Cellular/Molecular

Neuroprotection by Nicotine in Mouse Primary Cortical Cultures Involves Activation of Calcineurin and L-Type Calcium Channel Inactivation

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Regulation of intracellular calcium influences neuronal excitability, synaptic plasticity, gene expression, and neurotoxicity. In this study, we investigated the role of calcium in mechanisms underlying nicotine-mediated neuroprotection from glutamate excitotoxicity. Neuroprotection by nicotine in primary cortical cultures was not seen in knock-out mice lacking the β2 subunit of the nicotinic acetylcholine receptor (nAChR). Neuroprotection was partially blocked in wild-type cultures by α-bungarotoxin, an antagonist of the α7 nAChR subtype, suggesting a potential cooperative role for these subtypes. Pretreatment with nicotine decreased glutamate-mediated calcium influx in primary cortical cultures by 41%, an effect that was absent in cultures from knock-out mice lacking the β2 subunit of the nAChR. This effect was dependent on calcium entry through L-type channels during nicotine pretreatment in wild-type cultures. The ability of nicotine to decrease glutamate-mediated calcium influx was occluded by cotreatment with nifedipine during glutamate application, suggesting that nicotine pretreatment decreased subsequent activity of L-type calcium channels. Treatment with the calcineurin antagonists FK506 and cyclosporine during pretreatment eliminated both nicotine-mediated neuroprotection and the effects of nicotine on L-type channels. We conclude that neuroprotective effects of nicotine in cortical neurons involve both β2- and α7-containing nAChRs, activation of calcineurin, and decreased intracellular calcium via L-type channels.

Key words: **calcium imaging; cell death; glutamate; FK506; nifedipine; nicotinic acetylcholine receptors**

Introduction

Nicotine, the primary active agent in tobacco smoke, is protective against neurotoxicity initiated by excitatory amino acids *in vivo* (Borlongan et al., 1995) and *in vitro* (Akaike et al., 1994; Marin et al., 1994; Dajas-Bailador et al., 2000). In addition, smoking is negatively correlated with development of the neurodegenerative disorders Parkinson's and Alzheimer's disease (Fratiglioni and Wang, 2000). The primary targets for nicotine in the CNS are the neuronal nicotinic acetylcholine receptors (nAChRs), a diverse family of ligand-gated ion channels that are associated with gene transcription, neurotransmitter release, addiction, and neuroprotection (Jones et al., 1999; Picciotto et al., 2001; Rezvani and Levin, 2001).

The identity of the nAChR subtype(s) responsible for the protective effects of nicotine is controversial. In hippocampal neurons, nicotine-induced neuroprotection is blocked by α-bungarotoxin (α-BTX), a specific antagonist of α7 subunit-containing nAChRs (Dajas-Bailador et al., 2000). However, in cortical neurons, $\alpha4/\beta2^{\star}$ nAChRs appear to be involved in nicotine-mediated protection against glutamate excitotoxicity (Akaike et al., 1994), although α7-type nAChRs might also contribute to these effects (Kaneko et al., 1997; Kihara et al., 1998). Furthermore, β2 nAChR subunit knock-out mice ($\beta2-/-$) that lack high-affinity nicotine binding sites (Picciotto et al., 1995) show increased cortical atrophy during aging (Zoli et al., 1999) and increased cortical susceptibility to ibotenic acid lesion (Laudenbach et al., 2002), suggesting that endogenous neuroprotection is reduced as a result of loss of β2 subunit-containing nAChRs. The signal transduction mechanism(s) underlying nAChR-mediated neuroprotection in all cell types remains unclear.

One potential mechanism for this effect is nicotinic modulation of glutamate-mediated increases in intracellular calcium (Ca^{2+}) levels. Maintenance of intracellular Ca^{2+} homeostasis is crucial for cell survival and synaptic plasticity. The site, magnitude, and kinetics of Ca^{2+} changes determine the biological consequences of Ca^{2+} signaling in the neuron (Shieh et al., 1998; Tao et al., 1998; Shoop et al., 2002). Because nAChRs can increase intracellular Ca^{2+} directly (McGehee and Role, 1996; Girod et al., 2003) or indirectly through activation of voltage-gated Ca^{2+} channels or release of Ca^{2+} from intracellular stores (Tsuneki et al., 2000; Chang and Berg, 2001), we hypothesized that one mechanism underlying nicotine-induced protection against excitotoxicity could be modulation of glutamate-mediated Ca^{2+} entry. We hypothesized further that one mechanism that could be affected by low levels of Ca^{2+} entry because of nAChR activation

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could be activation of the high-affinity Ca^{2+}-dependent phosphatase calcineurin, which could, in turn, modulate Ca^{2+}-dependent signaling pathways that mediate nicotine neuroprotection. In this paper, we demonstrate that nicotine pretreatment alters Ca^{2+} influx in cortical neurons, and that β2 subunit-containing nAChRs are essential for this effect. The mechanisms elucidated have implications for neuroprotection but also for the effects of nicotine on synaptic plasticity, learning, and memory.

Materials and Methods

All animal procedures were conducted in strict accordance with National Institutes of Health Care and Use of Laboratory Animals Guidelines and were approved by the Yale Animal Care and Use Committee. Reagents were purchased from Sigma (St. Louis, MO) unless stated otherwise.

Cell culture. Mixed cortical cultures were made from wild-type or β2−/− fetal mice [embryonic day 16 (E16)–E18]. Neurons were dissociated by incubating minced cortices in PDD [0.01% papain (Worthington, Freehold, NJ), 0.1% dispase (Roche Products, Hertforshire, UK), and 0.01% DNase (Sigma)] in HBS [composed of the following (in mM): 10 HEPES, 10 glucose, 140 NaCl, and 5 KCl, pH 7.4] for 15 min at 37°C. Cells were resuspended in Neurobasal media supplemented with 5% FBS, B27 supplement (Invitrogen, San Diego, CA), sodium pyruvate, glutamine, and 2 mM HEPES. Dissociated cortical neurons were cultured on poly-L-lysine–laminin-coated 15 mm glass coverslips at 8×10^4 to 1.0×10^5 cells/ml. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and fed once per week. Mixed cortical cultures were used for Ca^{2+} imaging and cell toxicity experiments between days 11 and 13 after plating. Glial cells were present in the cultures but were <10% of the overall population. The few neurons that grew on top of glial cells were excluded from the imaging studies because accurate changes in fluorescence would not be obtained. β2−/− cortical cultures were obtained from mice that were back-crossed >14 generations onto the C57BL/6J background.

Ca^{2+} imaging. After nicotine or HBS pretreatment, cells were loaded with 2 μM fluo-3 (Molecular Probes, Eugene, OR) for 10 min at room temperature in HBS 0/0 and washed twice with HBS 2/1 (HBS with 2 mM $CaCl_2$ and 1 mM $MgCl_2$). The dye was not saturated under the conditions used here because raising the extracellular Ca^{2+} level from 2 to 3 mM resulted in a significant increase in the calcium signal (data not shown). Although this experiment shows that fluo-3 was not saturated with calcium under conditions of glutamate stimulation, fluo-3 emission may be in a sublinear range under these conditions; thus our results must be considered only semiquantitative. In the time course study, one group of cultures was treated with nicotine for 5–10 min after loading. Inhibitors [in μM : 100 APV, 50 nifedipine, 200 cadmium, 50 2,3-dihydroxy-6-nitro-7-sulfonyl-benzo[*f*]quinoxaline (NBQX), or 50 dantrolene] were added to the loading solution after pretreatment with nicotine or HBS for 1 hr and were present throughout the experiment. In experiments blocking effects of nicotine during the 1 hr preincubation period, 100 nM FK506 (Research Biochemicals, Natick, MA) was added 10 min before the 1 hr incubation and washed out before glutamate treatment. α-BTX was added 20 min before addition of nicotine or HBS. To remove Ca^{2+} during nicotine pretreatment, cells were incubated in HBS (0 $CaCl_2$, 3 mM MgCl) with or without 10 μM nicotine for 1 hr, loaded, and assayed for glutamate response in HBS (2.1). Solutions were exchanged using heated bath perfusion (37°C) with a flow rate of 2 ml/min in a volume of ~75 μl. All images were obtained using a 20× objective on a TE300 inverted microscope (Nikon, Tokyo, Japan) equipped with a cooled CCD camera (orca; Hamamatsu, Bridgewater, NJ). Acquired images were adjusted for the level of background intensity, digitally colored, and analyzed using IPLab software (Scanalytics, Fairfax, VA). Images of the fluo-3 loaded cells were obtained over a period of 1 min at 2 sec intervals. Each experiment was repeated a minimum of three times with comparable results.

Quantitation. Change in fluorescence was quantitated as described previously (Vijayaraghavan et al., 1992). Ca^{2+} influx was determined as the change from basal levels after glutamate stimulation [(stimulated level − basal level)/basal level of fluorescence]. Stacked images from 0 to 60 sec were collected for each experiment. A region including the entire soma of an individual neuron was selected, and the change in pixel density was averaged over this area for every frame. A change in basal fluorescence was seen in studies using 200 μM cadmium (average level, 45.0 ± 8.9), as has been reported in studies from other laboratories (Sharma and Vijayaraghavan, 2001). No change in basal fluorescence was seen for any other treatments (wild-type cultures pretreated with HBS or nicotine, average basal level of fluorescence 21.79 ± 11.2 and 22.38 ± 12.5, respectively; β2−/− cultures pretreated with HBS or nicotine, average basal level of fluorescence 26.56 ± 7.9 and 26.33 ± 8.6, respectively). On average, 9–12 cells were examined per condition for each culture. Change in fluorescence was determined for each cell and averaged across cells in each culture. These studies were conducted in at least three different cultures, and an overall average from each culture was plotted for each graph. *n* represents the number of cultures used for each experiment.

Assessment of neurotoxicity. For cell-death assays, nicotine was added to the media to a final concentration of 10 μM. The same volume of HBS 2.1 was added to control cultures, and cells were incubated for 1 hr at 37°C in a humidified atmosphere of 5% CO_2. In experiments using FK506, the drug was added 10 min before nicotine or HBS preincubation. Media containing 100 μM glutamate was added after preincubation, and cultures were assessed for cell death 18–20 hr after the initiation of glutamate treatment. To minimize cell death attributable to perturbation of the cultures, media were not changed once glutamate was added. In control cells, the same volume of HBS 2.1 was added instead of glutamate. Cells were treated with 0.16 mM calcein–AM–0.36 mM ethidium homodimer in HBS 2.1 for 40 min at room temperature. Live cells were stained green throughout the soma, and projections and dead cells had red staining only in the nucleus or highly punctate green staining. Neurotoxicity was calculated as percentage survival [live cells/(live + dead cells)], and these values were expressed relative to the percentage survival of control cells. Four to six coverslips were examined per treatment, and a total of 10–15 randomly selected fields (10× objective) were captured using IPLab software. At least 200 cells were counted per condition, and each experiment was repeated in at least four different cultures.

Statistical analysis. Fluorescence measurements are reported as change in fluorescence over baseline. Data points were normalized and expressed as mean ± SEM. All data were analyzed using repeated-measures ANOVA. The peak values for each curve were used for ANOVA (at least five time points were used). *Post hoc* analyses were conducted when appropriate using the least significant difference (LSD) test. $p < 0.05$ was considered statistically significant.

Results

Primary cortical cultures from wild-type or β2−/− mice were used to examine nicotine-mediated neuroprotection. A combination of calcein–AM and ethidium homodimer was used to visualize live and dead neurons after glutamate-induced excitotoxicity (Fig. 1*a*,*c*). The number of live cells and the percentage of dead cells were equivalent between wild-type and β2−/− cultures treated with glutamate alone. Nicotine pretreatment significantly increased viability from 49 to 74.5% after glutamate treatment in wild-type cultures (Fig. 1*b*) but resulted in no change in cell death in β2−/− cultures (Fig. 1*d*), suggesting that high-affinity nAChRs are necessary for the neuroprotective effects of nicotine in cortical cultures. The α7-selective antagonist α-BTX partially blocked nicotine-mediated neuroprotection in wild-type cultures but had no effect in β2−/− cultures, suggesting that cooperative effects of α7 and β2 subunit-containing nAChRs might mediate the protective effect of nicotine in wild-type neurons (Fig. 1*e*,*f*).

To determine the cellular mechanism(s) responsible for the protective effect of nicotine, the high-affinity Ca^{2+}-sensitive dye fluo-3 was used to measure glutamate-mediated Ca^{2+} influx in neurons. Similar experiments were performed using the low-affinity Ca^{2+}-sensitive dye BTC, but by using this dye, the changes in fluorescence observed during glutamate treatment

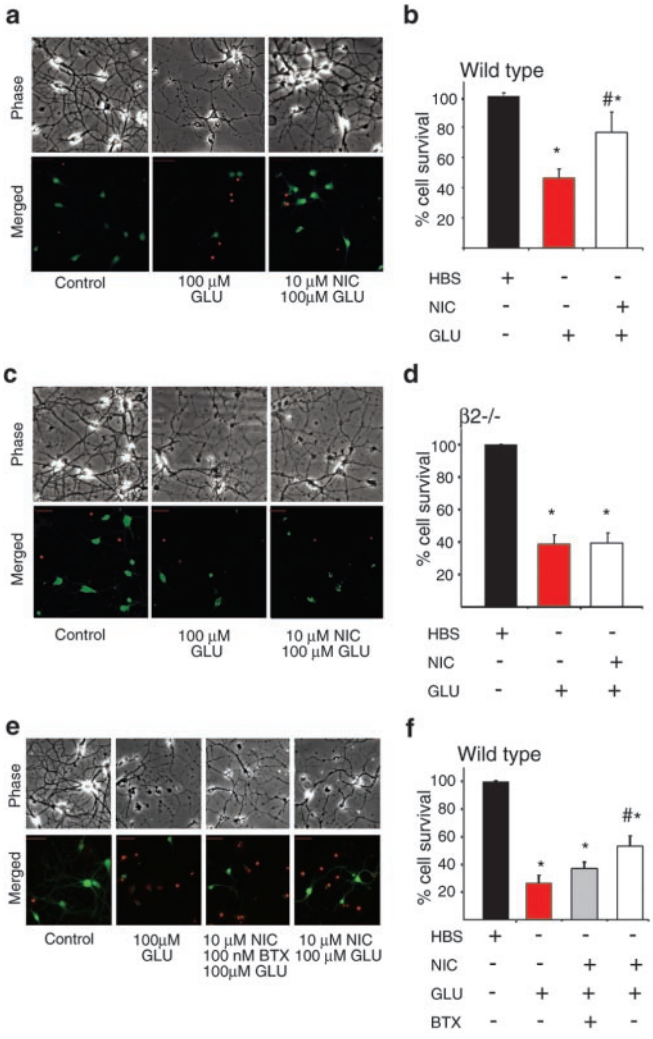

Figure 1. Nicotine-mediated neuroprotection against glutamate toxicity is abolished in primary cortical cultures from $\beta 2-/-$ mice. *a*, Phase pictures and merged fluorescence images of calcein–AM and ethidium-stained cells demonstrate a reduction in live cells (green) and an increase in dead cells (red) in glutamate-treated cultures. NIC, Nicotine; GLU, glutamate. *b*, Glutamate treatment reduced cell survival (49%), and nicotine protected neurons from glutamate-mediated cell death (74.5%; $n = 7$). *c*, Phase pictures and merged images from $\beta 2-/-$ cultures demonstrate that nicotine is not protective in the absence of high-affinity nAChRs. *d*, In cultures from $\beta 2-/-$ mice, glutamate significantly reduced cell survival (38.67%) even after pretreatment with 10 μM nicotine (39.45%) ($p < 0.005$; $n = 5$). *e*, Phase pictures and merged images from wild-type cultures show that coincubation of α-BTX with nicotine resulted in an intermediate cell death between the glutamate and nicotine. *f*, In wild-type cultures, coincubation of BTX and nicotine resulted in an intermediate 36.94% survival compared with glutamate (26.50%) and 10 μM nicotine (52.22%; $n = 5$). Asterisks indicate that cell survival after both glutamate treatment and nicotine pretreatment is statistically different from control ($p < 0.05$). Number signs indicate that cell survival in nicotine and glutamate-treated cultures is statistically different from survival of cultures treated with glutamate alone ($p < 0.05$; LSD *post hoc* test). Data are shown as mean ± SEM.

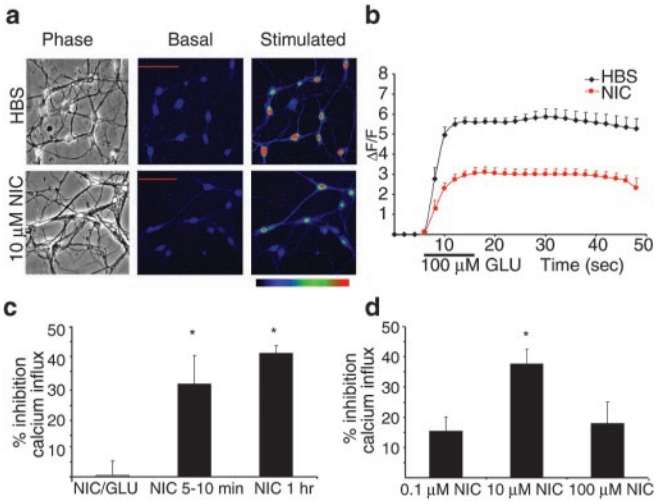

Figure 2. Nicotine pretreatment decreases glutamate-mediated Ca^{2+} entry into cortical primary cultured neurons. *a*, Examples of [Ca^{2+}] measurements in cortical neurons using fluo-3. NIC, Nicotine. Shown are neurons pretreated for 1 hr with either HBS (top) or 10 μM nicotine (bottom), at rest (basal), or during application of 100 μM glutamate (stimulated). Phase contrast images of the respective cells are shown on the left. Scale bar, 50 μm. *b*, Quantitation of glutamate-mediated Ca^{2+} entry into neurons, with or without nicotine pretreatment ($n = 7$). GLU, Glutamate. *c*, Time course of nicotine-induced decrease in glutamate-mediated Ca^{2+} influx. A significant decrease in Ca^{2+} influx was observed after 5–10 min, but maximal reduction in Ca^{2+} influx was observed after 1 hr of nicotine treatment. *d*, Dose–response relationship for the ability of nicotine pretreatment to reduce glutamate-mediated Ca^{2+} entry ($n = 4$). Maximal inhibition (41%) occurred with 10 μM nicotine. Percentage inhibition was calculated as [(control − treatment)/control] value of Ca^{2+} influx. *$p < 0.05$. Data are shown as mean ± SEM.

were too small to allow reliable detection of changes in Ca^{2+} levels (data not shown). Cells were phase-bright and well differentiated as shown by extensive dendritic outgrowth (Fig. 2*a*). Cultures were preincubated for 1 hr with either control media (HBS) or 10 μM nicotine and then stimulated with 100 μM glutamate for 10 sec. Glutamate stimulation induced a substantial increase in intracellular Ca^{2+} levels that decreased by 41% after pretreatment with nicotine ($p < 0.00001$) (Fig. 2*b*). Coincubation with nicotine and glutamate for 10 sec was ineffective in altering Ca^{2+} influx; however, nicotine application 5–10 min before glutamate treatment caused a significant and sustained decrease in glutamate-mediated Ca^{2+} influx (Fig. 2*c*). The maximal effect was observed with 1 hr of nicotine pretreatment. In addition, dose–response experiments established that a maximal effect on glutamate-mediated Ca^{2+} entry was achieved at 10 μM nicotine (Fig. 2*d*), so all additional experiments were performed at this dose.

The ability of nicotine to increase intracellular Ca^{2+} was essential for its effects on glutamate-mediated Ca^{2+} entry. Cells were incubated with either HBS containing no calcium (HBS 0.3) or 10 μM nicotine in HBS 0.3 for 1 hr (3 mM MgCl was used to counteract the effect of lowering extracellular Ca^{2+} on resting membrane resistance and excitability) and were then assayed for $\Delta F/F$. Removal of Ca^{2+} during nicotine exposure abolished the ability of nicotine to modulate glutamate-mediated Ca^{2+} influx (Fig. 3*a*). Fluorescence appears to decrease more rapidly in cells pretreated in calcium-free medium, which could represent increased susceptibility to cell death in cultures after this destabilizing treatment, but this difference was not significant. The ability of nicotine to increase intracellular calcium was also assessed. A 10 μM concentration of nicotine did not produce increases in Ca^{2+} that could be resolved under the conditions used, although a higher concentration of nicotine (1 mM) did produce a small increase in Ca^{2+} when a higher extracellular calcium concentration was used (data not shown).

Several previous studies have suggested that voltage-gated Ca^{2+} channels are critical for the effects of nAChR activation in many cell types (Damaj et al., 1993; Chang and Berg, 2001; Shoop et al., 2001). We therefore hypothesized that voltage-gated Ca^{2+} channel activity occurring during nicotine pretreatment was critical for the subsequent decrease in glutamate-mediated

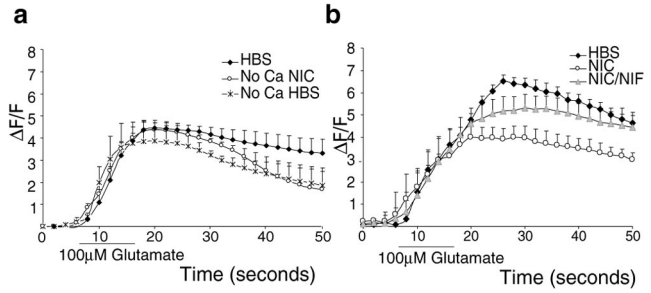

Figure 3. The effect of nicotine on glutamate-mediated Ca^{2+} entry is dependent on the presence of Ca^{2+} during nicotine pretreatment. *a*, The effect of nicotine on glutamate-mediated Ca^{2+} influx was abolished when extracellular Ca^{2+} was removed during nicotine pretreatment. Cells were treated in HBS 0.3 (0 mM $CaCl_2$, 3 mM $MgCl_2$) with or without 10 μM nicotine and then assayed for changes in Ca^{2+} in HBS 2.1. Control cells were treated with HBS 2.1 for 1 hr before assaying for glutamate response ($n = 3$). *b*, Coincubation with 50 μM nifedipine (NIC/NIF) reversed the ability of nicotine (10 μM) to alter glutamate-mediated Ca^{2+} entry ($n = 4$). Data are shown as mean ± SEM.

Ca^{2+} entry. L-type Ca^{2+} channels were blocked with 50 μM nifedipine during nicotine pretreatment, nifedipine was washed out, and cells were loaded with fluo-3 and treated with glutamate. Coincubation with nicotine and nifedipine significantly reduced the effect of nicotine on subsequent glutamate-mediated Ca^{2+} influx compared with nicotine pretreatment alone (Fig. 3*b*). We cannot rule out that 50 μM nifedipine may act as an nAChR channel blocker, but it is likely that the predominant effect during pretreatment is on Ca^{2+} channel blockade (Tsuneki et al., 2000). In addition, a small amount of nifedipine may still be present during glutamate treatment, but this concentration is likely to be below the K_i for L-type channel antagonism. Nifedipine is a reversible channel blocker and, thus, is not likely to result in a persistent blockade of L-type channels after washout. Together, these studies demonstrate the importance of Ca^{2+} entry during nicotine treatment for its subsequent effects on glutamate-mediated Ca^{2+} entry. In addition, these studies identify a role for nAChR and L-type Ca^{2+} channels during the nicotine preincubation period.

The ability of nicotine to decrease glutamate-mediated Ca^{2+} entry was absent in $\beta2-/-$ cultures, suggesting that high-affinity nAChRs are critical for this effect (Fig. 4*a*,*b*). The $\alpha7$-selective antagonist α-BTX (100 nM) was added to wild-type and $\beta2-/-$ cultures to examine the possible contribution of the $\alpha7$ nAChR. Whereas glutamate-mediated Ca^{2+} influx was reduced significantly by nicotine pretreatment, coincubation with nicotine and α-BTX resulted in an intermediate Ca^{2+} response to glutamate that was not significantly different from HBS-treated ($p > 0.1$) or nicotine-treated ($p > 0.3$) cultures (Fig. 4*c*,*e*). Coincubation of $\beta2-/-$ cultures with nicotine and α-BTX did not significantly alter Ca^{2+} response to glutamate compared with nicotine pretreatment alone ($p > 0.06$), although there was a trend toward a small decrease in Ca^{2+} levels compared with nicotine alone that could reflect a role for $\alpha7$ nAChRs in this effect (Fig. 4*d*,*f*).

Next, we determined whether ionotropic glutamate receptors, Ca^{2+} channels, or internal stores were sources for glutamate-mediated increases in intracellular Ca^{2+}. Cadmium (200 μM) increased basal fluorescence but abolished Ca^{2+} influx with glutamate stimulation (Fig. 5*a*). At the concentration used, cadmium is known to block L-, P-, and T-type Ca^{2+} channels and to inhibit NMDA receptors but not AMPA or kainate receptors (Mayer et al., 1989), suggesting that voltage-sensitive Ca^{2+} channels or NMDA receptors are dominant sources for the cytosolic

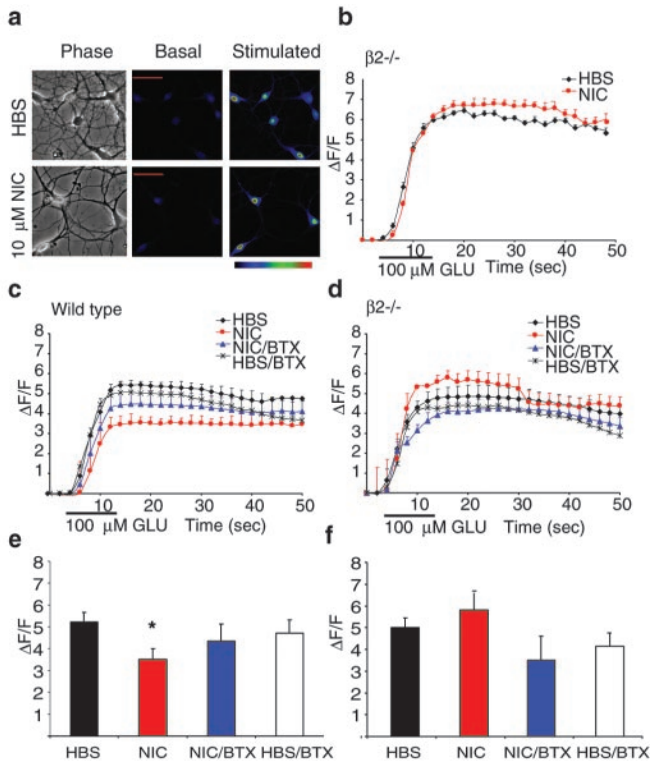

Figure 4. High-affinity nAChRs are involved in the effect of nicotine on glutamate-mediated Ca^{2+} entry. *a*, Examples of $\beta2-/-$ cortical neurons at rest or stimulated with 100 μM glutamate pretreated with either 10 μM nicotine (bottom) or HBS (top). Phase contrast images of respective fields are shown on the left. Scale bar, 50 μm. *b*, Quantitation of glutamate-mediated Ca^{2+} influx in $\beta2-/-$ cultures. Shown are averages from $n = 5$ cultures. *c*, Effect of the $\alpha7$ nAChR blocker α-BTX on wild-type cortical neurons. Cultures ($n = 4$) were pretreated with either α-BTX or nicotine or coincubated with nicotine and α-BTX. Pretreatment with 10 μM nicotine was the only treatment that significantly reduced Ca^{2+} influx. *d*, α-BTX does not significantly alter Ca^{2+} influx in cultures from $\beta2-/-$ mice. The small increase in Ca^{2+} influx observed in $\beta2-/-$ cultures treated with nicotine was not significant ($p > 0.06$; $n = 4$). *e*, *f*, Average $\Delta F/F$ for each treatment from 20 to 40 sec for wild-type and knock-out cultures. NIC, Nicotine; GLU, glutamate. *$p < 0.05$. Data are shown as mean ± SEM.

Ca^{2+} increase in response to glutamate. A 50 μM concentration of NBQX, a blocker of AMPA-type glutamate receptors, did not alter glutamate-dependent Ca^{2+} entry, suggesting that AMPA channels do not play a substantial role in glutamate-evoked Ca^{2+} entry in cortical cultures (Fig. 5*b*). In contrast, treatment with an NMDA receptor antagonist (100 μM APV) significantly decreased glutamate-mediated Ca^{2+} influx (~50%) (Fig. 4*c*). The specific L-type channel blocker nifedipine (50 μM) also resulted in a ~50% decrease in glutamate-mediated Ca^{2+} influx (Fig. 5*d*). Thus, both NMDA-type glutamate receptors and L-type Ca^{2+} channels contribute significantly to the Ca^{2+} influx after glutamate stimulation. Combined treatment with nifedipine and APV did not block all Ca^{2+} entry attributable to glutamate (Fig. 5*e*); however, a ryanodine receptor antagonist that blocks release of Ca^{2+} from intracellular stores (50 μM dantrolene) caused a decrease in glutamate-stimulated Ca^{2+} entry that was similar in magnitude to the residual Ca^{2+} after nifedipine and APV treatment (Fig. 5*f*). Together, these data suggest that glutamate treatment in cortical neurons results in activation of L-type Ca^{2+} channels and NMDA receptors as well as in release of Ca^{2+} from intracellular stores through ryanodine receptors. APV and dantrolene did not change the ability of nicotine to inhibit glutamate-induced Ca^{2+} influx, because each agent, when combined with nicotine pretreatment, significantly attenuated the response to

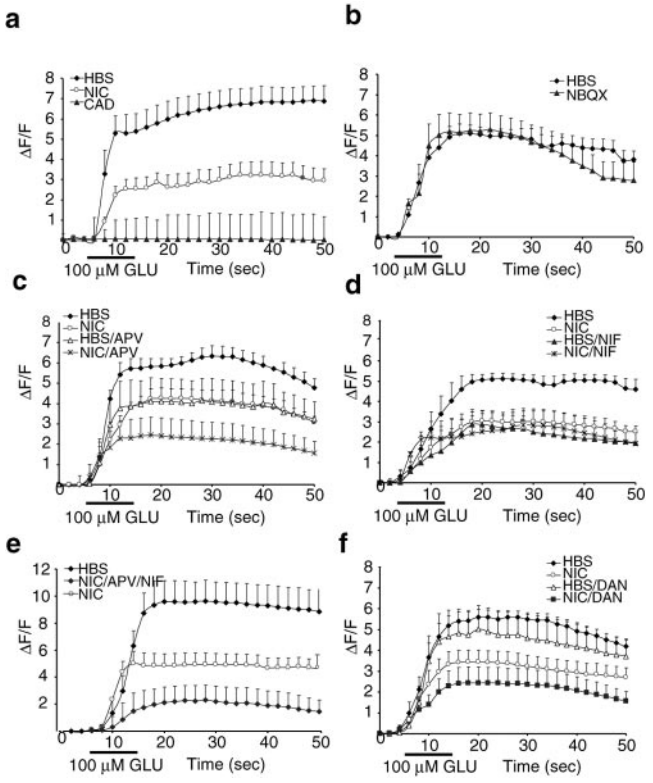

Figure 5. Nicotine pretreatment abolishes glutamate-induced Ca^{2+} entry through L-type Ca^{2+} channels. *a*, A 200 μM concentration of cadmium (CAD) abolished glutamate-mediated Ca^{2+} influx ($n = 3$). *b*, A 50 μM concentration of NBQX had no effect on Ca^{2+} influx, suggesting that AMPA currents do not contribute to glutamate-mediated Ca^{2+} entry in neurons. *c*, A 100 μM concentration of APV decreased glutamate-evoked Ca^{2+} influx by 50% (HBS/APV). Nicotine and APV inhibition of Ca^{2+} entry was additive (NIC/APV). NIC/APV was significantly different from HBS, NIC, and HBS/APV treatment ($n = 4$). *d*, A 50 μM concentration of nifedipine (HBS/NIF) significantly reduced glutamate-mediated Ca^{2+} influx. Nifedipine did not further reduce the glutamate-mediated Ca^{2+} entry after nicotine pretreatment (NIC/NIF) ($n = 4$). *e*, A residual glutamate-mediated Ca^{2+} influx was observed when both NMDA receptors and L-type channels were blocked, most likely because of internal stores ($n = 3$). *f*, A 50 μM concentration of dantrolene (HBS/DAN) decreased glutamate-mediated Ca^{2+} influx slightly but significantly ($n = 4$). The effects of nicotine and dantrolene (NIC/DAN) on glutamate-mediated Ca^{2+} entry were additive. $^*p < 0.05$ using LSD *post hoc* test. Data are shown as mean ± SEM.

glutamate compared with nicotine pretreatment alone (Fig. 5*c*,*e*). In contrast, nicotine occluded the inhibition of glutamate-evoked Ca^{2+} entry by nifedipine (Fig. 5*d*), suggesting that nicotine pretreatment decreases the activity of L-type Ca^{2+} channels in cortical neurons.

L-type channels can be inactivated by the Ca^{2+}-dependent phosphatase calcineurin (Hernandez-Lopez et al., 2000; Day et al., 2002). We therefore examined whether inhibition of calcineurin could abolish the nicotine-mediated decrease in Ca^{2+} influx. Preincubation with nicotine and FK506 (100 nM), a calcineurin inhibitor, completely abolished the subsequent decrease in glutamate-mediated Ca^{2+} influx (Fig. 6*a*). Coincubation with FK506 (100 nM) during the pretreatment period also blocked the protective effect of nicotine (Fig. 6*b*). In contrast, neurons pretreated with nicotine for 1 hr and then subsequently treated with FK506 and glutamate showed intact nicotine-mediated neuroprotection. Finally, treatment with FK506 alone, which continued through the period of glutamate application, was neuroprotective. Thus, calcineurin activation before glutamate treatment (as is achieved by nicotine pretreatment) is neuroprotective. In contrast, once glutamate is present, calcineurin blockade is neuroprotective. To verify the specificity of the involvement of calcineurin in the effects of nicotine, these experiments were repeated using a second calcineurin blocker, cyclosporine. Coincubation with 10 μM nicotine and 1 μM cyclosporine completely abolished the subsequent decrease in glutamate-mediated Ca^{2+} influx (Fig. 6*c*). Like FK506, pretreatment with cyclosporine (1 μM) for 1 hr before glutamate treatment was neuroprotective, but coincubation blocked the protective effect of nicotine (Fig. 6*d*). Thus, both cyclosporine and FK506 treatment abolished the decrease in glutamate-mediated Ca^{2+} entry attributable to nicotine and also reversed nicotine-mediated neuroprotection. This suggests that the neuroprotective effects of nicotine might be mediated through calcineurin-dependent inactivation of L-type Ca^{2+} channels.

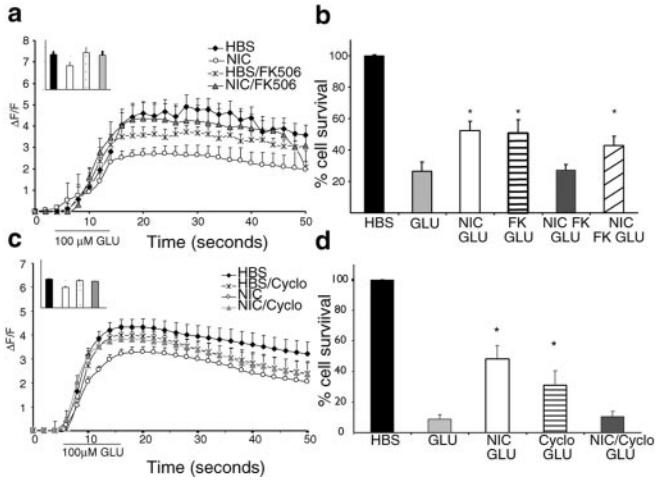

Figure 6. Blockade of calcineurin counteracts the effects of nicotine (NIC) on glutamate (GLU)-mediated Ca^{2+} entry and neuroprotection. *a*, Treatment with FK506 during nicotine preincubation (NIC/FK506) reversed the decrease in glutamate-mediated Ca^{2+} influx observed with nicotine pretreatment ($n = 5$). *b*, Nicotine (NIC–GLU) and FK506 (FK–GLU) treatment alone were significantly neuroprotective against glutamate toxicity in neurons (cell survival compared with control cultures was 52.2 and 50.6%, respectively) ($p < 0.05$). Cell survival after pretreatment with both nicotine and FK506 (NIC FK–GLU) was not statistically different from glutamate treatment alone. Treatment with nicotine and subsequently with FK506 plus glutamate showed a level of neuroprotection equivalent to nicotine treatment alone ($n = 5$). *c*, Treatment with cyclosporine (1 μM) during nicotine preincubation reversed the decrease in glutamate-mediated Ca^{2+} influx observed with nicotine pretreatment ($n = 6$). *d*, Coincubation with nicotine and cyclosporine (NIC/Cyclo) significantly attenuated nicotine-mediated neuroprotection. The peak values for each curve were used for ANOVA (12–22 sec). *Post hoc* analyses were conducted when appropriate using the LSD test. $^*p < 0.05$. Data are shown as mean ± SEM.

Discussion

The mechanisms underlying nicotine-mediated neuroprotection from glutamate excitotoxicity were investigated in this study. We demonstrate that nicotine pretreatment reduces glutamate-mediated Ca^{2+} influx into cortical neurons and identify a signaling pathway leading from nicotine treatment to decreased glutamate-mediated excitotoxicity (Fig. 7). Nicotine treatment activates nAChRs (including β2 subunit-containing receptors); influx of low levels of Ca^{2+} during this treatment might occur directly through nAChRs but also occurs indirectly through activation of voltage-gated Ca^{2+} channels. Ca^{2+} influx activates calcineurin, which has been shown to decrease L-type Ca^{2+} channel activity (Lukyanetz et al., 1998; Day et al., 2002). This L-type channel inactivation results in decreased Ca^{2+} entry into the neuron with glutamate stimulation, which might decrease excitotoxicity.

Several studies have identified biochemical responses to nico-

tine that might contribute to its neuroprotective effects, including increased levels of neuronal growth factors (Belluardo et al., 2000), decreased nitric oxide generation (Shimohama et al., 1996), decreased arachidonic acid release (Marin et al., 1997), activation of akt kinase (Kihara et al., 2001), and decreased caspase signaling (Meyer et al., 2002). However, a direct link between these pathways and nicotine-mediated neuroprotection has yet to be identified. The experiments described here show that high-affinity nicotinic receptors containing the β2 subunit are required for decreased glutamate-mediated Ca^{2+} entry. Both *in vivo* and *in vitro* studies have shown that $\alpha4/\beta2^*$ nAChRs contribute to the neuroprotective effects of nicotine. For example, mice lacking the α4 subunit of the nAChR do not show the neuroprotective effects of nicotine against methamphetamine-induced neurodegeneration in striatal neurons (Ryan et al., 2001). Similarly, $\alpha4/\beta2$-type nAChRs are required for nicotine-induced neuroprotection against neonatal ibotenic acid lesion of the cortex (Laudenbach et al., 2002). We propose that the mechanisms reported here could also explain nicotine-induced neuroprotection against methamphetamine or ibotenate neurotoxicity.

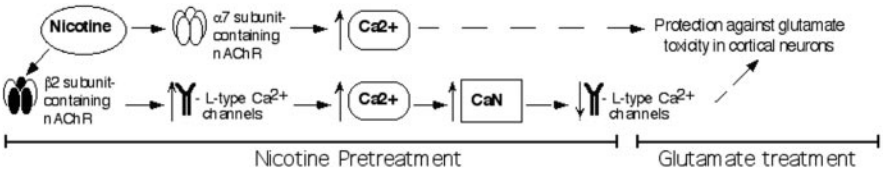

Figure 7. Schematic diagram of the mechanism underlying the neuroprotective effects of nicotine in primary cortical cultures. During pretreatment with nicotine, both α7- and $\alpha4/\beta2$-type nAChRs are activated. Both types of neuronal nAChR are permeable to Ca^{2+}, which might contribute to activation of calcineurin (CaN). In addition, nAChRs depolarize the neuron and activate L-type Ca^{2+} channels. Low concentrations of Ca^{2+} activate calcineurin. This phosphatase might act directly to dephosphorylate and inactivate L-type Ca^{2+} channels or might act on other substrates to decrease L-type activity. Inactivation of L-type channels results in decreased Ca^{2+} influx after glutamate stimulation and appears to result in neuroprotection against glutamate-mediated excitotoxicity.

Our data are consistent with a cooperative involvement of β2 subunit-containing and α7 nAChRs in the neuroprotective effects of nicotine. One possible explanation for these data are that α7-containing nAChRs are critical for direct Ca^{2+} influx, whereas β2 subunit-containing nAChRs are required for depolarization and subsequent activation of voltage-gated Ca^{2+} channels. Recent reports also suggest that α7 and β2 can form functional heteromeric nAChRs (Khiroug et al., 2002). However, the mechanisms of β2- and α7-mediated neuroprotection appear to be at least somewhat distinct. Knock-out of the β2 subunit abolished the effect of nicotine on glutamate-mediated Ca^{2+} entry, whereas α-bungarotoxin treatment did not alter this effect significantly. This is consistent with previous studies showing that α7-mediated neuroprotection in the hippocampus did not involve alteration of glutamate-mediated Ca^{2+} entry (Dajas-Bailador et al., 2000). The effect of α7 on nicotine-mediated neuroprotection might be downstream of Ca^{2+} entry and might involve activation of the MAP kinase cascade (Dajas-Bailador et al., 2002). Our data suggest that more than one subtype of nAChR is involved in the neuroprotective effects of nicotine, and the contribution of each subtype might depend on the type of cytotoxic insult, the region of the brain studied, and the neuronal types involved.

The data presented here suggest that nicotine acting through β2 subunit-containing nAChRs activates calcineurin and leads to inactivation of L-type Ca^{2+} channels. A role for calcineurin as a downstream mediator of nAChR stimulation is plausible, because calcineurin is activated by low levels of Ca^{2+} (Yakel, 1997). After nicotine treatment, nAChRs are activated and desensitized rapidly; however, after desensitization, there is a significantly reduced response to nicotine that is not abolished (Fenster et al., 1999). Thus, nAChR stimulation is likely to result in a low level of Ca^{2+} entry that can activate a high-affinity Ca^{2+} sensor (calcineurin) but is less able to activate low-affinity Ca^{2+} sensors (such as the Ca^{2+}-dependent kinases) (Lisman, 1989).

Consistent with this possibility, we have shown that inhibition of calcineurin blocks the effects of nicotine on glutamate-evoked Ca^{2+} influx through L-type channels and abolishes the protective effect of nicotine in cortical neurons. The effect of nicotine on glutamate-mediated Ca^{2+} entry is already seen after 5 min of nicotine pretreatment and is sustained after 1 hr of nicotine treatment. In contrast, cotreatment with nicotine and glutamate did not result in a change in glutamate-mediated Ca^{2+} entry, suggesting that nicotinic activation of calcineurin only during the period when glutamate is present is not sufficient to result in changes in Ca^{2+} influx.

FK506 and cyclosporine, when present during glutamate exposure, were protective against excitotoxicity, as has been seen in several other studies (H. G. Wang et al., 1999; Guo et al., 2001); in contrast, cotreatment with FK506 or cyclosporine and nicotine during the 1 hr before glutamate exposure results in blockade of the protective effects of nicotine against excitotoxicity. This suggests that calcineurin has a differential role in neuroprotection in the period before exposure to an excitotoxic agent and during the period the agent is present. Similar paradoxical observations have been made using tumor necrosis factor-α and nicotine (Carlson et al., 1998). The actions of calcineurin extend well beyond regulation of L-type Ca^{2+} channels. For example, calcineurin increases the activity of nitric oxide synthase (Dawson et al., 1993), an effector implicated in events downstream of nicotine-induced neuroprotection (Shimohama et al., 1996), dephosphorylates and activates the proapoptotic protein BAD (Bcl-2-associated death protein) (H. G. Wang et al., 1999), and downregulates NMDA receptors (Shi et al., 2000). Phosphorylation of BAD can be either neuroprotective (H. X. Wang et al., 1999) or proapoptotic (Konishi et al., 2002), depending on the site of phosphorylation, suggesting another point for differential regulation of neuroprotection by calcineurin. Given the multitude of effectors downstream of calcineurin (potentially including nAChRs), it is conceivable that different levels or temporospatial patterns of calcineurin activity modulate signaling cascades that have either neuroprotective or neurotoxic effects.

These studies show that nicotine treatment modulates glutamate signaling in cortical neurons through activation of calcineurin. Both glutamate receptors and calcineurin are known to be critical for changes in synaptic strength such as long-term potentiation and long-term depression (Malenka and Nicoll, 1999; Riedel, 1999). Thus, although the current studies have focused on a mechanism underlying nicotine-mediated neuroprotection, the signal transduction pathways delineated here could also be responsible for effects of nicotine on plastic processes, such as learning or addiction.

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