Nicotinic Regulation of Calcium/Calmodulin-Dependent Protein Kinase II Activation in the Spinal Cord

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ABSTRACT
Recent studies have implicated the involvement of Ca\(^{2+}\)-dependent mechanisms, in particular, calcium/calmodulin-protein kinase II in nicotine-induced antinociception during 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 \(\beta_2\) nicotinic receptor subunit plays a central role in nicotine-induced spinal antinociception via calcium/calmodulin-dependent calmodulin protein kinase II activation. The antinociceptive effects of i.t. nicotine in the tail-flick test did not significantly differ in wild-type and \(\alpha_2\) knockout (KO) animals but were lost in \(\beta_2\) knockout mice. When calcium/calmodulin-dependent calmodulin protein kinase II activity in the lumbar spinal cord after acute i.t. administration of nicotine was investigated in wild-type and \(\beta_2\) and \(\alpha_2\) knockout mice, the increase in calcium/calmodulin-dependent calmodulin protein kinase II activity was not significant reduced in \(\alpha_2\) KO mice but was eliminated in the \(\beta_2\) KO mice. In addition, L-type calcium channel blockers nimodipine and verapamil but not the \(N\)-methyl-D-aspartate antagonist MK-801 (dizocilpine maleate) blocked the increase in the kinase activity induced by nicotine. Taken together, these results are consistent with the hypothesis that increases in extracellular 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 \(\beta_2\)-containing acetylcholine nicotinic receptors in the spinal cord can activate calcium/calmodulin-dependent calmodulin protein kinase II and produce nicotinic analgesia, which may require L-type calcium voltage and gated channels but not the intervention of glutamatergic transmission.

Activation of cholinergic pathways by nicotine elicits antinociceptive effects in a variety of acute and chronic pain models (Mattila et al., 1968; Aceto et al., 1986). 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 (Mattila et al., 1968; Aceto et al., 1986; Iwamoto, 1991; Iwamoto and Marion, 1993; Khan et al., 1997; Damaj et al., 1998; Scott Bitner et al., 1998). Furthermore, nicotinic agonists have well documented antinociceptive properties when administered i.t. in mice (Damaj et al., 1998). Studies with knockout mice deficient in the \(\alpha_4\) and \(\beta_2\) nicotinic acetylcholine receptor subunits suggest an important role of \(\alpha_2\beta_2\) 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 \(\beta_2\) and \(\alpha_2\) 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, 2000). In addition, calcium/calmodulin-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/calmodulin-dependent calmodulin protein kinase II.

The present study was undertaken to examine and char-

ABBREVIATIONS: nAChR, acetylcholine nicotinic receptor; NMDA, \(N\)-methyl-D-aspartate; MK-801, dizocilpine maleate; \(\alpha\)-BGTX, \(\alpha\)-bungarotoxin; MLA, methyllycaconitine; %MPE, % maximal possible effect; LCC, L-type calcium channel; CREB, cAMP response element-binding protein.

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acterize the effect of acute nicotine exposure on the activity of calcium/calmodulin-dependent calmodulin protein kinase II in the spinal cord and investigate whether the regulation of calcium/calmodulin-dependent calmodulin protein kinase II by nicotine occurs directly through nAChRs or indirectly through NMDA receptors or L-type calcium channels. We first investigated the effects of i.t. injection of acute nicotine on calcium/calmodulin-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/calmodulin-dependent calmodulin protein kinase II activity after nicotine administration. Investigation of the role of major nicotinic subtypes (β2 and α7 nAChRs subtypes) in nicotine-induced changes in calcium/calmodulin-dependent calmodulin protein kinase II followed using two approaches for this characterization: classic pharmacological methods (use of nicotinic antagonists) and genetically modified mice (β2 and α7 knockout mice). After characterizing the changes in calcium/calmodulin-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/calmodulin-dependent calmodulin protein kinase II activation.

Materials and Methods

**Animals.** Mice lacking the α7 nicotinic receptor (C57BL/6 background) and wild-type littermates were purchased from The Jackson Laboratories (Bar Harbor, ME; B6.129S7-charnax7tm1bay, no. 003232). Breeding pairs of mice lacking the β2 nicotinic receptor (C57BL/6 background) and wild-type littermates were shipped from Institut Pasteur (Paris, France). Homozygous α7 and β2 mutant and wild-type controls were obtained from crossing heterozygote mice. Male mice approximately 8 to 12 weeks old (together with age- and sex-matched littermates wild-type controls) were used for the kinase experiments. Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care-approved facility in groups of three and had free access to food and water. Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care-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-BTX), methyllycaconitine (MLA), and dihydro-β-erythroidine were purchased from RBI (Natick, MA). Nimodipine was a gift from Miles, and supernatant was retained (membrane fraction). Standard phosphorylation reaction solutions contain 15 μg of extract protein, 100 μM calcium/calmodulin-dependent calmodulin protein kinase II specific substrate peptide (Autocamtide-2), 0.25 μM protein kinase inhibitors (0.25 μM each of protein kinase A and protein kinase C inhibitor peptides), 75 mM magnesium acetate, 500 μM ATP, 20 mM HEPES, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM CaCl2, and 5 μg of 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 (five times) followed by a brief acetone rinse before analysis by scintillation counting. Calcium/calmodulin-dependent calmodulin protein kinase II activity was expressed in picomoles phosphate per minute per microgram and determined using the following calculations: [(count-control)/(10 × corrected factor)] × (specific radioactivity) × time (10 min).
Statistical Analysis. Statistical analysis of all analgesic studies was performed using either Student’s t test or analysis of variance with Tukey’s test post hoc test when appropriate. All differences were considered significant at $p < 0.05$.

**Results**

Calcium/Calmodulin-Dependent Calmodulin Protein Kinase II Activity in Lumbar Spinal Cord Tissues after Acute i.t. Injection of Nicotine in Mice: Time Course and Dose-Response Effects. The activity of calcium/calmodulin-dependent calmodulin protein kinase II in the lumbar spinal cord after i.t. 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 picomoles of $^{32}$P incorporated into calmodulin protein kinase II substrate peptide per minute per milligram of protein in the presence or absence of calcium) in the membrane was measured. The onset of action for nicotine (20 $\mu$g/animal) on the calcium-dependent activity of calmodulin protein kinase II in the lumbar spinal cord was rapid, with maximal activation occurring between 0 and 5 min (Fig. 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 (Fig. 1B). A similar time course was seen at lower doses of nicotine (data not shown).

A dose-response relationship was then established for nicotine (20 $\mu$g/animal) on the calcium-dependent activity of calmodulin protein kinase II in the lumbar spinal cord was rapid, with maximal activation occurring between 0 and 5 min (Fig. 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 (Fig. 1B). A similar time course was seen at lower doses of nicotine (data not shown).

Nicotine-Induced Antinociception in $\beta_2$ and $\alpha_7$ Knockout Mice after i.t. Injection. The antinociceptive effects of i.t. nicotine in the tail-flick test are shown in Fig. 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 $\beta_2$ and $\alpha_7$ nAChR subunit is not essential in the perception of acute thermal nociception (data not shown). Nicotine given at a dose of 20 $\mu$g/mouse showed a 87 ± 7%MPE in wild-type and 90 ± 5%MPE in $\alpha_7^{-/-}$ mice. In contrast, $\beta_2^{-/-}$ mice did not exhibit a significant antinociceptive response to nicotine (Fig. 3).

Calcium/Calmodulin-Dependent Calmodulin Protein Kinase II Activity in Lumbar Spinal Cord Tissues in $\beta_2$ and $\alpha_7$ Knockout Mice and after i.t. Injection of Nicotine. The activity of calcium/calmodulin-dependent calmodulin protein kinase II in the spinal cord after acute i.t. administration of nicotine in mice was investigated in wild-type and $\beta_2$ and $\alpha_7$ knockout mice. Animals received either saline or nicotine (20 $\mu$g/mouse i.t.) and were sacrificed 15 min after injection. Spinal cord tissues were dissected, and the calcium-dependent and -independent activity of calcium/calmodulin-dependent calmodulin protein kinase II in the membrane was measured. As shown in Fig. 4, A and B, a...
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 ± S.E. of six to 12 mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dependent Activity</th>
<th>Independent Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle/vehicle</td>
<td>4.7 ± 0.07</td>
<td>0.6 ± 0.02</td>
</tr>
<tr>
<td>Vehicle/nicotine (20 μg)</td>
<td>11.9 ± 0.3*</td>
<td>3.37 ± 0.09*</td>
</tr>
<tr>
<td>Mecamylamine (20 μg)/nicotine (20 μg)</td>
<td>5.5 ± 0.2</td>
<td>0.7 ± 0.05</td>
</tr>
<tr>
<td>Dihydro-β-erythroidine (20 μg)/nicotine (20 μg)</td>
<td>5.7 ± 0.3</td>
<td>0.5 ± 0.05</td>
</tr>
<tr>
<td>α-BGTX (2 μg)/nicotine (20 μg)</td>
<td>10.7 ± 0.5*</td>
<td>3.28 ± 0.02*</td>
</tr>
<tr>
<td>MLA (7.5 μg)/nicotine (20 μg)</td>
<td>11.5 ± 0.4*</td>
<td>3.31 ± 0.05*</td>
</tr>
</tbody>
</table>

* p < 0.05 from vehicle/vehicle.

Discussion

We previously reported that activation of neuronal nAChRs by systemic nicotine administration leads to an influx of calcium, which activates calcium/calmodulin-dependent calmodulin protein kinase II in the spinal cord (Damaj, 2000). In addition, selective calcium/calmodulin-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/calmodulin-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/calmodulin-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 Ca2+-dependent compared with Ca2+-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/calmodulin-dependent calmodulin protein kinase II activity was blocked by mecamylamine, a nonselective nicotinic antagonist, suggesting the involvement of spinal nAChRs in this effect. It is interesting to note that the increase in calcium/calmodulin-dependent calmodulin protein kinase II activity in the spinal cord was sustained until at least 60 to 120 min after a single injection of nicotine (Fig. 1). This sustained activation could potentially have important pharmacological and molecular consequences since calcium/calmodulin-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 β2 subunits in nicotine-induced antinociception, our data suggest a calcium-dependent kinase molecular mechanism mediating the activation of the β2-containing receptor subtype. We now propose that calcium/calmodulin-dependent calmodulin protein kinase II activation in the spinal cord mediates nicotine’s antinociceptive effects through the activation of β2− but not α7-containing nAChRs. Although calcium/calmodulin-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 α2, α3, α4, and β2 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 β4 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 β3-containing nAChRs, α4α6β2* 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 α7-containing nAChRs. On the other hand, it has been suggested that α7 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/calmodulin-dependent calmodulin protein kinase II activation as reported in our studies. Taken together, these results are consistent with the involvement of spinal calcium/calmodulin-dependent calmodulin protein kinase II in nicotine-induced antinociception via β2− containing nAChRs.

It is known that transduction changes can be set in motion by calcium influx through activation of ligand-gated ion channels such as 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 in ciliary ganglion neurons (Chang and Berg, 2001). Instead, calcium in-
flux through nAChRs themselves, together with calcium-induced calcium release from internal stores, sustained CREB phosphorylation levels in the ganglionic neurons. In contrast, stimulation of α2 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 β2-containing nAChRs in the spinal cord can activate calcium/calmodulin-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 β2-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 β2-containing nAChRs in the spinal cord is not sufficient to trigger enough calcium/calmodulin-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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References


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