ELSEVIER

European Journal of Pharmacology 404 (2000) 103–110

ejp

www.elsevier.nl/locate/ejphar

The involvement of spinal Ca^{2+}/calmodulin-protein kinase II in nicotine-induced antinociception in mice

M. Imad Damaj *

Department of Pharmacology and Toxicology, Medical College of Virginia, Virginia Commonwealth University, Box 980613, Richmond, VA 23298-0613, USA

Received 2 March 2000; received in revised form 25 July 2000; accepted 28 July 2000

Abstract

The nature of the signaling process activated by neuronal nicotinic receptors has not been fully defined; however, several recent studies have implicated the involvement of Ca^{2+} fluxes in the response to nicotine. In order to assess Ca^{2+}-dependent mechanisms in nicotine-induced antinociception, the Ca^{2+} channel antagonist nimodipine and several calcium/calmodulin-protein kinase II (CaM kinase II) inhibitors were evaluated for their effects on nicotine-induced antinociception. The results indicate that both of these antagonists dose-dependently blocked nicotine-induced antinociception after intrathecal (i.t.) injection. Indeed, three structurally unrelated CaM kinase II inhibitors blocked nicotine's effects in the tail-flick test in a dose-related manner. A second series of experiments assessed the effect of acute nicotine exposure on $[Ca^{2+}]_i$ and CaM kinase II activity in spinal cord tissues. Nicotine increased $[Ca^{2+}]_i$ in a concentration-dependent manner after application of the drug to spinal synaptosomes. Furthermore, a dose-dependent increase in the spinal cord membrane CaM kinase II activity was seen after acute injection of nicotine in mice. Taken together, these results are consistent with the hypothesis that nicotine binding to nicotinic receptors leads to channel opening and depolarization responses with an influx of Ca^{2+} ions, which would reach sufficient levels to activate Ca^{2+}-dependent/CaM kinase II. Neuronal Ca^{2+}, acting via Ca^{2+}-dependent CaM kinase II, appears to mediate nicotine-induced antinociception at the spinal level.

Keywords: Nicotine; Antinociception; CaM kinase II; (Mice); Ca^{2+} channels; Intrathecal

1. Introduction

Activation of cholinergic pathways by nicotine elicits antinociceptive effects in a variety of species and pain tests (Aceto et al., 1986; Mattila et al., 1968; Phan et al., 1973). There is a strong evidence that the antinociceptive effect of nicotine can occur via activation of acetylcholine nicotinic receptors expressed in a variety of central nervous system (CNS) loci. There appear to be several CNS regions that contain nicotinic receptors capable of producing antinociception. These include the medulla (Iwamoto and Marion, 1993), certain areas of the midbrain (Mattila et al., 1968), the thalamus and the pedunculopontine tegmental nucleus (Iwamoto, 1989), the nucleus raphe magnus (Iwamato, 1991; Scott Bitner et al., 1998) and the spinal cord (Aceto et al., 1986; Christensen and Smith, 1990; Damaj et al., 1998; Khan et al., 1997).

Nicotinic receptors found throughout the central and peripheral nervous system have a high Ca^{2+} permeability. Significant amounts of Ca^{2+} enter the cell following activation of certain neuronal nicotinic receptors, causing a rise in $[Ca^{2+}]_i$ concentration. Studies with chick ciliary ganglion neurons (Vijayaraghavan et al., 1992), medial habenular neurons (Mulle et al., 1992), primary cultures of cortical neurons (Fluhler et al., 1992) and primary cultures of hippocampal neurons (Barrantes et al., 1994; Castro and Albuquerque, 1995) report an increase in $[Ca^{2+}]_i$ after nicotine application. Neuronal nicotinic receptors can elevate intracellular Ca^{2+} both directly because of their permeability to Ca^{2+} and/or indirectly because of their ability to depolarize the membrane and activate voltage-gated Ca^{2+} channels. An increase in $[Ca^{2+}]_i$ concentration can initiate a second messenger cascade or result in transmitter release. Nicotine-induced activation of protein kinase C and extracellular signal-regulated kinase 2 in cultured

* Tel.: +1-804-828-1676; fax: +1-804-828-2117.
E-mail address: mdamaj@hsc.vcu.edu (M.I. Damaj).

0014-2999/00/$ - see front matter
PII: S0014-2999(00)00579-3

bovine adrenal cells was shown to be Ca^{2+}-dependent (Pavlovic-Surjancev et al., 1992; Tuominen et al., 1992). In addition, nicotine-induction of c-*fos* in differentiated PC12 cells has been shown to rely upon a flux of Ca^{2+} ions into the cell through voltage-gated Ca^{2+} channels (Hughes and Dragunow, 1995). In the CNS, nicotine-evoked neurotransmitter release is largely extracellular Ca^{2+}-dependent (Rapier et al., 1988; Rowell and Winkler, 1984). Influx of Ca^{2+} mediated by nicotinic receptors stimulates intracellular messenger systems such as the release of arachidonic acid in chick ganglionic neurons (Vijayaraghavan et al., 1995). Furthermore, an increase in $[Ca^{2+}]_i$ after stimulation of nicotinic receptors activates calcium/calmodulin-protein kinase II (CaM kinase II) in PC12 cells (MacNicol and Schulman, 1992), a major cellular target for neuronal Ca^{2+}. Furthermore, Ochoa and O'Shea (1994) showed a Ca^{2+}-dependent increase in the phosphorylation of an 80-kDa protein (which contains synapsin I, a target of CaM kinase II) in rat frontal cortex synaptosomes after stimulation with nicotine. CaM kinase II is the most abundant protein kinase in the brain, comprising 0.5–1% of total brain protein. It is distributed throughout the forebrain neurons, but is especially concentrated in the cytoskeletal fractions and in the postsynaptic density. CaM kinase II has target substrate enzymes that include tyrosine hydroxylase, tryptophan hydroxylase, and cytoskeletal proteins such as synapsin I and transcription factors such Cyclic AMP Response Element Binding (CREB). CaM kinase II plays an important role in the regulation of neuronal excitability and synaptic transmission and is tightly associated with synaptic vesicles in the presynaptic terminal.

The purpose of the present study was to investigate the involvement of Ca^{2+}-dependent mechanisms in nicotine-induced antinociception in mice at the spinal level, in particular L-voltage-gated Ca^{2+} channels and CaM kinase II. For that, both behavioral and biochemical approaches were used. We first established an increase in spinal $[Ca^{2+}]_i$ after nicotine application and then investigated the effect of acute administration of nicotine on spinal CaM kinase II activity. Finally, we took advantage of the availability of Ca^{2+} channel antagonists and CaM kinase II membrane-permeable inhibitors and tested their effects on nicotine's antinociceptive actions.

2. Materials and methods

2.1. Animals

Male ICR mice (20–25 g) obtained from Harlan Laboratories (Indianapolis, IN) were used throughout the study. They were housed in groups of six 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.

2.2. Drugs

1-[*N*,*O*-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62), (*N*-(2-[*N*-[4-chlorocinnamyl]-*N*-methylaminomethyl]phenyl)-*N*-2-hydroxyethyl)-4-methoxybenzenesulfonamide) (KN-93), (2-[*N*-(4-methoxybenzenesulfonyl)]amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine, phosphate) (KN-92), (8*R**,9*S**,11*S**)-(−)-9-hydroxy-9-methoxycarbonyl-8-methyl-14-*n*-propoxy-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7*b*,11*a*-triazadibenzo[*a*,*g*]cycloocta[*cde*]trinden-1-one (KT-5926) and 1-[*N*,*O*-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine derivative (KN-04) were purchased from Calbiochem (San Diego, CA). Morphine and Δ^9-tetrahydrocanabinoid (Δ^9-THC) were supplied by the National Institute on Drug Abuse (Washington, DC). Nicotine enantiomers were synthesized and converted to the ditartrate salt as described by Aceto et al. (1979). All drugs were dissolved in physiological saline (0.9% sodium chloride). Nimodipine, KN-62, KN-93, KN-92, KT-5926 and KN-04 were prepared in dimethylsulfoxide (DMSO). All doses are expressed as the free base of the drug.

2.3. Intrathecal (i.t.) injections

I.t. injections 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.

2.4. Antinociceptive assay

Antinociception was assessed by the tail-flick method of D'Amour and Smith (1941) as modified by Dewey et al. (1970). A control response (2–4 s) was determined for each animal before treatment, and a test latency was determined after drug administration. In order to minimize tissue damage, a maximum latency of 10 s was imposed. Antinociceptive response was calculated as the percentage of maximum possible effect (%MPE), where $\%MPE = [(test - control)/(10 - control)] \times 100$. Groups of 8–12 animals were used for each dose and for each treatment. The mice were tested 5 min after i.t. injection of nicotine. Antagonism studies were carried out by pretreating the mice i.t. with either vehicle, CaM kinase II inhibitors or

nimodipine, 5 min before nicotine. The animals were tested 5 min later.

2.5. Synaptosomal preparation

Synaptosomes were prepared from mouse spinal cord using subcellular fractionation techniques described by McGovern et al. (1973). Male ICR mice were decapitated and spinal cord was removed and homogenized in 0.32 M sucrose in Kreb's buffer (pH 7.4–7.5) on ice (1 g of tissue/20 ml sucrose). The Kreb's buffer was composed of (mM): NaCl 120; KCl 5; NaH_2PO_4 1.2; $MgCl_2$ 1.2; $CaCl_2$ 1; glucose 10, $NaHCO_3$ 20, and was brought to pH 7.5 with 1 N HCl. The homogenate was centrifuged at $1000 \times g$ for 10 min. The supernatant was then removed and centrifuged at $18{,}000 \times g$ for 20 min. The P2 pellet, containing synaptosomes in a crude mitochondrial pellet, was used to obtain adequate protein for further assays.

2.6. Measurement of intracellular Ca^{2+} in synaptosomal preparations

The synaptosome pellet was resuspended in well-oxygenated assay buffer (KCl, 150 mM; $MgCl_2$, 3 mM; HEPES, 20 mM; sodium azide, 0.5 mM; phosphocreatine, 10 mM; creatine phosphokinase, 20 U/ml; pH adjusted to 7.4 with Tris base). Synaptosomes were loaded with 5 μM 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxyl]-2-(2″-amino-5″-methylphenoxy)-ethane-*N*,*N*,*N*,*N*-tetraacetic acid-pentaacetoxymethyl ester (fura-2/AM) at room temperature for 1 h. The synaptosomes were then washed and resuspended in the assay buffer, which was modified as needed to evaluate changes in Ca^{2+} concentrations. Stimulation of the release of Ca^{2+} was initiated by the addition of nicotine at different concentrations. The fura-2 signal in the presence of 100-fold excess EGTA (prepared in 3 M Tris, pH 8.3) was used to determine the background value of relative fluorescence (F_{min}). The maximal fluorescence (F_{max}) was determined when fura-2/AM is maximally bound to Ca^{2+} following exposure of the cells to 0.1% Triton-X. $[Ca^{2+}]_i$ was calculated using the method of Grynkiewicz and Iverson (1985) and 224 nm for the K_D for fura-2. $[Ca^{2+}]_i$ were quantitated using the Spex System CM-3 Cation Measurement System which allows for the measurement of Ca^{2+} in suspensions placed in quartz cuvettes. Autofluorescence was quantitated for all compounds added.

2.7. Cam kinase II phoshorylation assays

Calcium-dependent calmodulin-protein kinase II activity was measured using an assay kit (Upstate biotechnology, Lake Placid, NY). Briefly, following experimental treatment, spinal cord tissues were homogenized in calcium-free Tris buffer that contains 1 mM PMSF. Homogenates were normalized for protein concentration. Samples were centrifugated in order to separate the membrane and the cytosol containing-kinase. Standard phosphorylation reaction solutions contains 70 μg protein, 10 mM $MgCl_2$, 1 μCi of [^{32}P]ATP, 10 mM Pipearzine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) (pH 7.4), 5 μM $CaCl_2$ and 5 μg calmodulin. Standard reactions were performed in a shaking water bath at 30°C. CaM kinase activity was determined using the following calculations: [(count-specific binding) × (correcting factor)]/[(specific radioactivity) × time (10 min)].

2.8. Statistical analysis

Data were analyzed statistically by an analysis of variance followed by the Fisher's least significant difference (PLSD) multiple comparison test. The null hypothesis was rejected at the 0.05 level. Antagonist dose 50% (AD_{50}) values with 95% confidence limits (CL) for antinociception data were calculated by unweighted least-squares linear regression, as described by Tallarida and Murray (1987).

3. Results

3.1. Effect of nicotine on $[Ca^{2+}]_i$ levels in spinal synaptosomes

Synaptosomes were prepared from mouse spinal cord and loaded with the fluorescent chelator fura-2/AM for the measurement of $[Ca^{2+}]_i$. Free Ca^{2+} within the synaptosomes after application of saline or nicotine at different concentrations was measured. As shown in Fig. 1, nicotine increased $[Ca^{2+}]_i$ in a concentration-dependent manner. At 10 μM of nicotine, $[Ca^{2+}]_i$ were increased four-fold compared to saline. Nicotine-induced increase in $[Ca^{2+}]_i$ is receptor-mediated since it was completely

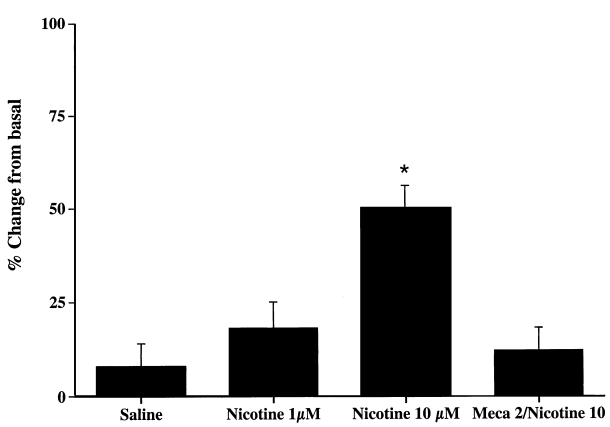

Fig. 1. Effect of nicotine on $[Ca^{2+}]_i$ in spinal synaptosomes. The average %change (increase) in $[Ca^{2+}]_i$ over basal $[Ca^{2+}]_i$ was calculated for 3–5 separate preparations of synaptosomes. $^{*}P < 0.05$ from saline. Meca 2 = mecamylamine at 2 μM.

blocked by mecamylamine, a nicotinic antagonist, at 2 μM.

3.2. Cam kinase II activity in spinal cord tissues after acute injection of nicotine in mice

The activity of CaM kinase II in the spinal cord after acute administration of nicotine in mice was investigated. Animals received either saline or nicotine (1 or 3 mg/kg, s.c.) and were sacrificed 2 min after injection. Spinal cord tissues were dissected and the activity of CaM kinase II (expressed as the number of pmol ^{32}P incorporated into CaM kinase II substrate peptide min^{-1} mg^{-1} of protein) in the membrane and cytosol was measured. As shown in Fig. 2, a dose-dependent increase in the membrane kinase activity was seen after acute injection of nicotine. However, no significant change was observed in the cytosolic fraction of the enzyme. The increase in the kinase activity induced by nicotine was blocked by mecamylamine pretreatment. These results are consistent with the notion that CaM kinase II activation after acute nicotine exposure is a receptor-mediated event.

3.3. Effects of Cam kinase II inhibitors on nicotine-induced antinociception in mice

To further investigate the involvement of CaM kinase II in nicotine's effects, various CaM kinase II inhibitors were evaluated for their ability to alter the antinociceptive effect of nicotine. KN-62, a CaM kinase II inhibitor, given i.t. inhibited the antinociceptive responses of spinally injected nicotine in a dose-dependent manner (Fig. 3A). As illustrated, increasing doses of KN-62 produced a gradual

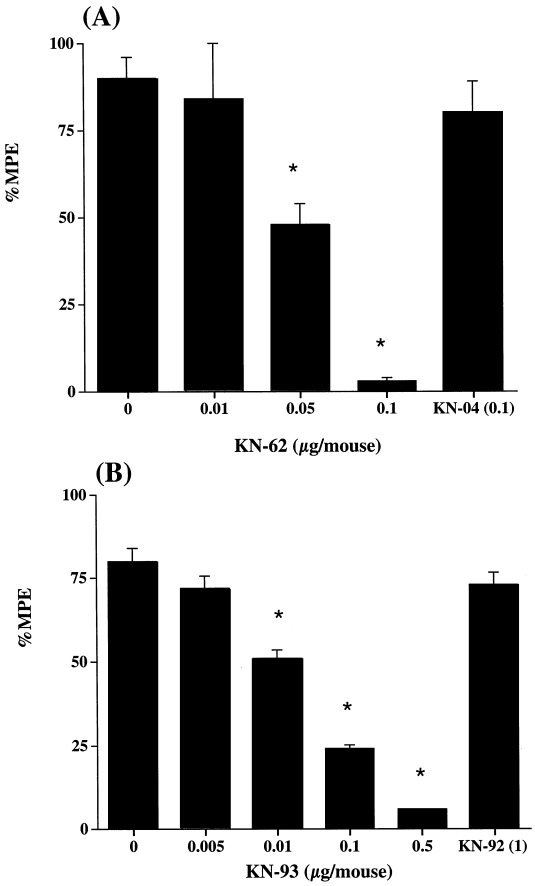

Fig. 3. Effects of (A) KN-62 and (B) KN-93 and their inactive analogs (KN-04 and KN-92, respectively) on the antinociceptive effect of nicotine after i.t. administration. Mice were pretreated i.t.with different doses of CaM kinase II inhibitors 5 min before nicotine (20 μg/animal) and tested 5 min after the second injection in the tail-flick test. Each point represents the mean %MPE ± S.E. for 6–8 mice. * Statistically different from vehicle (dose 0) at $P < 0.05$.

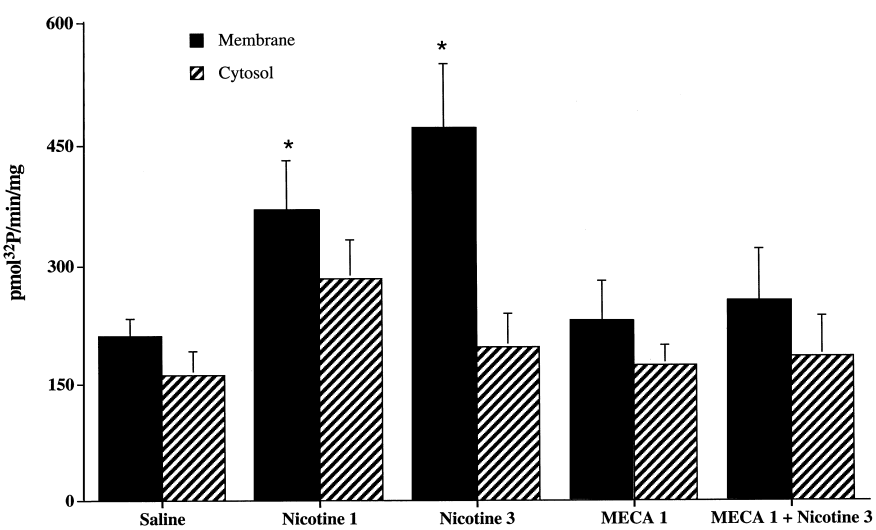

Fig. 2. Increase in spinal membrane CaM kinase II activity after acute injection of nicotine (1 and 3 mg/kg, s.c.) in mice. The increase was blocked by pretreatment with mecamylamine (1 mg/kg), a nicotinic antagonist. Animals were sacrificed 2 min after nicotine or saline injection. Meca = mecamylamine. Each point represents the mean ± S.E. of 5–6 mice (* $P < 0.05$ from saline).

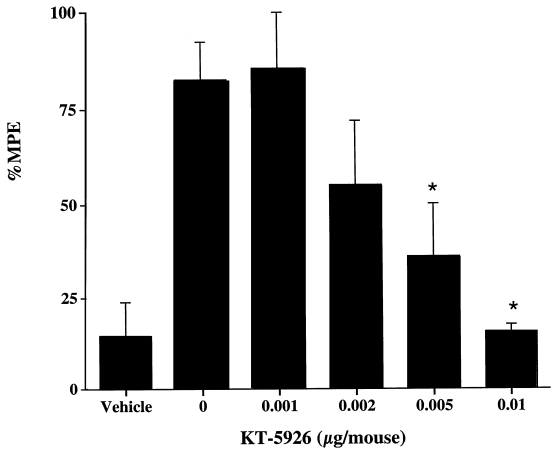

Fig. 4. Effects of KT-5926, a CaM kinase II inhibitor, on the antinociceptive effect of nicotine after i.t. administration. Mice were pretreated i.t. with different doses of KT-5926 5 min before nicotine (20 μg/animal) and tested 5 min after the second injection in the tail-flick test. Each point represents the average %MPE ± S.E. for 6–8 mice. * Statistically different from vehicle (dose 0) at $P < 0.05$.

inhibition of the antinociceptive response to 20 μg of nicotine, with an AD_{50} of 0.05 [(0.01–0.065)] μg/mouse (0.07 nmol). KN-62 alone did not significantly alter the tail-flick latencies produced following administration of any of the doses tested in this experiment. No external sign of toxicity was observed after i.t. injection of KN-62. On the other hand, KN-04 (0.1 μg/animal), an analog of KN-62 that does not block CaM kinase II, failed to significantly attenuate the antinociceptive effect of 20 μg/i.t of nicotine. Similarly, KN-93, another CaM kinase II inhibitor which is structurally unrelated, given i.t. blocked nicotine-induced antinociception in a dose-related manner (Fig. 3B). The AD_{50} of KN-93 was 0.035 [(0.008–0.060)] μg/mouse (0.06 nmol). KN-93 alone did not

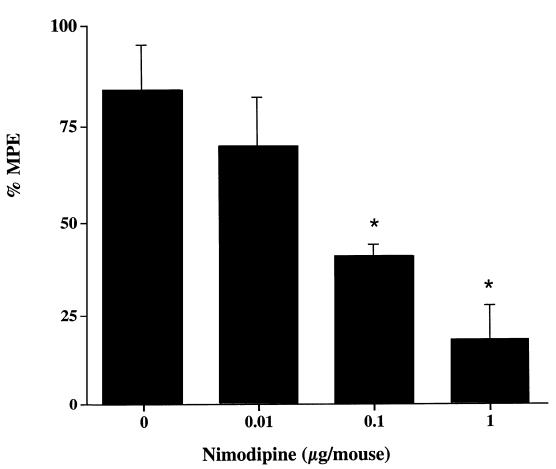

Fig. 5. Effects of nimodipine, a calcium channel blocker, on the antinociceptive effect of nicotine after i.t. administration. Mice were pretreated i.t. with different doses of nimodipine 5 min before nicotine (20 μg/animal) and tested 5 min after the second injection in the tail-flick test. Each point represents the average %MPE ± S.E. for 6–8 mice. * Statistically different from vehicle (dose 0) at $P < 0.05$.

Table 1
Lack of blockade of morphine and Δ^9-THC-induced antinociception by KN-62 after i.t. injection in mice using the tail-flick test. Mice were pretreated with KN-62 (1 μg/i.t.) 5 min before morphine (1 μg/i.t.) or Δ^9-THC (70 μg/i.t.). Each point represents the mean ± S.E. of 6–12 mice

Treatment	%MPE ± S.E.M.
Vehicle/Vehicle	7 ± 2
KN-62 (1 μg)/Vehicle	18 ± 9
Vehicle/Morphine (1 μg)	84 ± 10
KN-62 (1 μg) + Morphine (1 μg)	73 ± 17
Vehicle/Δ^9-THC (7 μg)	78 ± 9
KN-62 (1 μg) + Δ^9-THC (7 μg)	73 ± 17

significantly alter the tail-flick latencies produced following administration of any of the doses tested in this experiment. Pretreatment with KN-92 (1 μg/animal, i.t.), an analog of KN-93 that does not block CaM kinase II, did not significantly attenuated the antinociceptive effect of 20 μg/i.t of nicotine (Fig. 3B). Finally, KT-5926, a very potent inhibitor of CaM kinase II, was five times more potent than KN-62 in blocking the antinociceptive effects of nicotine in vivo (AD_{50} = 0.006 μg/mouse or 0.01 nmol) (Fig. 4).

3.4. Effects of nimodipine on nicotine-induced antinociception in mice

Nimodipine, a Ca^{2+} channel blocker, given i.t. inhibited the antinociceptive responses of nicotine given i.t. (Fig. 5) with an AD_{50} of 0.12 (0.1–0.3) μg/animal. By itself, nimodipine did not cause antinociception at the indicated doses and times.

3.5. Effects of Cam kinase II inhibitors on other antinociceptive agents

The effects of KN-62 on the antinociceptive effects of morphine and Δ^9-THC were investigated after i.t. administration. As shown in Table 1, KN-62 at a dose 20 times higher than AD_{50} for nicotine blockade, failed to significantly block the effect of an active dose of morphine and Δ^9-THC in the tail-flick test. Thus, the effect of KN-62 appears not to be generalized to all analgesic substances since it did not block the effects of morphine and Δ^9-THC i.t. administration.

4. Discussion

The data in this study indicate that the blockade of L-type Ca^{2+} channels in the spinal cord impairs the expression of nicotine-induced antinociception. Likewise, inhibition of CaM kinase II blocked nicotine's antinociceptive effects. In addition, acute exposure to nicotine increases $[Ca^{2+}]_i$ as well as the activity of CaM kinase II at

the spinal cord. Taken together, these results are consistent with the hypothesis that increases in cytosolic Ca^{2+} result in an enhancement in Ca^{2+}-mediated second messengers in the spinal cord that play an important role in nicotine-induced antinociception as measured in the tail-flick test.

Our in vivo data specifically suggest the involvement of CaM kinase II in nicotine's effects. Indeed, three structurally unrelated CaM kinase II inhibitors, dose-dependently blocked nicotine-induced antinociception. In addition, a relationship exists between their potency in blocking nicotine and their affinity to CaM kinase II. For example, KT-5926, a very potent inhibitor of CaM kinase II ($K_i = 4.4$ vs. 900 nM for KN-62), was five times more potent than KN-62 in blocking the effects of nicotine in vivo ($AD_{50} = 0.006 \mu g/mouse$). Furthermore, KN-04 and KN-92, respective analogs of KN-62 and KN-93 that do not block CaM kinase II, failed to significantly attenuate nicotine's effect in the tail-flick test. These CaM kinase II blockers are not competitive nicotinic blockers since they do not bind to [^{3}H]-nicotine binding sites with high affinity (data not shown). It is important to note that all the CaM kinase II inhibitors tested have no inhibitory effect on protein kinase A, protein kinase C, myosin light chain kinase, or casein kinase I, and they are not calmodulin antagonists (Hashimoto et al., 1991; Sumi et al., 1991; Tokumitsu et al., 1990). However, KT-5926 was reported to inhibit both Ca^{2+}/calmodulin-dependent and -independent smooth muscle myosin light chain kinases to a similar extent (Nakanishi et al., 1990). These kinase inhibitors have been used previously as specific inhibitors for CaM kinase II in various in vitro and in vivo neuronal systems (Suh et al., 1997; Tohda et al., 1991). The inhibitory effect of these antagonists observed in vivo was validated by the fact that acute injection of nicotine increased dose-dependently the activity of CaM kinase II in the spinal cord. Furthermore, the effect of KN-62 appears not to be generalized to all analgesic substances since it did not block the effects of morphine and Δ^9-THC i.t. administration. The lack of effect of KN-62 on morphine acute analgesia is similar to that previously reported with Suh et al. (1997), further supporting the hypothesis that morphine and nicotine produce their antinociception by activating different pain-inhibitory systems. Thus, our data provide clear and direct evidence of the involvement of CaM kinase II in nicotine-induced antinociception. Consistent with this finding, increase in the activity of CaM kinase II in PC12 cells which contain nicotinic receptors, have been reported after exposure to nicotinic agonist DMPP (MacNicol and Schulman, 1992).

We recently reported that changes in Ca^{2+} homeostasis could modulate nicotine-induced antinociception in mice. Indeed, Ca^{2+} channel blockers, such as nifedipine, attenuate nicotine-induced antinociception and hypomotility, whereas Ca^{2+}, thapsigargin and 1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluromethy)-phenyl]-3-pyridine (BAYK 8644), a Ca^{2+} channel agonist, dramatically potentiate these activities of nicotine (Damaj et al., 1993; Damaj and Martin, 1993). Our present results with nimodipine confirm the involvement of L-type Ca^{2+} channels in the spinal effect of nicotine.

Although neuronal nicotinic receptors found throughout the brain and peripheral nervous system have high Ca^{2+} permeability (Barrantes et al., 1994; Castro and Albuquerque, 1995; Mulle et al., 1992; Vijayaraghavan et al., 1992), we report here for the first time a rise in $[Ca^{2+}]_i$ following the activation of spinal nicotinic receptors. Binding studies have shown nicotinic receptor sites to be present in the spinal cord, in particular the ventral and the dorsal horns (Gillberg and Aquilonius, 1985; Khan et al., 1994). Two classes of binding sites differing in affinities and total number of binding sites were reported in the spinal cord membranes using (±)-[^{3}H]-epibatidine as a radioligand. Molecular biology studies confirmed the possible existence of multiple nicotinic receptors 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).

The present data demonstrate that Ca^{2+} channels antagonists and CaM kinase II inhibitors were capable of attenuating nicotine-induced antinociception at the spinal level. These data are consistent with the hypothesis that nicotine binding to nicotinic receptors leads to channel opening and depolarization responses with an influx of Ca^{2+} ions, which would reach sufficient levels to activate Ca^{2+}-dependent/CaM kinase II. The Ca^{2+}-dependent protein kinase can influence down-stream events such as serotonin and/or norepinephrine release and thus provide mechanisms for nicotine's antinociceptive effect at different sites of the CNS. However, our data cannot rule out that activation of nicotinic receptors results in neurotransmitter release in the spinal cord which then evokes Ca^{2+} signals postsynaptically, resulting in CaM kinase II activation.

Several neurotransmitter systems are involved in mediating the antinociceptive effects of nicotinic agonists. Activation of nicotinic receptors may lead to the activation of multiple, descending inhibitory pathways which involve release of acetylcholine (which activate spinal muscarinic receptors), norepinephrine (which activate spinal α_2 adrenoceptors) and serotonin (which activate serotonergic spinal receptors) (Iwamato, 1991; Iwamoto, 1989; Iwamoto and Marion, 1993; Rogers and Iwamoto, 1993). Such descending pathways explain nicotinic antinociceptive effects observed in the tail-flick test in which a spinal reflex initiated does not require the involvement of higher CNS regions. In fact, nicotine receptors both pre- and post-synaptic neural elements may mediate the local regulation of transmitter release. We recently reported that changes in Ca^{2+} homeostasis could modulate nicotine-induce

antinociception in mice. Indeed, i.t. administration of Ca^{2+} channel blockers, such as nifedipine and nimodipine, attenuate nicotine-induced antinociception, whereas Ca^{2+}, thapsigargin and BAYK 8644, a calcium channel agonist, dramatically potentiate the effect of nicotine (Damaj et al., 1993; Damaj and Martin, 1993).

In summary, our results suggest that intracellular Ca^{2+} concentrations and Ca^{2+}-dependent events in the spinal cord play an important role in mediating nicotine-induced antinociception.

Acknowledgements

The authors greatly appreciate the technical assistance of Tie Han, Kim Creasy and Gray Patrick. This work was supported by the National Institute on Drug Abuse grant # DA-05274.

References

Aceto, M.D., Martin, B.R., Uwaydah, I.M., May, E.L., Harris, L.S., Izazola-Conde, C., Dewey, W.L., Vincek, W.C., 1979. Optically pure (+)-nicotine from (±)-nicotine and biological comparisons with (−)-nicotine. J. Med. Chem. 22, 174–177.

Aceto, M.D., Bagley, R.S., Dewey, W.L., Fu, T.-C., Martin, B.R., 1986. The spinal cord as a major site for the antinociceptive action of nicotine in the rat. Neuropharmacology 25, 1031–1036.

Barrantes, G.E., Westwick, J., Wonnacott, S., 1994. Nicotinic acetylcholine receptors in primary cultures of hippocampal neurons: pharmacology and Ca^{++} permeability. Biochem. Soc. Trans. 22, 195S–294S.

Castro, N., Albuquerque, E., 1995. a-Bungarotoxin-sensitive hippocampal nicotinic receptor channel has a high calcium permeability. Biophys. J. 68, 516–524.

Christensen, M.-K., Smith, D.F., 1990. Antinociceptive effects of the stereoisomers of nicotine given intrathecally in spinal rats. J. Neural Transm. 80, 189–194.

D'Amour, F.E., Smith, D.L., 1941. A method for determining loss of pain sensation. J. Pharmacol. Exp. Ther. 72, 74–79.

Damaj, M.I., Martin, B.R., 1993. Calcium agonists and antagonists of the dihydropyridine type: effect on nicotine-induced antinociception and hypomotility. Drug Alcohol Depend. 32, 73–79.

Damaj, M.I., Welch, S., Martin, B.R., 1993. Involvement of calcium and L-type channels in nicotine-induced antinociception. J. Pharmacol. Exp. Ther. 266, 1330–1338.

Damaj, M.I., Fei-Yin, M., Dukat, M., Glassco, W., Glennon, R.A., Martin, B.R., 1998. Antinociceptive responses to nicotinic acetylcholine receptor ligands after systemic and intrathecal administration in mice. J. Pharmacol. Exp. Ther. 284, 1058–1065.

Dewey, W.L., Harris, L.S., Howes, J.S., Nuite, J.A., 1970. The effect of various neurohormonal modulations on the activity of morphine and the narcotic antagonists in tail-flick and phenylquinone test. J. Pharmacol. Exp. Ther. 175, 435–442.

Dineley-Miller, K., Patrick, J., 1992. Gene transcripts for the nicotinic acetylcholine receptor subunit, Beta4, are distributed in multiple areas of the rat central nervous system. Mol. Brain Res. 16, 339–344.

Fluhler, E., Lippiello, P., Fernandes, K., 1992. Localization and function of high-affinity nicotine receptors on cultured cortical neurons from rat brain. In: Lippiello, P.M., Collins, A.C., Gray, J.A., Robinson, J.H. (Eds.), The Biology of Nicotine — Current Research Issues. Raven Press, New York, pp. 23–38.

Gillberg, P., Aquilonius, S., 1985. Cholinergic, poioid and glycine receptor binding sites localized in human spinal cord by in vitro autoradiography. Acta Neurol. Scand. 72, 299–306.

Grynkiewicz, G.L., Iverson, L.L., 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. 260, 3440–3450.

Hashimoto, Y., Nakayama, T., Teramoto, T., Kato, H., Watanabe, T., Kinoshita, M., Tsukamoto, K., Tokunaga, K., Kurokawa, K., Nakanishi, S., 1991. Potent and preferential inhibition of Ca^{2+}/calmodulin-dependent protein kinase II by K252a and its derivative, KT5926. Biochem. Biophys. Res. Commun. 181, 423–429.

Hughes, P., Dragunow, M., 1995. Induction of immediate-early genes and the control of neurotransmitter-regulated gene expression within the nervous system. Pharmacol. Rev. 47, 133–178.

Hylden, J.L., Wilcox, G.L., 1980. Intrathecal morphine in mice: a new technique. Eur. J. Pharmacol. 67, 313–316.

Iwamoto, E.T., 1989. Antinociception after nicotine administration into the mesopontine tegmentum of rats: evidence for muscarinic actions. J. Pharmacol. Exp. Ther. 251, 412–421.

Iwamato, E.T., 1991. Characterization of the antinociception induced by nicotine in the pedunculus tegmental nucleus and the nucleus raphe magnus. J. Pharmacol. Exp. Ther. 257, 120–133.

Iwamoto, E.T., Marion, L., 1993. Adrenergic, serotonergic and cholinergic components of nicotinic antinociception in rats. J. Pharmacol. Exp. Ther. 265, 777–789.

Khan, I., Yaksh, T., Taylor, P., 1994. Ligand specificity of nicotinic acetylcholine receptors in rat spinal cord: studies with nicotine and cytisine. J. Pharmacol. Exp. Ther. 270, 159–166.

Khan, I.M., Yaksh, T.L., Taylor, P., 1997. Epibatidine binding sites and activity in the spinal cord. Brain Res. 753, 269–282.

MacNicol, M., Schulman, H., 1992. Multiple Ca^{2+} signaling pathways converge on CaM kinase in PC12 cells. FEBS Lett. 304, 237–240.

Mattila, M.J., Ahtee, L., Saarnivaara, L., 1968. The analgesic and sedative effects of nicotine in white mice, rabbits and golden hamsters. Ann. Med. Exp. Fenn. 46, 78–84.

McGovern, S., Maguire, M.E., Gurd, R.S., Mahler, H.R., Moore, W.J., 1973. Separation of adrenergic and cholinergic synaptosomes from immature rat brain. FEBS Lett. 31, 193–198.

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

Nakanishi, S., Yamada, K., Iwahashi, K., Kuroda, K., Kase, H., 1990. KT5926, a potent and selective inhibitor of myosin light chain kinase. Mol. Pharmacol. 37, 482–488.

Ochoa, E.L., O'Shea, S.M., 1994. Concomitant protein phosphorylation and endogenous acetylcholine release induced by nicotine: dependency on neuronal nicotinic receptors and desensitization. Cell. Mol. Neurobiol. 14, 315–340.

Pavlovic-Surjancev, B., Cahill, A.L., Perlman, R.L., 1992. Nicotinic agonists, phorbol esters, and growth factors activate 2 extracellular signal-regulated kinases, ERK1 and ERK2, in bovine chromffin cells. J. Neurochem. 59, 2134–2140.

Phan, D.V., Doda, M., Bite, A., György, L., 1973. Antinociceptive activity of nicotine. Acta Physiol. Acad. Sci. Hung. 44, 85–93.

Rapier, C., Lunt, G.G., Wonnacott, S., 1988. Stereoselective nicotine-induced release of dopamine from striatal synaptosomes: concentration dependence and repetitive stimulation. J. Neurochem. 50, 1123–1130.

Rogers, D., Iwamoto, E., 1993. Multiple spinal mediators in parenteral nicotine-induced antinociception. J. Pharmacol. Exp. Ther. 267, 341–349.

Rowell, P.P., Winkler, D.L., 1984. Nicotinic stimulation of [^{3}H]acetylcholine release from mouse cerebral cortical synaptosomes. J. Neurochem. 43, 1593–1598.

Scott Bitner, R.L.N.A., Curzon, P., Arneric, S.P., Bannon, A.W., Decker, M.W., 1998. Role of the nucleus raphe magnus in antinociception

produced by ABT-594: immediate early gene responses possibly linked to neuronal nicotinic acetylcholine receptors on serotonergic neurons. J. Neurosci. 18, 5426–5432.

Suh, H.W., Song, D.K., Choi, S.R., Huh, S.O., Kim, Y.H., 1997. Differential effects of w-conotoxin GVIA, nimodipine, calmidazolium and KN-62 injected intrathecally on the antinociception induced by β-endorphin, morphine and enkephalin administered intracerebroventricularly in the mouse. J. Pharmacol. Exp. Ther. 282, 961–966.

Sumi, M., Kiuchi, K., Ishikawa, T., Ishii, A., Hagiwara, M., Nagatsu, T., Hidaka, H., 1991. The newly synthesized selective Ca^{2+}/calmodulin dependent protein kinase II inhibitor KN-93 reduces dopamine contents in PC12 cells. Biochem. Biophys. Res. Commun. 181, 968–975.

Tallarida, R.J., Murray, R.B., 1987. Manual of Pharmacological Calculations with Computer Programs. Springer-Verlag, New York.

Tohda, M.J.K., Hidaka, H., Nomura, Y., 1991. Inhibitory effects of KN-62, a specific inhibitor of Ca/calmodulin-dependent protein kinase II, on serotonin-evoked Cl^- current and $^{36}Cl^-$ efflux in Xenopus oocytes. Neurosci. Lett. 129, 47–50.

Tokumitsu, H., Chijiwa, T., Hagiwara, M., Mizutani, A., Terasawa, M., Hidaka, H., 1990. KN-62, 1-[*N*,*O*-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine, a specific inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II. J. Biol. Chem. 265, 4315–4320.

Tuominen, R.K., McMillian, M.K., Ye, H., Stachowiak, M.K., Hudson, P.M., Hong, J.S., 1992. Long-term activation of protein kinase C by nicotine in bovine adrenal chromaffin cells. J. Neurochem. 58, 1652–1658.

Vijayaraghavan, S., Pugh, P.C., Zhang, Z.-W., Rathouz, M.M., Berg, D.K., 1992. Nicotinic receptors that bind α-bungarotoxin on neurons raise intracellular free Ca^{2+}. Neuron 8, 353–362.

Vijayaraghavan, S., Huang, B., Blumenthal, E.M., Berg, D.K., 1995. Arachidonic acid as a possible negative feedback inhibitor of nicotinic acetylcholine receptors on neurons. J. Neurosci. 15, 3679–3687.

Wada, E., Wada, K., Boulter, J., Deneris, E., Heinemann, S., Patrick, J., Swanson, L., 1989. Distribution of alpha2, alpha3, alpha4, and beta2 neuronal nicotinic receptor subunit mRNAs in the central nervous system: a hybridization histochemical study in the rat. J. Comp. Neurol. 284, 314–335.