JPET Fast Forward. Published on August 12, 2005 as DOI: 10.1124/jpet.105.092460
This article has not been copyedited and formatted. The final version may differ from this version.

JPET #92460

Calcium-acting drugs modulate expression and development of chronic tolerance to nicotine-induced antinociception in mice

M. I. DAMAJ

Department of Pharmacology and Toxicology, Medical Campus, Virginia Commonwealth University, Richmond, VA 23298-0613 (MID)

Running title: Effects of calcium channels modulators on nicotine tolerance

* Address all correspondence to:

Dr. M. Imad Damaj

Department of Pharmacology and Toxicology,

Virginia Commonwealth University,

Box 980613

Richmond, VA 23298-0613.

Tel # (804) 828 1676

Fax # (804) 828 2117

E-mail: mdamaj@hsc.vcu.edu

Text pages: 20

Tables: 1

Figures: 5

References: 27

Abstract: 249 words

Introduction: 591 words

Discussion: 811 words

ABBREVIATIONS : nAChR, Acetylcholine nicotinic receptor; CNS, Central nervous system; s.c., subcutaneous injection; i.t., Intrathecal; i.p., Intraperitioneal.

Abstract – Initial studies in our laboratory suggested that tolerance to nicotine is thought to involve neuronal adaptation not only at the level of the drug-receptor interaction, but at post-receptor events such as calcium-dependent second messengers. The present study was undertaken to investigate the hypothesis that L-type calcium channels and calcium/dependent calmodulin protein Kinase II are involved in the development and expression of nicotine tolerance. To that end, the effects of modulation of L-type calcium channels (through the use of inhibitors or activators) as well calcium/dependent calmodulin protein Kinase II inactivation were studied in a mouse model of tolerance where mice were infused with nicotine in minipumps (24 mg/kg/day) for 14 days. In addition, the activity of calcium/dependent calmodulin protein Kinase II in the lumbar spinal cord region obtained from nicotine-tolerant mice was measured. Our data showed that chronic administration of L-type calcium channels antagonists nimodipine (1 and 5 mg/kg) and verapamil (10 mg/kg) prevented the development of tolerance to nicotine-induced antinociception. In contrast, chronic exposure of BAYK8644, a calcium channel activator, enhanced nicotine's tolerance. Moreover, a significant increase in both dependent and independent calcium/dependent calmodulin protein Kinase II activity was seen in the spinal cord in nicotine-tolerant mice. Finally, spinal administration of KN-62, a calcium/dependent calmodulin protein Kinase II antagonist, reduced the expression of tolerance to nicotine-induced antinociception in mice. In conclusion, our data indicate that calcium-dependent mechanisms such as L-type calcium channels and calcium/dependent calmodulin protein Kinase II activation are involved in the expression and development of nicotine tolerance.

Introduction

Abundant clinical and experimental data revealed that chronic exposure to nicotine produces tolerance and leads to physical dependence. Tolerance to nicotine is thought to play an important role in the development and maintenance of dependence. Chronic exposure to nicotine can result in a loss of sensitivity to nicotine lasting a number of days. Following chronic treatment with nicotine, animals exhibit decreases in sensitivity to acute challenges with the drug in numerous physiological and behavioral measures, including respiration, locomotor activity, body temperature, sensitivity to seizures (Marks et al., 1986; Collins et al., 1988; Miner and Collins, 1988) and to antinociception (Damaj and Martin, 1996). The mechanisms underlying tolerance to nicotine are not well known. Initially, the development of tolerance was thought to be related to changes in nicotinic receptor binding after chronic exposure of the drug. Indeed, repeated exposure to nicotine produces significant increases in the number of ^{3}H-nicotine binding sites ($\alpha_4\beta_2$* nAChRs subtypes) in several rat and mouse brain regions (Schwartz and Kellar, 1985; Marks et al., 1986). Receptor desensitization was suggested to be the primary event leading to up-regulation of CNS nicotinic receptors and a compensatory response to chronic desensitization following prolonged agonist treatment (Peng et al., 1994). It has recently been proposed that receptor upregulation results from binding of nicotine to β_2 subunits in immature intracellular nAChRs (Sallette et al., 2004). However, several reports suggest that tolerance to nicotine cannot be completely explained by changes in receptor number (Marks et al., 1987; Pauly et al., 1992). Therefore tolerance to nicotine is thought to involve neuronal adaptation not only at the level of the drug-receptor interaction, but at post-receptor events such as intracellular messengers cascades and gene expression.

Initial studies in our laboratory showed that chronic exposure of nicotine in mice altered the behavioral sensitivity to calcium-modulating drugs such as BAYK8644 in mice, which suggests that functional changes in L-type calcium channels occur after chronic treatment with nicotine (Damaj, 1997). Pretreatment with L-type calcium channels antagonists reduced the expression of locomotor sensitization to nicotine (Biala and Weglinska, 2004) and mecamylamine-precipitated nicotine withdrawal in mice (Biala and Welginska, 2005). Taken together these data suggest a role for neuronal L-type calcium channels in the different aspects of nicotine dependence. Other calcium-mediated events such as calcium-dependent calmodulin protein Kinase II may also be involved. We have recently reported that nicotine increases calcium/dependent calmodulin protein Kinase II activity in the spinal cord membrane after acute exposure of nicotine in mice (Damaj, 2000). Collectively, these studies suggest the possible involvement of calcium-dependent mechanisms in the development tolerance to nicotine.

The present study was undertaken to investigate the hypothesis that L-type calcium channels and calcium/dependent calmodulin protein Kinase II are involved in the development and expression of nicotine tolerance. To that end, the effects of modulation of L-type calcium channels (through the use of inhibitors or activators) were studied in a mouse model of nicotine tolerance. The L-type calcium channels antagonists nimodipine and verapamil, were tested to prevent the development of tolerance to nicotine-induced antinociception when co-administered with nicotine chronically. The chronic co-exposure of BAYK8644, an L-type calcium channel activator, was tested to enhance nicotine's chronic tolerance. Finally, the activity of calcium/dependent calmodulin protein Kinase II in the lumbar spinal cord region obtained from nicotine-tolerant mice was measured to investigate whether the activation of this kinase could be changed during the development of tolerance to chronic nicotine. In addition, acute spinal administration of KN-62, a calcium/dependent calmodulin protein Kinase II antagonist, was investigated for its ability to prevent expression of tolerance to nicotine-induced antinociception in mice.

Materials and Methods

Animals. Male ICR (Institute for Cancer Research) mice (20-25 g, 10-weeks old) obtained from Harlan (Indianapolis, IN) were used throughout the study. Animals were housed in an AALAC approved facility in groups of three and had free access to food and water. The study was approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

Drugs. (-)-Nicotine was obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI) and converted to the ditartrate salt as described by Aceto et al. (1979). Other drugs were obtained as follows: Nimodipine, verapamil, (±)-1,4-Dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluromethyl)-phenyl]-3-pyridine carboxylic acid methyl ester (BAYK8644) were obtained from Sigma/RBI (Natick, MA, USA). 1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl--tyrosyl]-4-phenylpiperazine (KN-62), and 1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl--tyrosyl]-4-phenylpiperazine derivative (KN-04) were purchased from Calbiochem (San Diego, CA). Nicotine ditartrate and verapamil were dissolved in physiological saline (0.9% sodium chloride). KN-62 and KN-04 were prepared in dimethylsulfoxide (25%DMSO). Nimodipine and BAYK8644 were prepared in emulphor/ethanol/saline (1:1:18). Emulphor (EL620) was obtained from Rhone Poulenc (Crambury, NJ, USA). Solutions of BAYK8644 were refrigerated in foil-lined containers. 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.

Tolerance Induction. Mice were implanted with Alzet osmotic minipumps [model 2002 (14 days); Alza Corporation, Palo Alto, CA] filled with either (-)-nicotine or sterile physiological saline solutions. The concentration of nicotine solution in minipumps was adjusted according to animal weight, resulting in 24 mg/kg/day for 14 days. The minipumps were surgically implanted s.c. under sterile conditions with pentobarbital anesthesia (50 mg/kg, i.p.). An incision was made in the back of the animals, and a pump was inserted. The wound was closed with wound clips, and the animal was allowed to recover before being returned to its home cage. At day 15, animals

were injected with various doses of nicotine. To test the effects of L-type calcium channels modulators on nicotine tolerance development, mice implanted with minipumps received vehicle (1:1:18) or nimodipine (1 and 5 mg/kg, i.p.), verapamil (10 mg/kg, i.p.) and BAYK8644 (0.1 and 0.5 mg/kg, i.p.) twice a day for 14 days. For the studies with Calcium/dependent calmodulin protein Kinase II inhibitors, minipumps were removed at day 15 and 12 hrs later animals were injected with different intrathecal doses of Calcium/dependent calmodulin protein Kinase II inhibitors. Mice were 12 hrs later challenged with a dose of 2.5 mg/kg of nicotine.

Tail-Flick Test. 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 injection of nicotine.

Calcium/dependent calmodulin protein Kinase II phosphorylation assay

Calcium/dependent calmodulin protein Kinase II activity was measured using a modified assay kit (Upstate Biotechnology, Lake Placid, NY). Mice implanted with either saline or nicotine minipumps (24 mg/kg/day) for 14 days were used to measure calcium/dependent calmodulin protein Kinase II activation. At day 15, 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. Supernatant is retained (cytosolic fraction). The pellet is resuspended in homogenization buffer plus 1% NP-40 (IGEPAL) and allowed to incubate on ice 1 hour. The tubes are spun again and supernatant is retained (Membrane fraction). Standard phosphorylation reaction solutions contains 15 µg

extract protein, 100 µM CaM 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 CaCl2 and 5 µg calmodulin for the measurement of calcium-dependent activity. In aliquots used for calcium-independent activity, 5 mM EGTA was added and CaCl2 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 $p < 0.05$. ED_{50} values with 95% CL for behavioral data were calculated by unweighted least-squares linear regression as described by Tallarida and Murray (1987). Tests for parallelism were calculated according to the method of Tallarida and Murray (1987). If confidence limit values did not overlap, then the shift in the dose-response curve was considered significant.

Results

Tolerance to nicotine-induced antinociception: Effects of L-type calcium channel antagonists

Tolerance to nicotine's effect in the tail-flick test was seen in mice infused with nicotine for 14 days (24 mg/kg/day). Dose-response curves for the nicotine-induced antinociception in chronic nicotine and saline-treated animals are presented in Fig. 1. Animals that received chronic nicotine were less sensitive to the acute nicotine challenge in the tail-flick test and nicotine's dose-response curve was shifted to the right compared to the vehicle-treated group. The ED_{50} values (and 95% CL) for saline-treated and nicotine-treated animals were 1.05 (0.5-2.0) and 4.1 (3.4-5.3) mg/kg, respectively (Table 1). In nicotine-infused mice that are chronically treated with nimodipine, tolerance to nicotine was prevented in a dose-dependent manner (Fig. 1). The ED_{50} value for nicotine-treated animals was reduced from 4.1 to 2.5 and 1.7 mg/kg after chronic treatment with nimodipine at 1 and 5 mg/kg, respectively. A similar prevention of nicotine tolerance was observed with verapamil, a non-dihydropyridine L-type calcium channel antagonist (Fig. 2). The ED_{50} value for nicotine-treated animals was reduced from 4.1 to 1.6 mg/kg after chronic treatment with verapamil at 10 mg/kg.

Tolerance to nicotine-induced antinociception: Effects ofBAYK8644, an L-type calcium channel activator

In contrast to L-type calcium channel antagonists that prevented the development of tolerance, BAYK8644 increased the degree of tolerance after chronic administration in nicotine tolerant mice. Dose-response curves for the nicotine-induced antinociception in chronic administration of BAYK8644 are presented in Fig. 3. In nicotine-infused mice that are chronically treated with BAYK8644, tolerance to nicotine was enhanced in a dose-dependent manner. The ED_{50} value for nicotine-treated animals was increased from 4.3 to 5.3 and 7.7 mg/kg after chronic treatment with BAYK8644 at 0.1 and 0.5 mg/kg, respectively (Table 1). The increase in the degree of tolerance was only significant at the higher dose of BAYK8644 (0.5 mg/kg) since the confidence limits between the nicotine-treated mice and the BAYK8644-treated animals did not overlap.

Calcium/dependent calmodulin protein Kinase II activity in lumbar spinal cord tissues after chronic administration of nicotine.

To further investigate the involvement of calcium/dependent calmodulin protein Kinase II in nicotine's tolerance, the activity of calcium/dependent calmodulin protein Kinase II in the spinal cord after chronic exposure to nicotine

in mice was investigated in mice. Animals received either saline or nicotine ((24 mg/kg/day) for 14 days and were sacrified at day 15 min. Spinal cord tissues were dissected and the calcium-dependent and -independent activity of calcium/dependent calmodulin protein Kinase II (expressed as the number of pmol ^{32}P incorporated into calcium/dependent calmodulin protein Kinase II substrate peptide/min/mg of protein) in the membrane was measured. As shown in Fig. 4, a significant increase in both dependent and independent calcium/dependent calmodulin protein Kinase II activity in the spinal cord was seen after chronic exposure of nicotine in mice.

Prevention of expression of tolerance to nicotine-induced antinociception by calcium/dependent calmodulin protein Kinase II antagonists

Tolerance to nicotine's effect in the tail-flick test was seen in mice infused with nicotine for 14 days (24 mg/kg/day) after a challenge dose of 2.5 mg/kg of nicotine at day 15 (Fig. 4). To further investigate the involvement of calcium/dependent calmodulin protein Kinase II in nicotine's tolerance, an inhibitor of the enzyme was evaluated for its ability to alter the tolerance to nicotine-induced antinociception. KN-62, a calcium/dependent calmodulin protein Kinase II inhibitor, given i.t. inhibited the expression of nicotine tolerance in a dose-dependent manner (Fig. 5). As illustrated, increasing doses of KN-62 produced a gradual inhibition of chronic tolerance to nicotine. KN-62 alone did not significantly alter the tail-flick latencies produced following administration of any of the doses tested in this experiment. On the other hand, KN-04 (0.1 μg/animal), an analog of KN-62 that does not block calcium/dependent calmodulin protein Kinase II, failed to significantly attenuate the expression of nicotine tolerance.

Discussion

The present study examined the role of L-type calcium channels and calcium-mediated second messenger systems in the development and expression of chronic tolerance to nicotine. We have shown that chronic pretreatment with L-type calcium channels antagonists nimodipine and verapamil, which decrease the intracellular calcium level, prevented the development of tolerance to nicotine's antinociceptive effects. In contrast, chronic exposure to L-type calcium channels activator BAYK8644, which increases the intracellular calcium level, enhanced the degree of tolerance to nicotine. In addition, the present study demonstrates that inhibition of spinal calcium/dependent calmodulin protein Kinase II by intrathecal injection of its specific inhibitor KN-62 strongly attenuated the expression of nicotine tolerance in response to chronic administration of the drug. Furthermore spinal calcium/dependent calmodulin protein Kinase II activity was significantly increased in nicotine-tolerant mice as compared with those in saline-infused mice. Taken together, these findings indicate a substantial role of activated calcium/dependent calmodulin protein Kinase II in the development of tolerance to nicotine-induced antinociception. This contention can be strongly supported by the fact that KN-62 inhibited expression to tolerance induced by nicotine. The present study extends our previous findings in which we reported that L-type calcium channels and calcium/dependent calmodulin protein Kinase II inhibitors reduced nicotine antinociception after acute administration (Damaj et al., 1995; Damaj, 2000). Furthermore, pretreatment with L-type calcium channels antagonists were reported to reduce the expression of locomotor sensitization to nicotine (Biala 2003) and mecamylamine-precipitated nicotine withdrawal in mice (Biala and Welginska, 2005). The involvement of L-type calcium channels in nicotine's dependence and tolerance is similar to that reported with other drugs of abuse such as alcohol, morphine, and sedatives (Little, 1991; Littleton and Brennan, 1993)

The mechanisms by which L-type calcium channels and calcium/dependent calmodulin protein Kinase II affect nicotine tolerance remain unclear. Although there are few reports on the contributions of calcium-dependent signaling pathways in nicotine tolerance, recent studies have focused on the modulation of CREB following chronic treatment with nicotine, often with dissimilar results. Brunzell et al. (2003) reported that phosphorylated CREB decreased in the nucleus accumbens in mice following chronic oral consumption of nicotine, while Pluzarev and Pandey (2004) showed that, in rats, nicotine withdrawal (but not chronic treatment with nicotine itself) significantly reduced the levels of CREB in rat cortex and amygdala. More recently, chronic exposure to nicotine was reported to increase Ca^{2+} entry into cortical neurons through L-type calcium channels and levels of [^{3}H]-verapamil binding sites after chronic nicotine exposure (Katsura et al., 2002). Moreover, chronic injection of nicotine in mice

increased [^{3}H]-diltiazem binding sites and expression of α_1 and α_2/δ_1 subunits in the cerebral cortex. Consistent with these findings, L-type calcium channels are found on the terminals of dopaminergic afferents in the basal forebrain and activation of nAChRs are known to increase calcium conductance of membranes of central neurons resulting in dopamine release (Marshall et al., 1997). In addition, studies with neuronal preparations showed that significant amounts of Ca^{2+} enter the neuron following activation of nAChRs causing a rise in $[Ca^{2+}]_i$ concentration (Mulle et al., 1992; Barrantes et al., 1995). This effect is sufficient to activate calcium-dependent protein kinases like calcium/dependent calmodulin protein Kinase II (Damaj, 2000). Furthermore, nAChRs receptor-mediated depolarization can activate voltage-operated Ca^{2+} channels, which augments the primary Ca^{2+} signals generated by nicotinic receptors (Dajas-Bailador and Wonnacott, 2004). We therefore can hypothesize that after chronic exposure to nicotine a greater calcium influx, through voltage-operated Ca^{2+} channels and possibly NMDA receptors, leads to an activation of calcium-dependent kinases such as calcium/dependent calmodulin protein Kinase II during tolerance. Also, given that chronic nicotine exposure causes an increase in the number of L-type calcium channels in different brain regions, antagonists could prevent the induction of synaptic neuroadaptation processes and the expression of nicotine tolerance syndrome in mice. It is almost certain that enhanced calcium/dependent calmodulin protein Kinase II activity can phosphorylate and modulate functions of other proteins that could play a role in modulating nicotine tolerance. For example, one can speculate that sustained activation of calcium/dependent calmodulin protein Kinase II modulates the onset and recovery of nAChR desensitization. Desensitization of nAChRs after chronic nicotine leads to a decrease in direct calcium influx through these receptors. This decrease will prompt adpatative changes and functional upregulation in other calcium regulating mechanisms such as voltage-gated calcium channels and the intracellular calcium stores, and in turn, triggers an increase in intracellular calcium and a sustained calcium/dependent calmodulin protein Kinase II activation. Taken together, our data strongly support the idea that the long-lasting activation of calcium/dependent calmodulin protein Kinase II in the spinal cord may lead to the synaptic plasticity associated with the development and/or maintenance of nicotine tolerance.

In conclusion, our data indicate that calcium-dependent mechanisms such as L-type calcium channels and calcium/dependent calmodulin protein Kinase II activation are involved in the expression and development of nicotine tolerance. Since L-type calcium channels antagonists can reduce tolerance to nicotine, they offer potential new strategies for treating nicotine dependence.

Acknowledgments. The authors greatly appreciate the technical assistance of Tie Han and Travis Roberts.

References

Aceto MD, Martin BR, Uwaydah IM, May EL, Harris LS, Izazola-Conde C, Dewey WL, Bradshaw TJ and Vincek WC (1979) Optically pure (+)-nicotine from (+/-)-nicotine and biological comparisons with (-)-nicotine. *J Med Chem* **22**:174-177.

Barrantes GE, Murphy CT, Westwick J and Wonnacott S (1995) Nicotine increases intracellular calcium, in rat hippocampal neurons via voltage-gated calcium channels. *Neurosci Lett* **196**:101-104.

Biala G and Weglinska B (2004) Calcium channel antagonists attenuate cross-sensitization to the rewarding and/or locomotor effects of nicotine, morphine and MK-801. *J Pharm Pharmacol* **56**:1021-1028.

Biala G and Weglinska B (2005) Blockade of the expression of mecamylamine-precipitated nicotine withdrawal by calcium channel antagonists. *Pharmacol Res* **51**:483-488.

Brunzell DH, Russell DS and Picciotto MR (2003) In vivo nicotine treatment regulates mesocorticolimbic CREB and ERK signaling in C57Bl/6J mice. *J Neurochem* **84**:1431-1441.

Collins AC, Romm E and Wehner JM (1988) Nicotine tolerance: an analysis of the time course of its development and loss in the rat. *Psychopharmacology* **96**:7-14.

D'Amour FE and Smith DL (1941) A method for determining loss of pain sensation. *J Pharmacol Exp Ther* **72**:74-79.

Dajas-Bailador F and Wonnacott S (2004) Nicotinic acetylcholine receptors and the regulation of neuronal signalling. *Trends Pharmacol Sci* **25**:317-324.

Damaj MI (1997) Altered sensitivity of mice to calcium-modulating dugs after chronic nicotine administration. *Eur J Pharmacol* **322**:129-135.

Damaj MI (2000) The involvement of spinal Ca^{2+}/calmodulin-protein kinase II in nicotine-induced antinociception in mice. *Eur J Pharmacol* **404**:103-110.

Damaj MI and Martin BR (1996) Tolerance to the antinociceptive effect of epibatidine after acute and chronic administration in mice. *Eur J Pharmacol* **300**:51-57.

Damaj MI, Welch SP and Martin BR (1995) Involvement of calcium and L-type channels in nicotine-induced antinociception. *J Pharmacol Exp Ther* **266**:1330-1338.

Hylden JL and Wilcox GL (1980) Intrathecal morphine in mice: A new technique. *Eur J Pharmacol* **67**:313-316.

Katsura M, Mohri Y, Shuto K, Hai-Du Y, Amano T, Tsujimura A, Sasa M and Ohkuma S (2002) Up-regulation of L-type voltage-dependent calcium channels after long term exposure to nicotine in cerebral cortical neurons. *J Biol Chem* **277**:7979-7988.

Little HJ (1991) The role of neuronal calcium channels in dependence on ethanol and other sedatives/hypnotics. *Pharmacol Ther* **50**:347-365.

Littleton J and Brennan C (1993) Adaptive changes in numbers of calcium channels in drug dependence. *Biochem Soc Symp* **59**:193-203.

Marks MJ, Romm E, Gaffney DK and Collins AC (1986) Nicotine-induced tolerance and receptor changes in four mouse strains. *J Pharmacol Exp Ther* **237**:809-819.

Marks MJ, Stitzel JA and Collins AC (1987) Influence of kinetics of nicotine administration on tolerance development and receptor levels. *Pharmacol Biochem Behav* **27**:505-512.

Marshall DL, Redfern PH and Wonnacott S (1997) Presynaptic nicotinic modulation of dopamine release in the the three ascending pathways studied by in vitro microdialysis: comparison of naive and chronic nicotine-treated rats. *J Neurochem* **68**:1511-1519.

Miner LL and Collins AC (1988) The effect of chronic nicotine treatment on nicotine-induced seizures. *Psychopharmacology* **95**:52-55.

Mulle C, Choquet D, Korn H and Changeux J-P (1992) Calcium influx through nicotinic receptor in rat central neurons: Its relevance to cellular regulation. *Neuron* **8**:135-143.

Pauly JR, Grun EU and Collins AC (1992) Tolerance to nicotine following chronic treatment by injections: A potential role for corticosterone. *Psychopharmacology* **108**:33-39.

Peng X, Gerzanich V, Anand R, Whiting PJ and Lindstrom J (1994) Nicotine-induced increase in neuronal nicotinic receptors results from a decrease in the rate of receptor turnover. *Mol Pharmacol* **46**:523-530.

Pluzarev O and Pandey SC (2004) Modulation of CREB expression and phosphorylation in the rat nucleus accumbens during nicotine exposure and withdrawal. *J Neurosci Res* **77**:884-891.

Sallette J, Bohler S, Benoit P, Soudant M, Pons S, Le Novere N, Changeux JP and Corringer PJ (2004) An extracellular protein microdomain controls up-regulation of neuronal nicotinic acetylcholine receptors by nicotine. *J Biol Chem* **279**:18767-18775.

Schwartz RD and Kellar KJ (1985) In vivo regulation of [^{3}H]acetylcholine recognition sites in brain by nicotinic cholinergic drugs. *J Neurochem* **45**:427-433.

Tallarida RJ, and Murray RB (1987) *Manual of pharmacological calculations with computer programs*: Springer-Verlag, New York.

FOOTNOTES

This work was supported by National Institute on Drug Abuse grants # DA-12610 and DA-05274.

FIGURE LEGENDS

Fig. 1 Blockade of the development of tolerance to nicotine-induced antinociception by nimodipine, an L-type calcium channels blocker, after chronic administration. Dose-response curves of nicotine antinociceptive effects in mice that were infused with nicotine (24 mg/kg for 14 days) and chronically injected with either vehicle or different doses of nimodipine. Mice were tested on day 15 using the tail-flick test. Each point represents the mean %MPE ± S.E. for 8-10 mice.

Fig. 2 Blockade of the development of tolerance to nicotine-induced antinociception by verapamil, a non-dihydropyridine L-type calcium channels blocker, after chronic administration. Dose-response curves of nicotine antinociceptive effects in mice that were infused with nicotine (24 mg/kg for 14 days) and chronically injected with either vehicle or verapamil (10 mg/kg, i.p.). Mice were tested on day 15 using the tail-flick test. Each point represents the mean %MPE ± S.E. for 8-10 mice.

Fig. 3 Enhancement of the development of tolerance to nicotine-induced antinociception by BAYK8644, an L-type calcium channels activator, after chronic administration. Dose-response curves of nicotine antinociceptive effects in mice that were infused with nicotine (24 mg/kg for 14 days) and chronically injected with either vehicle or different doses of BAYK8644. Mice were tested on day 15 using the tail-flick test. Each point represents the mean %MPE ± S.E. for 8-10 mice.

Fig. 4 Increase in spinal Calcium/dependent calmodulin protein Kinase II activity (membrane) after chronic administration of nicotine (24 mg/kg/day for 14 days.) or vehicle. Each point represents the mean activity ± S.E. of 6 mice (*$P < 0.05$ vs Saline).

Fig. 5 Effects of KN-62, a Calcium/dependent calmodulin protein Kinase II inhibitor, on the expression of tolerance to nicotine-induced antinociception. Mice were infused with nicotine (24 mg/kg for 14 days) and injected i.t. with either vehicle, KN-62 or KN-04 (inactive analog) at day 15. Mice were tested 12 hrs later in the tail-flick after a challenge dose of nicotine (2.5 mg/kg). Each point represents the mean %MPE ± S.E. for 8-10 mice.

Table 1: Effect of calcium channels agonists and antagonists after chronic administration on the degree of tolerance to nicotine-induced antinociception in mice using the tail-flick test.

Treatment	Potency (ED_{50} mg/kg)	Potency Ratio (Treatment/Vehicle)[a]
Vehicle/Saline/Nicotine	1.05 (0.5-2.1)	-
Vehicle/Nicotine/Nicotine	4.1 (3.4-5.3)[b]	3.9
Nimodipine 1/Nicotine/Nicotine	2.5 (1.9-3.7)	2.4
Nimodipine 5/Nicotine/Nicotine	1.7 (1.3-2.1)	1.6
Verapamil 10/Nicotine/Nicotine	1.6 (1.1-2.3)	1.5
BAYK8644 0.1/Nicotine/Nicotine	5.3 (4.8-5.8)[b]	5.0
BAYK8644 0.5/Nicotine/Nicotine	7.7 (6.1-8.3)[b, c]	7.3

ED_{50} values ± Confidence limits (CL) were calculated from the dose-response curve of the respective treatment and expressed as mg/kg. Each dose group included 8 to 10 animals.

[a] Potency Ratio = Treatment ED_{50} values/Vehicle ED_{50} values

[b] ED_{50} value significantly different from Vehicle/Saline/Nicotine

[c] ED_{50} value significantly different from Vehicle/Nicotine/Nicotine

Figure 1

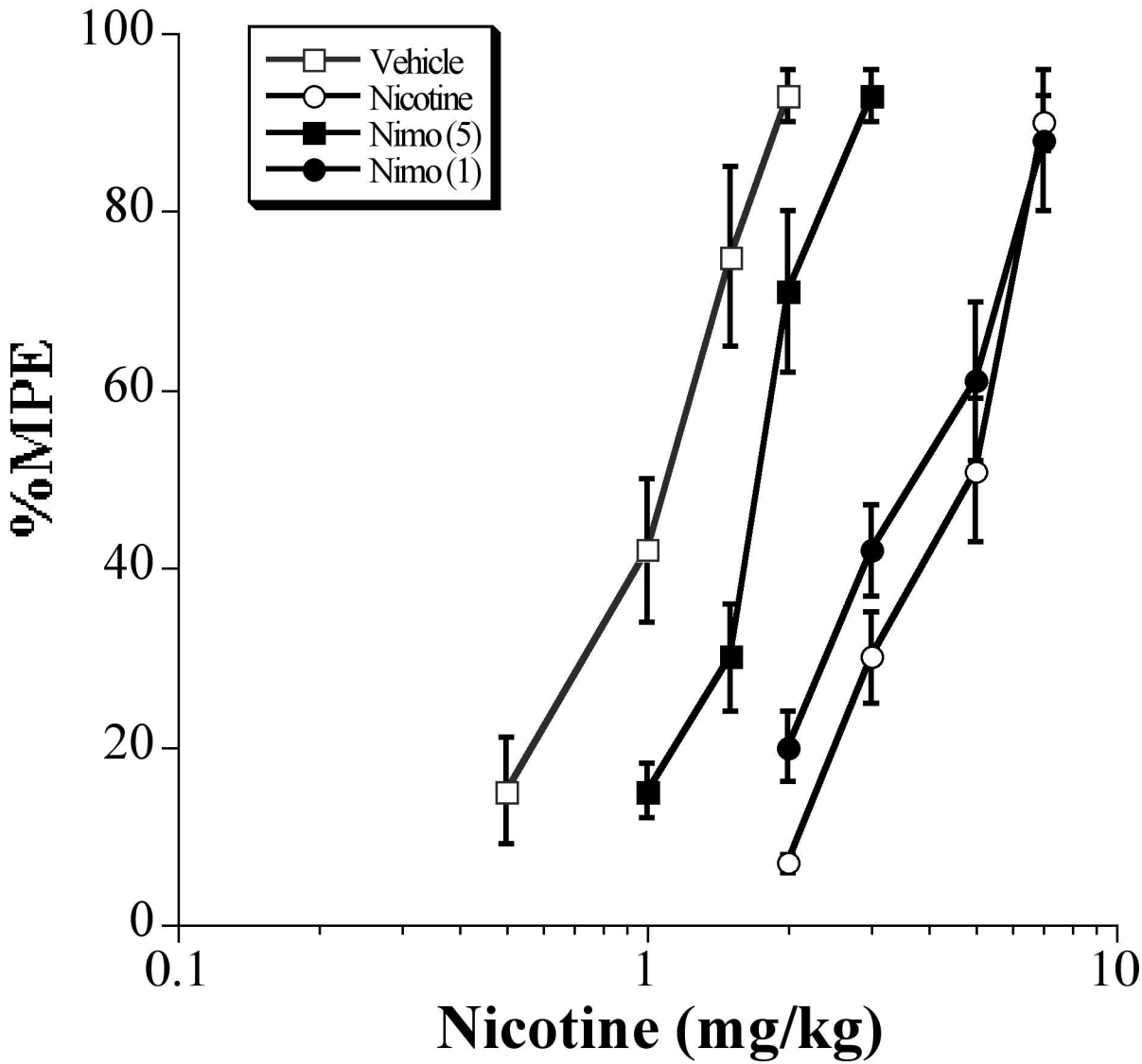

Vehicle
Nicotine
Nimo (5)
Nimo (1)
%MPE
100
80
60
40
20
0
0.1
1
10
Nicotine (mg/kg)

Figure 2

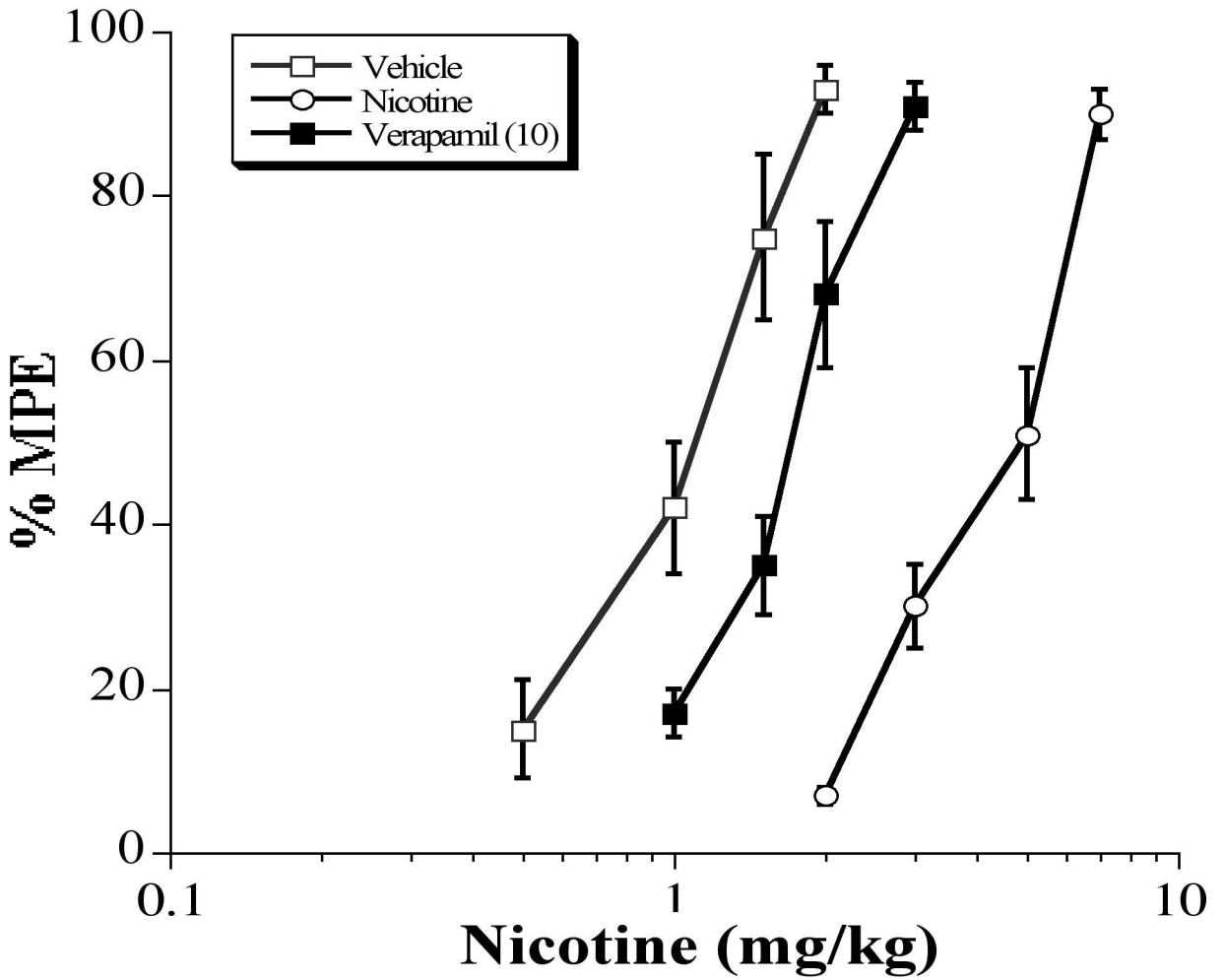
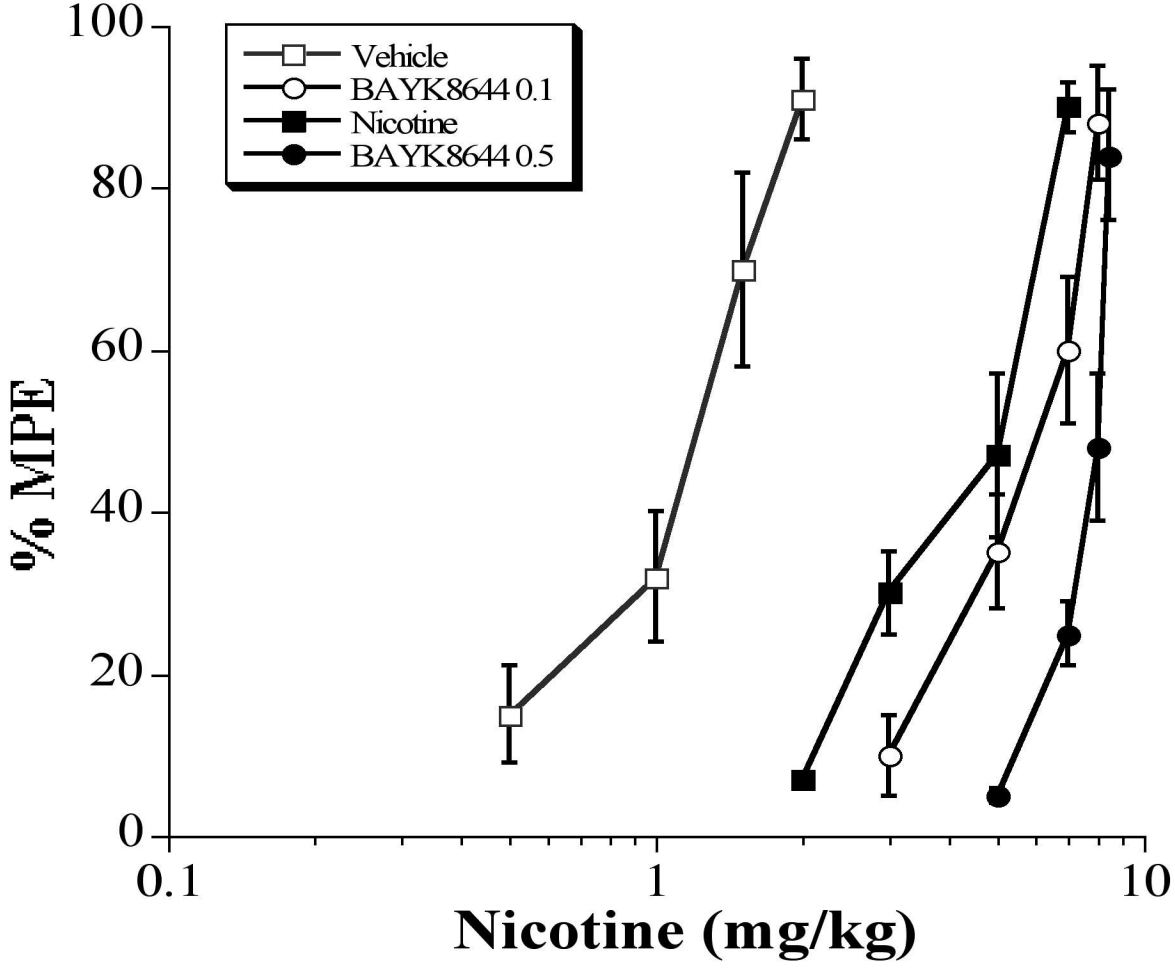

100
80
60
40
20
0
% MPE
Vehicle
Nicotine
Verapamil (10)
0.1
1
10
Nicotine (mg/kg)

Figure 3


100
80
60
40
20
0
% MPE
Vehicle
BAYK8644 0.1
Nicotine
BAYK8644 0.5
0.1
1
10
Nicotine (mg/kg)

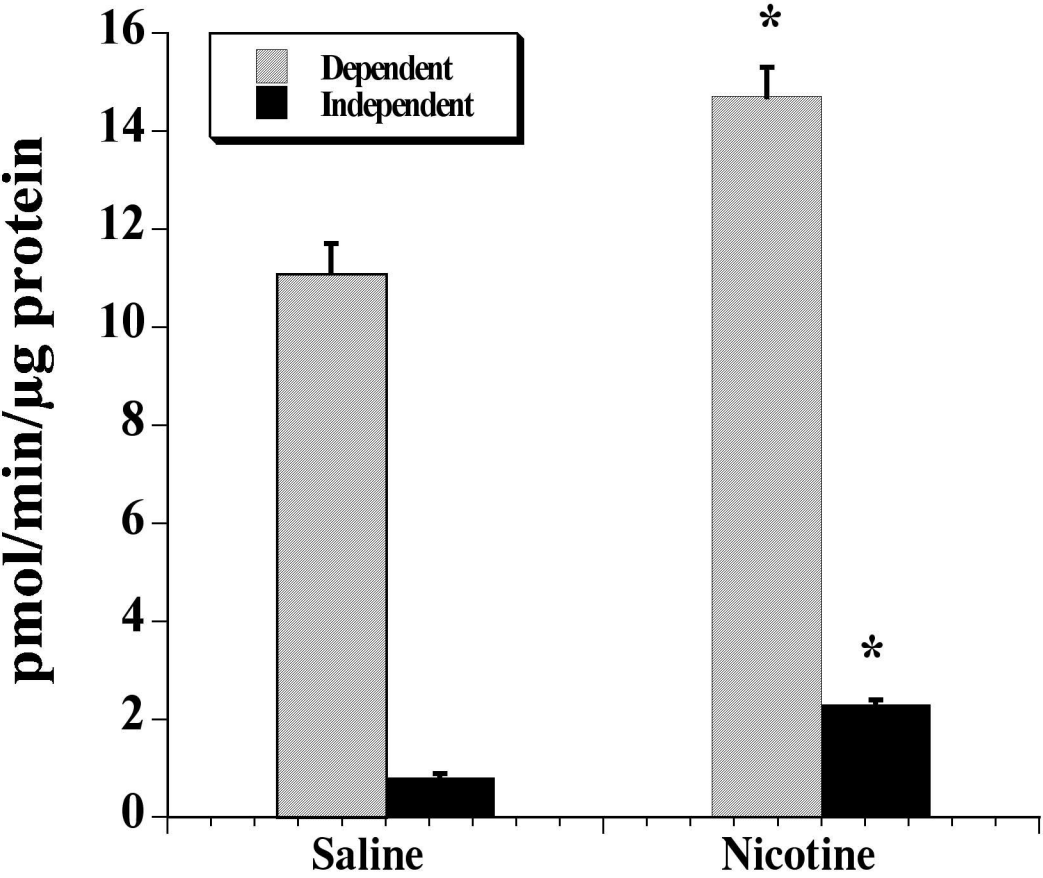
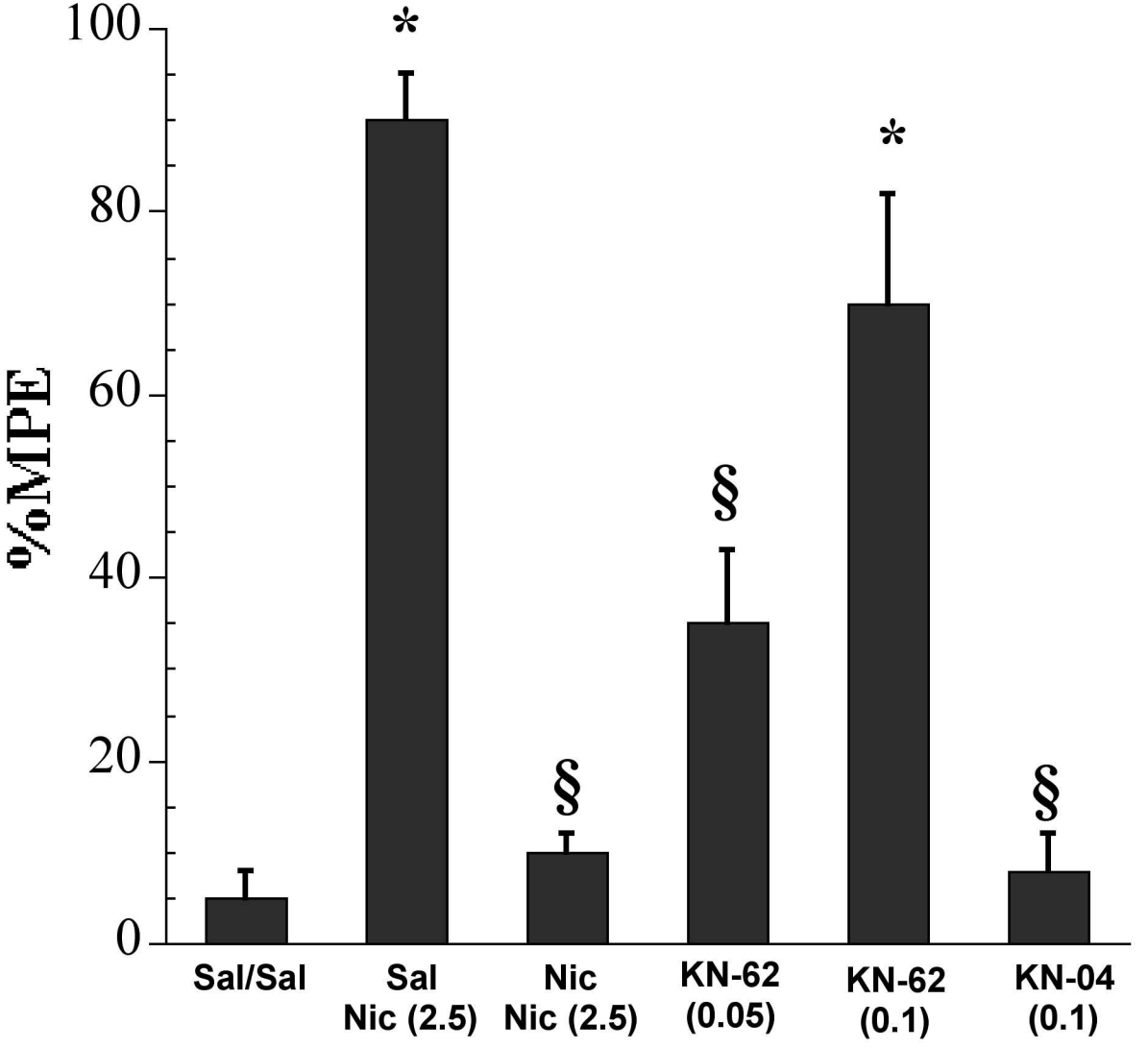

16
14
12
10
8
6
4
2
0
pmol/min/µg protein
Dependent
Independent
*
*
Saline
Nicotine

Figure 4

Figure 5


100
80
60
40
20
0
%MPE
*
*
§
§
§
Sal/Sal
Sal
Nic (2.5)
Nic
Nic (2.5)
KN-62
(0.05)
KN-62
(0.1)
KN-04
(0.1)