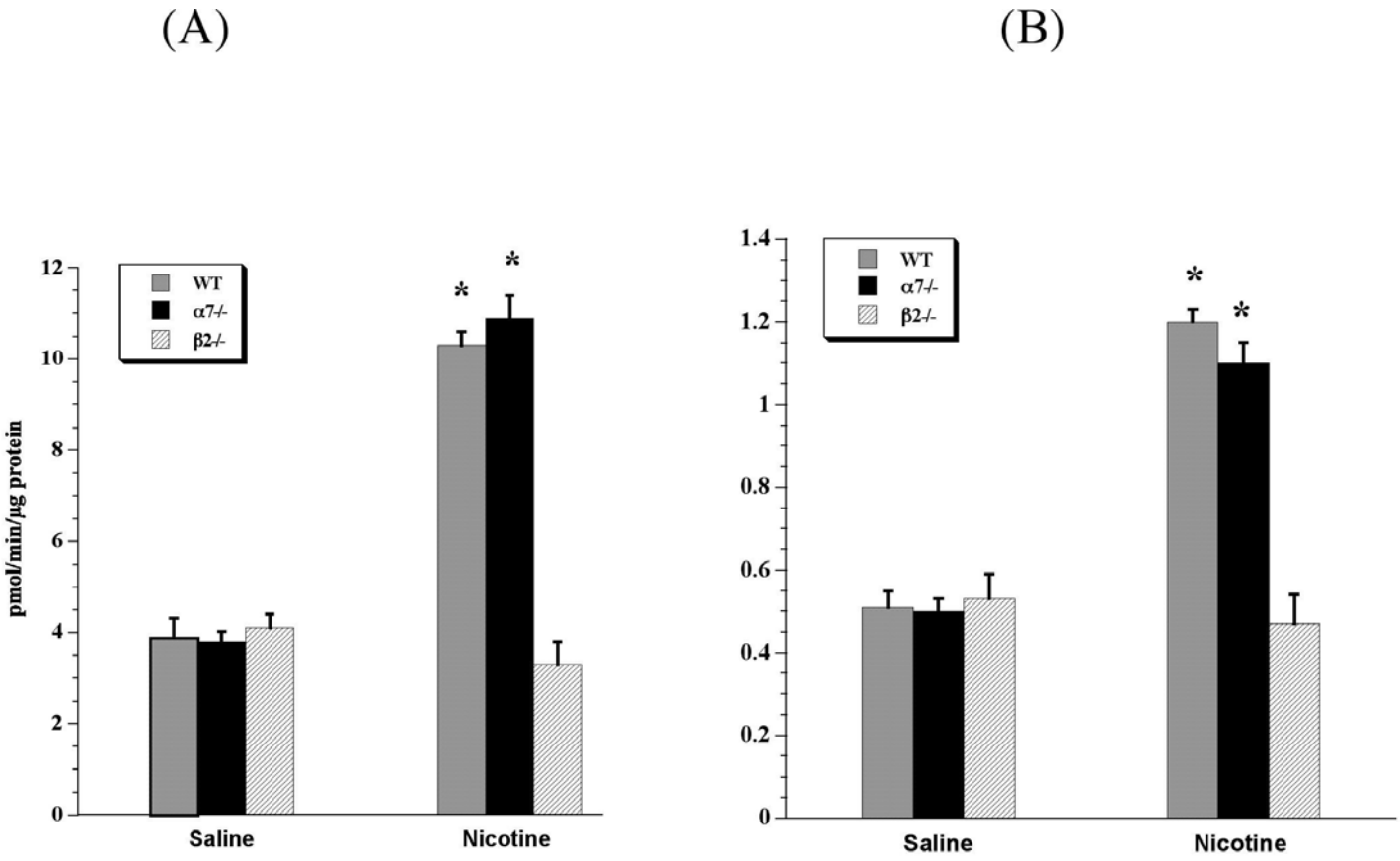

Figure 4

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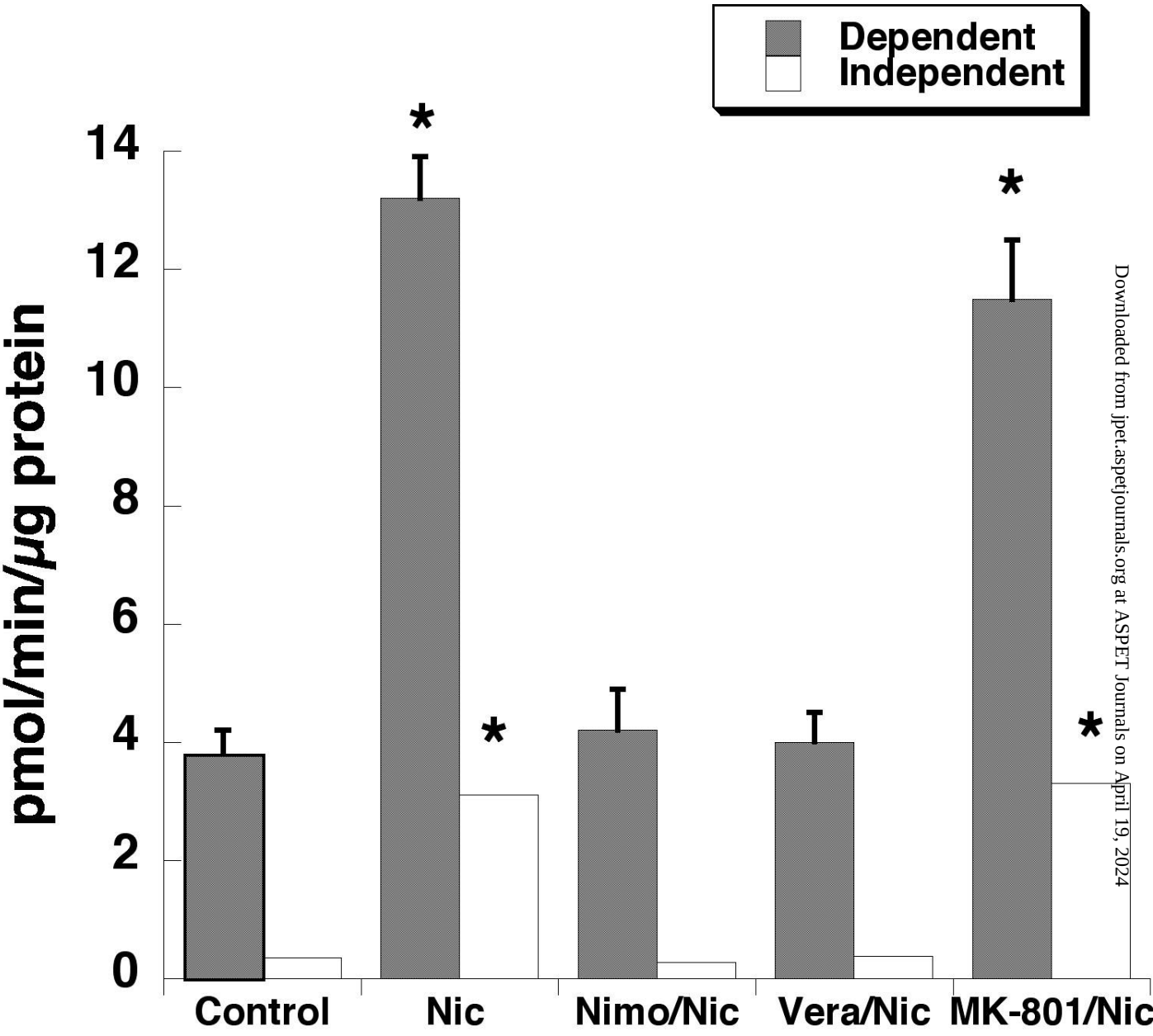

Figure 5