Differing temporal roles of Ca\(^{2+}\) and cAMP in nicotine-elicited elevation of tyrosine hydroxylase mRNA

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Gueorguiev, Volodia D., Richard J. Zeman, Bhargava Hiremagalur, Ana Menezes, and Esther L. Sabban. Differing temporal roles of Ca\(^{2+}\) and cAMP in nicotine-elicited elevation of tyrosine hydroxylase mRNA. Am. J. Physiol. 276 (Cell Physiol. 45): C54–C65, 1999.—The involvement of cAMP- and Ca\(^{2+}\)-mediated pathways in the activation of tyrosine hydroxylase (TH) gene expression by nicotine was examined in PC-12 cells. Extracellular Ca\(^{2+}\) and elevations in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) were required for nicotine to increase TH mRNA. The nicotine-elicited rapid rise in [Ca\(^{2+}\)]\(_i\) was inhibited by blockers of either L-type or N-type, and to a lesser extent P/Q-, but not T-type, voltage-gated Ca\(^{2+}\) channels. With continual nicotine treatment, [Ca\(^{2+}\)]\(_i\) returned to basal levels within 3–4 min. After a lag of 5–10 min, there was a smaller elevation in [Ca\(^{2+}\)]\(_i\), which persisted for 6 h and displayed different responsiveness to Ca\(^{2+}\) channel blockers. This second phase of elevated [Ca\(^{2+}\)]\(_i\) was blocked by an inhibitor of store-operated Ca\(^{2+}\) channels, consistent with the observed generation of inositol trisphosphate. 1,2-Bis(2-aminophenoxylethane-N,N,N',N' -tetraacetic acid-AM (BAPTA-AM), when added before or 2 h after nicotine, prevented elevation of TH mRNA. Nicotine treatment significantly raised cAMP levels. Addition of the adenyl cyclase inhibitor 2',5'-dideoxyadenosine (DDA) prevented the nicotine-elicited phosphorylation of cAMP response element binding protein. DDA also blocked the elevation of TH mRNA only when added after the initial transient rise in [Ca\(^{2+}\)]\(_i\) and not after 1 h. This study reveals that several temporal phases are involved in the induction of TH gene expression by nicotine, each of them with differing requirements for Ca\(^{2+}\) and cAMP.

EXPOSURE TO NICOTINE, a major component of cigarette smoke, produces many physiological changes and increases the risk of coronary and peripheral vascular disease. As a potent agonist of nicotinic acetylcholine receptors, nicotine triggers rapid secretion of catecholamines. The nicotine-triggered elevations in plasma catecholamine levels from the sympathetic nerves and the adrenal medulla are associated with alterations in heart rate and arterial pressure (13). Nicotine treatment also increases catecholamine biosynthesis by phosphorylation and rapid activation of tyrosine hydroxylase (TH), the first and major rate-limiting enzyme in catecholamine biosynthesis (23). In addition, prolonged exposure to nicotine for several days was found to elicit elevations in gene expression of rat adrenomedullary catecholamine biosynthetic enzymes as well as of several neuropeptides (neuropeptideY and enkephalin) and other constituents of chromaffin vesicles that can be co-released with the catecholamines (17, 18, 20). In the rat adrenal medulla, transcriptional mechanisms were shown to be involved in the induction of TH gene expression (9).

Cultured cells of adrenomedullary origin (bovine chromaffin and PC-12 cells) have been used to examine the underlying mechanisms by which nicotine activates gene expression. In these cells, as was found in vivo, nicotine increased the levels of mRNA for TH, as well as for dopamineβ-hydroxylase, proenkephalin, preproenkephalin, and several soluble proteins of chromaffin granule cores (4, 16, 36, 40). However, the precise mechanism for nicotine-driven gene expression is still unclear, and conflicting results have been reported. Several signaling pathways have been implicated in mediating the effect of nicotine on gene expression in cells of adrenomedullary origin. These pathways include the activation of protein kinase C (PKC), Ca\(^{2+}\)/calmodulin-dependent protein kinases, and/or protein kinase A (PKA), which can phosphorylate cAMP response element binding protein (CREB) and lead to its transactivation. Several transcription factors also respond to nicotine treatment. Nicotine not only elicits the phosphorylation of CREB but also rapidly enhances c-fos transcription, which precedes a slower rise in c-jun and junB mRNA levels (11, 36, 40). C-fos has been proposed to induce nicotine-stimulated proenkephalin transcription (40). However, the nicotine induction of TH gene transcription is reportedly independent of c-fos gene activation (5). Experiments using transient transfection of PC-12 cells with reporter constructs of the TH promoter mapped the nicotine response to the cAMP/Ca\(^{2+}\) response element binding protein (CRE/CaRE) (16). Similarly, CRE sites in the chromogranin A and proenkephalin promoters also mediated the nicotine-induced activation of these genes (36, 40).

Upon nicotinic stimulation, an influx of extracellular Ca\(^{2+}\) and Na\(^{+}\) occurs via nicotinic receptors, resulting in membrane depolarization and the recruitment of voltage-gated Ca\(^{2+}\) channels that promote Ca\(^{2+}\) entry, leading to a rapid increase in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) (33). Studies by Craviso et al. (4, 5) suggested that the influx of extracellular Ca\(^{2+}\) is necessary for the effect of nicotine on TH gene expression, since nitrendipine, an L-type Ca\(^{2+}\) channel blocker, prevented the elevation of c-fos and TH mRNA levels in bovine chromaffin cells treated with the nicotinic ago-

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nient 1,1-dimethyl-4-phenylpiperazinium (DMPP). The extent of induction depended on the extracellular Ca\(^{2+}\) concentration.

In addition to nicotine, a number of treatments that raise [Ca\(^{2+}\)], either from extracellular or intracellular sources, activate TH gene expression (31). Thus elevated levels of K\(^+\), veratridine, ionomycin, and bradykinin activate TH transcription (29, 30, 32). However, previous studies have also indicated discrepancies between the activating mechanisms of these compounds and that triggered by nicotine. Like nicotine, ionomycin and elevated K\(^+\) were found to increase TH promoter activity via the CRE/CaRE site (21, 31). The ionomycin-elicited induction of TH promoter activity and the phosphorylation of CREB were observed in normal and in PKA-deficient PC-12 cells (31). In contrast, the nicotine-triggered activation of TH gene expression did not occur in the PKA-deficient cell lines, suggesting that PKA is needed for the induction by nicotine. Consistent with this report, cAMP analogs and nictinic receptor agonists exhibit nonadditive effects on TH mRNA levels (5, 34) despite exerting additive effects on chromogranin A promoter activity (35).

In this study, we explored the involvement of cAMP-mediated events and increased [Ca\(^{2+}\)] in the nicotine-triggered induction of TH gene expression. The elevation of TH mRNA by nicotine was prevented by either chelation of extracellular Ca\(^{2+}\) or adenyl cyclase inhibition. The types of channels involved were examined with selective antagonists. The rise in [Ca\(^{2+}\)] is biphasic, with a second prolonged but moderate increase after the initial transient rise. Our results indicate that this second rise in [Ca\(^{2+}\)] is necessary for activation of TH gene expression and suggest that several temporal phases with different requirements for Ca\(^{2+}\) and cAMP are involved in the induction of TH gene expression by nicotine.

**MATERIALS AND METHODS**

Materials were obtained as follows: DMEM, streptomycin, penicillin, and the Select-Amine kit were obtained from GIBCO BRL (Gaithersburg, MD), tissue culture dishes were from Falcon (Lincoln Park, NJ), and Calcium Green-1-AM, 1,2-bis(2-aminophenoxy)ethane-N,N',N'-tetraacetic acid (BAPTA)-AM, and EGTA-AM were purchased from Molecular Probes (Eugene, OR). \(\omega\)-Conotoxins (GVIA, MVIIA, and MVIIIC) were from Alomone Labs (Jerusalem, Israel). The primary antibodies specific for CREB and phospho-CREB (P-CREB) (10) were purchased from Upstate Biotechnology (Lake Placid, NY). Alkaline phosphatase-conjugated secondary antibody was obtained from Promega (Madison, WI), and the enhanced 3,3'-diaminobenzidine tetrahydrochloride (DAB) substrate was purchased from Pierce (Rockford, IL). Fetal bovine serum and donor horse serum were obtained from JRH Biosciences (Lenexa, KS). W-7 and 2,5'-dideoxyadenosine (DAA) were from Calbiochem (San Diego, CA), [\(\alpha\)-\(^{32}\)P]dCTP and myo-[\(^{3}H\)]inositol were obtained from DuPont NEN Research Products (Boston, MA), and nicotine bi-d-tartrate was from RBI (Natick, MA). All other reagents were purchased from Sigma Chemical (St. Louis, MO) and were of reagent grade unless specified.

Treatment of cells. PC-12 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 5% heat-inactivated donor horse serum, 50 \(\mu\)g/ml streptomycin, and 50 IU/ml penicillin in a humidified atmosphere at 37°C and 7% CO\(_2\), as described previously (16). Cells were treated at a medium density (3 \times 10\(^5\) cells/cm\(^2\)). For nicotine treatment, nicotine solution in sterile water was added to a final concentration of 200 \(\mu\)M. For elevated K\(^+\) treatment, osmotically balanced medium with 50 \(\mu\)M K\(^+\) was prepared using the Select-Amine kit (GIBCO BRL) as previously described (21, 29). In some experiments, cells were pretreated with EGTA (5 \(\mu\)M), EGTA-AM or BAPTA-AM (10 \(\mu\)M), nifedipine (10 \(\mu\)M), ecaconazole (100 \(\mu\)M, 5 \(\mu\)M, or 10 \(\mu\)M), DDA (10 or 100 \(\mu\)M), caldiseptine (300 \(\mu\)M) or \(\omega\)-conotoxins (GVIA, MVIIA, or MVIIIC; 500 \(\mu\)M), or flunarizine (1 \(\mu\)M) for 10 min. For experiments with medium without Ca\(^{2+}\), the medium was prepared by using the Select-Amine kit (GIBCO BRL) with all the components except the Ca\(^{2+}\) salts. At least three or four duplicate cell culture plates were used in each experiment. All experiments were performed at least twice.

Northern blot analysis. At the times indicated, cells were washed once with PBS and pelleted. Total RNA was isolated, and Northern blot analysis was performed as previously described (16). Briefly, total RNA (15 \(\mu\)g) was fractionated through 1.3% agarose gels containing 2.2 M formaldehyde and 1× MOPS buffer (20 mM MOPS, 400 mM NaOH, 10 mM NaH\(_2\)PO\(_4\), and 1 mM EDTA), and 0.4% SDS at 42°C for 4 h. Hybridizations were then performed consecutively using a 1.1 kb EcoRI fragment from the rat TH cDNA and a DNA probe for 18S rRNA (as previously described in Ref. 16) labeled with \([\alpha\]-\(^{32}\)P]dCTP by using the random primer method (Megaprime, Amersham). The labeled probes were heat denatured (90°C, 5 min) and used for the prehybridization solution and hybridized at 42°C for 18 h. After hybridization, the filters were washed twice with 2× SSPE and once with 0.2× SSPE and 0.1% SDS at room temperature for 30 min. The filters were then exposed to X-ray films for various times. In addition, the autoradiographic images were captured with a charge-coupled device (CCD) camera (Datavision), and the ratio of TH DNA signal vs. 18S rRNA was quantified by performing densitometric analyses within the linear range of each captured signal by using the Image Pro Plus software (Media Cybernetics, Silver Spring, MD).

[Ca\(^{2+}\)]\(^{i}\) measurements. PC-12 cells were grown in 25-mm glass coverslip chambers (Nunc) previously coated with collagen. The cells were loaded with 3 \(\mu\)M fura 2-AM or 15 \(\mu\)M Calcium Green-1-AM for 30 min at 37°C. Alterations in [Ca\(^{2+}\)]\(^{i}\) were measured by analyzing the ratio of fura 2 fluorescence (480 nm) excited at 340 and 380 nm. Fluorescence images of fura 2-loaded PC-12 cells were captured with a Nikon Diaphot fluorescence microscope equipped with a Quantex QX-7 CCD camera and a digital imaging system, as previously described (31). The [Ca\(^{2+}\)]\(^{i}\) of individual cells was calculated as described by Grynkiewicz et al. (12) after the average values of pixels overlying each cell in ratioed (340 nm/380 nm) images were obtained. A value of 224 nm was used for the dissociation constant of fura 2-Ca\(^{2+}\). For confocal images, Calcium Green-1-loaded cells were visualized with a Bio-Rad MRC-1000 confocal microscope. Calcium Green-1 loaded cells were illuminated with an argon ion laser at a wavelength of 488 nm, the resulting fluorescence (515 nm) was imaged, and the average pixel value of each cell was obtained. As an indication of changes in [Ca\(^{2+}\)], the fluorescence of nicotine-treated cells was expressed relative to

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untreated cultures. At least four microscopic fields in two or three separate culture dishes were analyzed for each treatment.

Analysis of inositol phosphates. Measurement of inositol phosphates was performed as previously described (29). PC-12 cells were prelabeled with myo-[3H]inositol (6 µCi/ml) for 48 h at 37°C. Cells were incubated for 10 min in medium containing 10 mM LiCl to inhibit inositol phosphatases and were exposed to different time points to 200 µM nicotine, 50 mM K+, or 1 µM bradykinin. The supernatants after homogenization in 10% ice-cold TCA were extracted with diethyl ether, neutralized with NaOH (pH 6.5–7.5), and applied to a Dowex AG1-8X column to isolate the inositol monophosphate, inositol bisphosphate, and inositol trisphosphate (IP3) by a step gradient. The amount of newly synthesized inositol phosphates was determined by scintillation counting.

Immunocytochemistry. Cells were plated in triplicate on 24-well tissue culture plates and allowed to attach overnight. After treatment with nicotine or DDA for 10 min, the cells were rinsed once with PBS and fixed in 0.7 ml of 4% paraformaldehyde in PBS at room temperature for 20 min. The cells were then given three 5-min washings in PBS containing 10 mM glucose, and incubated with a 50% glycerol/PBS solution for 10 min. After a rinse in PBS and incubation in excess secondary antibody [goat anti-rabbit antibody at a 1:200 (vol/vol) dilution], cells were incubated at room temperature for 2 h in a PBS-based blocking solution containing 3% BSA. Subsequently, the cells were incubated with 0.7 µg/ml anti-P-CREB antibody for 24 h at 4°C. After the incubation, the cells were washed three times (5 min each wash) in PBS and incubated in excess secondary antibody [goat anti-rabbit antibody at a 1:200 (vol/vol) dilution] for 2 h at room temperature. After three washes of 5 min each in PBS at room temperature, incubations in enhanced DAB substrate for 5–10 min were performed. Finally, the cells were washed with tap water, mounted, and observed for nuclear staining. The anti-P-CREB antibodies used in this study were raised against a phosphopeptide corresponding to amino acids 123–136 of CREB (10).

cAMP determination. The cAMP content of the cells was measured as follows: individual PC-12 cell cultures were treated with 200 µM nicotine for 15 min and 24 h, the media were removed by aspiration, and 1 ml of 0.5% NP-40 at room temperature for 30 min. After a washing with PBS containing 5 mM sodium fluoride and 1 mM ammonium molybdate, cells were incubated at room temperature for 2 h in a PBS-based blocking solution containing 3% BSA. Subsequently, the cells were incubated with 0.7 µg/ml anti-P-CREB antibody for 24 h at 4°C. After the incubation, the cells were washed three times (5 min each wash) in PBS and incubated in excess secondary antibody [goat anti-rabbit antibody at a 1:200 (vol/vol) dilution] for 2 h at room temperature. After three washes of 5 min each in PBS at room temperature, incubations in enhanced DAB substrate for 5–10 min were performed. Finally, the cells were washed with tap water, mounted, and observed for nuclear staining. The anti-P-CREB antibodies used in this study were raised against a phosphopeptide corresponding to amino acids 123–136 of CREB (10).

RESULTS

Increased [Ca2+]i is required for nicotine-stimulated elevations of TH mRNA levels. Previous experiments revealed that treatment of PC-12 cells with 10 µM to 1 mM nicotine elicited rapid rises in [Ca2+]i. Concentrations of 50–200 µM nicotine caused maximal increases in the amounts of TH, chromogranin A, and c-fos mRNAs (11, 16, 36). To ascertain whether extracellular Ca2+ and increased [Ca2+]i were required for nicotine-triggered upregulation of TH mRNA, extracellular Ca2+ was reduced by using media either prepared without added Ca2+ or containing 5 mM EGTA. Both of these conditions prevented the induction of TH mRNA expression by nicotine (Fig. 1), indicating a requirement for extracellular Ca2+. Next, the effects of intracellular Ca2+ chelators were examined. In these experiments, pretreatment with 10 µM BAPTA-AM (Fig. 1) or EGTA-AM (not shown) prevented the nicotine-induced rise in [Ca2+]i. These concentrations of EGTA-AM or BAPTA-AM had no significant effect on basal TH mRNA levels. However, both agents completely prevented the elevation of TH mRNA levels in response to nicotine treatment, indicating that the rise of [Ca2+]i is necessary for the induction of TH mRNA in response to nicotine.

Ca2+ channels involved in nicotine-triggered rise in [Ca2+]i. In PC-12 cells, as well as in adrenal chromaffin cells, membrane depolarization by nicotine leads to an influx of extracellular Ca2+ via voltage-gated Ca2+ channels and the nicotinic channel (33). We determined whether activation of voltage-gated Ca2+ channels was required for TH induction as well as which type of channel was involved. PC-12 cells were treated with nicotine in the presence of two different L-type Ca2+ channel blockers. Results revealed that either the dihydropyridine blocker nifedipine (10 µM) or the inhibitory peptide calcsinetine (300 nM) (8) prevented the nicotine-induced rise in [Ca2+]i (Figs. 2 and 3A). Furthermore, Northern blot analysis showed that nifedipine prevented the rise in TH mRNA levels in the presence of nicotine, without affecting basal levels (Fig. 2).

The effects of other voltage-sensitive Ca2+ channel blockers on the rise of [Ca2+]i, triggered by nicotine were also examined. Pretreatment of cells with the N-type channel blocker ω-conotoxin GVIA (500 nM) also prevented the rise in [Ca2+]i, in the presence of 200 µM nicotine. Similarly, another N-type channel blocker, ω-conotoxin MVIIA (500 nM), greatly reduced the rise in [Ca2+]i caused by nicotine (Fig. 3B). However, the T-type channel blocker flunarizine (1 µM) had little effect on the extent of the rise, although the time course of the decay was more rapid than that seen in the control cells (Fig. 3C). A P/Q-type Ca2+ channel blocker, ω-conotoxin MVIIIC (500 nM), did not completely prevent the rise in [Ca2+]i but led to a substantial reduction of ~65% (Fig. 3D). These results indicate that blockage of L-type, N-type, and to some extent P/Q-type voltage-gated Ca2+ channels can eliminate or greatly reduce the rise in [Ca2+]i elicited by nicotine. For comparison, the effects of some of these inhibitors on the previously reported rapid rise in [Ca2+]i, induced by 50 mM K+ (29) were examined (Fig. 4). In contrast to their blockade of the nicotine-elicted rise, the same concentrations of calcsinetine or ω-conotoxin GVIA only partially prevented the elevation of [Ca2+]i in response to depolarization with elevated K+. On the other hand, the effect of ω-conotoxin MVIIIC was similar for both treatments.

Time course of the elevation of [Ca2+]i. To examine the long-term effect of continuous nicotine treatment on
[Ca\textsuperscript{2+}], we treated PC-12 cells with 200 µM nicotine for up to 6 h (Fig. 5A). Nicotine treatment elevated the [Ca\textsuperscript{2+}], within seconds of its addition, from a basal level of ~50 nM to a level between 200 and 450 nM (Figs. 1, 3, A–D, and 5, A and C). These increments were followed by a rapid decrease within several minutes. After a lag of ~5–10 min, a second elevation to 100–150 nM was observed. This second elevation was stable for relatively long periods of time, and [Ca\textsuperscript{2+}] remained elevated at 80–110 nM (Fig. 5A) after 6 h of continuous exposure to nicotine.

The dependence of this prolonged rise in [Ca\textsuperscript{2+}] on Ca\textsuperscript{2+} channels was examined (Fig. 5B). In contrast to its inhibition of the initial rise in [Ca\textsuperscript{2+}], induced by nicotine, calciseptine did not alter the [Ca\textsuperscript{2+}], under these conditions. However, addition of the N-type channel blocker (500 nM ω-conotoxin GVIA) to cells treated with nicotine for 6 h reduced the [Ca\textsuperscript{2+}] to basal levels. This inhibition was transient, and after several minutes the [Ca\textsuperscript{2+}] returned to the previous elevated levels. The reduced effectiveness of these Ca\textsuperscript{2+} channel blockers at these later times suggested that other Ca\textsuperscript{2+} channels, such as store-operated Ca\textsuperscript{2+} (SOC) channels, may contribute to the rise at 6 h. Therefore, econazole was added at a concentration (10 µM) that inhibits SOC channels (24). Econazole elicited a rapid and more sustained reduction in [Ca\textsuperscript{2+}]. Because econazole was effective in dissipating the sustained rise in Ca\textsuperscript{2+} with prolonged nicotine treatment, we examined its effects on the initial nicotine-triggered rise in [Ca\textsuperscript{2+}] (Fig. 5C). This inhibitor was partially effective at 100 nM, and at 5 µM it essentially prevented the initial rise in [Ca\textsuperscript{2+}].

Because capacitative influx via SOC channels is stimulated by depletion of IP\textsubscript{3}-sensitive intracellular Ca\textsuperscript{2+} stores, we examined whether nicotine treatment

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Fig. 1. Effect of reduction in extracellular or intracellular Ca\textsuperscript{2+} on nicotine-induced increases in intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]) and tyrosine hydroxylase (TH) gene expression. PC-12 cells were incubated for 6 h in absence or presence of 200 µM nicotine alone or in several conditions that reduce extracellular or intracellular Ca\textsuperscript{2+}. EGTA was at 5 mM, and 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA)-AM and EGTA-AM were at 10 µM. Ca\textsuperscript{2+}-free medium was prepared as described in MATERIALS AND METHODS. In all cases, cells were preincubated for 10 min before addition of nicotine and further incubated for 6 h. RNA was isolated, and levels of TH mRNA were determined by Northern blot analysis. Data are means ± SE. * P < 0.01 compared with control group. Inset: PC-12 cells were first loaded with 3 µM fura 2 for 30 min. [Ca\textsuperscript{2+}] was then measured in control cells without BAPTA-AM treatment and in cells pretreated with 10 µM BAPTA-AM before and at 1-min intervals after addition of 200 µM nicotine.

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Fig. 2. Effect of L-type Ca\textsuperscript{2+} channel blocker nifedipine on nicotine-elicited elevation in [Ca\textsuperscript{2+}] and TH mRNA levels. PC-12 cells preloaded with 15 µM Calcium Green-1-AM were pretreated with 10 µM nifedipine for 20 min, followed by treatment with 200 µM nicotine. [Ca\textsuperscript{2+}] was then monitored by confocal microscopy. Inset: representative Northern blot showing TH mRNA levels. PC-12 cells were treated with nifedipine (N), nicotine (N'), or nifedipine and nicotine (N + N') or were untreated (C). Equivalent amounts of total RNA loading were ensured by hybridization with a probe for 18S rRNA (not shown). * P < 0.01 compared with controls.
generated IP₃. Nicotine was found to elicit a prolonged elevation of IP₃, which peaked at 15 min (Fig. 6). This rise in IP₃ is about one-fourth of that generated by bradykinin (not shown). In contrast, depolarization with 50 mM K⁺ did not generate IP₃.

Temporal requirement of Ca²⁺ and role of adenylyl cyclase in nicotine-elicited elevation of TH mRNA. To determine the temporal requirement for increased [Ca²⁺]ᵢ in the induction of TH mRNA by nicotine, we treated the PC-12 cells with 10 µM BAPTA-AM at different time points before and after the addition of nicotine (Fig. 7). BAPTA-AM blocked the induction of TH mRNA by nicotine when added before or after the initial elevation of [Ca²⁺]ᵢ. Even when added 2 h after nicotine, BAPTA-AM still prevented the rise in TH mRNA levels. These data indicate that a sustained rise in [Ca²⁺]ᵢ is required for the elevation of TH mRNA by nicotine.

Previous results with PKA-deficient cells suggested that activation of PKA was required for nicotine-elicited gene transcription in PC-12 cells (16). To investigate this possibility, we measured the cAMP concentrations in untreated and nicotine-treated cells to examine whether inhibition of adenylyl cyclase affected the TH mRNA levels. Statistically significant increases in cAMP concentrations were observed in PC-12 cells exposed to nicotine for 15 min (means ± SE in pmol/3 × 10⁵ cells: control, 13 ± 0.4; nicotine, 16.4 ± 0.8; P < 0.05). The concentration of cAMP remained significantly elevated in cells exposed to nicotine for 24 h (means ± SE in pmol/3 × 10⁵ cells: control, 13.8 ± 0.4; nicotine, 15.2 ± 0.2; P < 0.05).

Cells were also exposed to 10 and 100 µM DDA, an adenylyl cyclase inhibitor, before and during the nicotine treatment. Northern blot analyses of RNA prepared from these cells are presented in Fig. 8. The addition of 10 µM DDA inhibited, but 100 µM DDA prevented, the nicotine-induced rise in TH mRNA levels. In contrast, 100 µM DDA did not prevent the phorbol ester-elicited induction of TH mRNA (Fig. 8), consistent with the specificity of DDA. These results suggest that cAMP is critically involved in the induction of TH gene expression by nicotine.
One of the steps mediating cAMP-induced activation of gene expression is phosphorylation of CREB, a transcription factor that binds the TH CRE/CaRE. Thus we further examined the phosphorylation of CREB in nicotine-treated cells (200 µM) in the presence or absence of DDA. Immunocytochemistry with anti-sera to P-CREB showed increased immunoreactivity in the nucleus of nicotine-treated cells (Fig. 9). Pretreatment with the adenylyl cyclase inhibitor DDA greatly reduced the immunoreactivity to P-CREB, indicating that the nicotine-elicited phosphorylation of CREB requires adenylyl cyclase activity.

Next, DDA was added at various times before and after nicotine addition to determine any temporal effects this inhibition may have (Fig. 10). DDA prevented the rise of TH mRNA after 6 h of treatment with nicotine when it was added 15 min before or 15 min after nicotine. However, when added 1 h after nicotine, DDA was no longer inhibitory. These results suggest that the temporal requirement for adenylyl cyclase activity differs from that of Ca²⁺.

DISCUSSION

Extracellular Ca²⁺. The present study investigated the involvement of Ca²⁺ and cAMP in the induction of TH mRNA expression caused by nicotine. We have shown that several distinct temporal phases exist in the nicotine-triggered elevation of TH mRNA levels that possess different requirements for cAMP or Ca²⁺. Nicotine elicited a rapid rise in [Ca²⁺], in PC-12 cells, and the absence of extracellular Ca²⁺ prevented the nicotine-stimulated induction of TH mRNA. This is in contrast to the induction of TH mRNA caused by bradykinin, which occurred in Ca²⁺-free medium or in the presence of EGTA (29). Upon nicotinic stimulation of PC-12 cells, an influx of extracellular Ca²⁺ and Na⁺ occurs via nicotinic receptors and results in membrane depolarization and the activation of voltage-gated Ca²⁺ channels. This promotes Ca²⁺ entry and leads to a rapid increase in [Ca²⁺] (33). Our results indicate that the influx of extracellular Ca²⁺ is essential for the induction of TH mRNA. In addition, we found that
nifedipine blocked nicotine's ability to increase TH mRNA levels, consistent with its inhibition of TH gene transcription in nifedipine-treated cultured bovine adrenal chromaffin cells treated with the nicotinic agonist DMPP (5). Dihydropyridines were also found to inhibit nicotine-stimulated activation of the chromogranin A promoter and expression of the proenkephalin gene (34). Collectively, these data imply that Ca$^{2+}$ channels are critically involved in the nicotine-induced increase of TH gene expression.

The blockage of more than one type of voltage-gated Ca$^{2+}$ channel was found to eliminate, or greatly reduce, the nicotine-elicited rise in [Ca$^{2+}$]. Inhibitors of either L-type (nifedipine or calciseptine) or N-type (ω-conotoxins GVIA or MVIIA) Ca$^{2+}$ channels essentially prevented any increase in [Ca$^{2+}$]. This is in contrast to the only partial inhibition of the rise in [Ca$^{2+}$] elicited by depolarization with elevated K$^{+}$ with the same concentrations of these N-type or L-type voltage-dependent Ca$^{2+}$ channel blockers.

There is some evidence that L-type Ca$^{2+}$ channel blockers, specifically the 1,4-dihydropyridines, may directly inhibit the nicotine receptor (25). In such a situation, these inhibitors would prevent all the downstream effects of nicotine, including the activation of N-type Ca$^{2+}$ channels and SOC channels. Such an effect would not alter the changes in [Ca$^{2+}$] by depolarization with elevated K$^{+}$. The same N-type channel blockers used in our study (ω-conotoxins GVIA and MVIIA) were previously found to inhibit nicotine-stimulated $^{45}$Ca$^{2+}$ entry into bovine adrenal chromaffin cells by ~25–30%. However, L-type blockers reduced a much larger percentage (53–89%) of the nicotine-triggered $^{45}$Ca$^{2+}$ entry (39). This is despite the somewhat smaller Ca$^{2+}$ currents attained with dihydropyridine-sensitive voltage-gated Ca$^{2+}$ currents compared with the N-type or P-type voltage-gated Ca$^{2+}$ channels (2). This would be consistent with an additional inhibitory effect of nifedipine on acetylcholine receptors. Various dihydropyridine inhibitors of L-type Ca$^{2+}$ channels were found to prevent $^{45}$Ca$^{2+}$ uptake or elevation in [Ca$^{2+}$] in fura 2-loaded bovine chromaffin cells (25) as well as to block DMPP-evoked catecholamine release. However, this nonspecific inhibition by L-type channel blockers is less likely to explain the findings in the present study, since calciseptine had an effect similar to that of nifedipine and is not a dihydropyridine (8).
Therefore, it is unlikely that the prevention of the rise in \([Ca^{2+}]_i\) by this L-type channel blocker is due to a nonspecific action on the nicotinic receptor.

The results indicate that nicotinic stimulation activates several different types of \(Ca^{2+}\) channels in PC-12 cells, with blockage of the L-type and N-type and, to some extent, the P/Q-type, each affecting the rise in \(Ca^{2+}\). Such a coordinated blockage may indicate that the inhibition of one type (N-type or L-type) is sufficient to allow the efflux and intracellular buffering of \(Ca^{2+}\) to overcome the influx of extracellular \(Ca^{2+}\) from the remaining activated channels and therefore may explain the complete inhibition by either L-type or N-type channel blockers alone. Another possibility is that there are interactions among the channels. For example, cAMP generated via activation of one channel may activate another type of \(Ca^{2+}\) channel. Consistent with this possibility, recruitment of dihydropyridine-sensitive, voltage-gated \(Ca^{2+}\) currents by cAMP has been observed in chromaffin cells (2).

However, the effect of the channel antagonists on the nicotine-elicited rise of \([Ca^{2+}]_i\), in contrast to the effect of elevated K\(^+\), is likely influenced by concurrent desensitization of the nicotinic receptors. Because of nicotinic receptor desensitization, the initial rise in \(Ca^{2+}\) does not achieve a steady level. This dynamic situation may magnify the effectiveness of blockade of specific \(Ca^{2+}\) channels.

Several temporal phases of elevation of \([Ca^{2+}]_i\). Many studies have confirmed the ability of nicotine to elicit rapid elevations in \([Ca^{2+}]_i\), however, the long-term effects are not well studied. Our experiments demonstrated that several minutes after the initial transient rise in \([Ca^{2+}]_i\), there was a second smaller elevation that was sustained for at least several hours. Evidence from a variety of sources indicates that nicotinic receptors exist in a number of functional states, including a closed resting state that is briefly converted to an open state upon agonist binding. The receptor can be converted to its desensitized or inactive state, remaining unresponsive to agonists, for extended times (6). The desensitization of nicotine receptors and the development of tolerance to catecholamine secretion have been examined in chromaffin cell cultures (3). These authors...
demonstrated that catecholamine release exhibited both acute and chronic tolerance to nicotine. Interestingly, the majority of the tolerance occurred within the first 10 min of nicotine exposure, the time frame of the first peak of elevated [Ca\(^{2+}\)]. The smaller, but sustained, subsequent rise in [Ca\(^{2+}\)] observed in the present study may be consistent with such desensitization and is consistent with the depression (but not abolition) of catecholamine release in chromaffin cells preexposed for several days to nicotine (3).

The second elevation of [Ca\(^{2+}\)] appears to be a necessary event, since the elevation in TH mRNA levels was inhibited by BAPTA-AM even when added 2 h after nicotine. The second sustained peak of elevated [Ca\(^{2+}\)] differed from the first initial transient rise in the effect of voltage-gated Ca\(^{2+}\) channel blockers. Thus pretreatment with calcineurin prevented the initial elevation of [Ca\(^{2+}\)]. However, when added at later times, after 6 h of nicotine treatment, it was no longer effective. An N-type channel blocker, \(\omega\)-conotoxin GVIA, reduced the long-term rise in [Ca\(^{2+}\)], but its effect was not sustained after several minutes. However, a sustained inhibition of the second as well as of the initial rise in [Ca\(^{2+}\)], in response to nicotine was observed with the imidazole-type blocker econazole (10 \(\mu\)M). At these concentrations, econazole inhibits SOC channels (24) as well as voltage-dependent Ca\(^{2+}\) channels (38). Because the effectiveness of calcineurin and \(\omega\)-conotoxin GVIA was lost or reduced after the early phase, it is likely that the effect of econazole on the long-term rise indicates a contribution of SOC channels. These results are consistent with the occurrence of time-dependent changes in the relative contribution of different types of Ca\(^{2+}\) channels to the elevation in [Ca\(^{2+}\)]. The L-type voltage-dependent Ca\(^{2+}\) channels appear to be involved only in the initial rise, whereas the SOC and N-type channels may contribute to the longer term effect, although the inhibition of the N-type blocker was transient, perhaps overcome by leak channels as well.

Although a prolonged rise of [Ca\(^{2+}\)] is needed for induction of TH mRNA by nicotine, such a sustained elevation was not required for induction by elevated K\(^{+}\) or bradykinin. TH mRNA induction by membrane depolarization with elevated K\(^{+}\) was blocked when EGTA was added within the first 10 min, but not after 30 min or longer (29). With bradykinin treatment, even the transient rise in [Ca\(^{2+}\)] within 5 min of exposure in the presence of EGTA was sufficient to elevate TH mRNA (29). Bradykinin, which mainly elevates [Ca\(^{2+}\)] by generating IP\(_3\), may more directly elevate nuclear Ca\(^{2+}\) concentration via IP\(_3\) receptors, some of which are known to be located on the inner nuclear membrane. This study found that nicotine, but not depolarization by elevated K\(^{+}\), also generated IP\(_3\). Activation of phospholipase C in nicotine-treated cells would lead to generation of IP\(_3\) and activation of PKC. Nicotine-simulated activation of PKC in PC-12 has been previously observed (35). Nevertheless, the amount of IP\(_3\) seen with nicotine treatment is a fraction of that generated with bradykinin. The requirement for prolonged exposure to nicotine for elevation of TH mRNA levels, compared with those more rapidly acting agents (bradykinin and elevated K\(^{+}\)) may involve a relatively lower ability to elevate nuclear Ca\(^{2+}\). Elevated K\(^{+}\) also increases cytosolic and nuclear Ca\(^{2+}\) by activating voltage-gated Ca\(^{2+}\) channels. However, its elevation of nuclear Ca\(^{2+}\) may be more sustained, since, in this case, Ca\(^{2+}\) channel activation does not involve acetylcholine receptors, which desensitize following stimulation with nicotine.

Nicotine receptor subtypes. There are a number of nicotinic receptor subtypes on PC-12 cells that may respond differently to prolonged exposure to nicotine, and the short- and long-term effects of nicotine observed here may be mediated by a different subset of receptors. The neuronal nicotinic acetylcholine receptors are diverse cationic ion channel complexes composed of two different types of subunits (\(\alpha\) and \(\beta\)). Recently, at least eight \(\alpha\)-subunits (\(\alpha_3\)–\(\alpha_9\)) and three \(\beta\)-subunits (\(\beta_2\)–\(\beta_3\)) have been identified (13). The PC-12 cells were shown to express genes for nicotinic receptor subunits \(\alpha_3\), \(\alpha_5\), \(\alpha_7\), \(\beta_2\), \(\beta_3\), and \(\beta_4\) (15, 19). The expression of nicotinic receptor subunits is reportedly regulated by cAMP and nerve growth factor (15, 27). However, there is conflicting evidence regarding the ability of nicotine to alter the expression of its receptors in PC-12 cells. Nicotine is reported to reduce the mRNA levels of \(\alpha_3\) and slightly increase those for \(\beta_2\) in wild-type, but not in PKA-deficient, PC-12 cells (28). Conversely, another study failed to find significant changes in the expression patterns of any of the nicotinic acetylcholine receptor mRNAs in PC-12 cells in response to long-term nicotine treatments, which elevated TH mRNA levels (19).

Involvement of cAMP. The crucial involvement of the PKA pathway in the elevation of TH mRNA levels was further supported by our results. A modest but significant rise in cAMP levels was observed in the nicotine-treated PC-12 cells. Pretreatment with the adenylyl cyclase inhibitor DDA prevented both the nicotine-elicted phosphorylation of CREB and the subsequent induction of TH mRNA. DDA appeared to act specifically, since it did not prevent the induction of TH mRNA by phorbol esters. We speculate that the activation of adenylyl cyclase by nicotine may be caused by microdomains of elevated Ca\(^{2+}\) near the membrane, in the proximity of the voltage-gated Ca\(^{2+}\) channels, since these cyclases are associated with sites of Ca\(^{2+}\) entry (37). Alternatively, activation of adenylyl cyclase may be coupled to the influx of Ca\(^{2+}\) through nicotinic receptors, leading to phosphorylation and activation of voltage-gated Ca\(^{2+}\) channels. There are eight isoforms of adenylyl cyclases, of which five (I, III, V, VI, and VIII) are reported to be Ca\(^{2+}\) sensitive based on in vitro assays (37). The type I adenylyl cyclase is a neuronal nicotinic acetylcholine receptor–mediated enzyme that couples [Ca\(^{2+}\)] to cAMP increases, and could be involved in the observed responses.

The inhibition of the rise in TH mRNA levels by DDA is consistent with other studies that suggested the involvement of cAMP-mediated pathways in nicotine-driven gene activation. Cholinergic regulation of cAMP...
pathways in bovine adrenal medullary cells has been reported by Anderson et al. (1). We found that the inhibition of TH gene expression was effective when DDA was added 15 min, but not 60 min, after nicotine. These results further demonstrate that the initial elevation of \([\text{Ca}^{2+}]_i\) within the first few minutes is not sufficient to lead to the induction of TH mRNA, because adding DDA after the first peak of \([\text{Ca}^{2+}]_i\) still prevented the induction of TH mRNA. However, after 1 h of nicotine treatment, DDA was no longer inhibitory, indicating that a requirement for cAMP exists within the first 1 h.

PKA-deficient cells treated with nicotine were unable to support many of the alterations in gene expression observed in normal cells treated similarly, including the elevation of TH mRNA levels (16, 28). However, surprisingly, PKA-deficient cells reportedly support the induction of chromogranin A promoter activity by nicotine, despite a CRE element being involved in this promoter’s activation (35). This difference may be related to the finding that the CRE in chromogranin A (TCACGTAA) is not identical to the consensus CRE/CaRE (TGACGTCA) of the TH and somatostatin promoters.

Although we found that DDA inhibited the phosphorylation of CREB, this may not be sufficient for nicotine to induce chromogranin A promoter activity. Previous experiments with dominant-negative CREB are confusing because, although dominant-negative CREB completely inhibited the activation of the chromogranin A promoter by cAMP, it reduced the induction of TH by phorbol esters or nicotine by ∼70% (35). We can speculate that different CRE-like elements may utilize different signaling pathways and transcription factors in response to nicotine. In this regard, it is interesting to note that the CRE-2 element in the enkephalin promoter, which is also transcriptionally activated by nicotine, binds primarily activator protein-1-like factors in chromaffin cells and CREB family members in the striatum (22, 26). Further experiments are needed to ascertain whether phosphorylation of CREB is di-

**Fig. 11.** Proposed signal transduction pathways for elevation of TH mRNA levels by nicotine in PC-12 cells. Binding of nicotine to nicotinic acetylcholine receptors leads to influx of Na\(^+\) and Ca\(^{2+}\). Resulting depolarization of cell membrane stimulates Ca\(^{2+}\) influx via L-type, N-type, P/Q-type, and T-type voltage-dependent Ca\(^{2+}\) channels, leading to further accumulation of [Ca\(^{2+}\)]. This accumulation can be further amplified by several mechanisms, including nicotine-stimulated IP\(_3\) formation, with subsequent release of Ca\(^{2+}\) from IP\(_3\)-sensitive stores, and capacitative Ca\(^{2+}\) influx via store-operated channels. Submembranous microdomains of Ca\(^{2+}\) from nicotinic receptors can lead to formation of cAMP by Ca\(^{2+}\)-sensitive forms of adenylyl cyclase (AC). Subsequent activation of protein kinase A (PKA) can further phosphorylate and activate dihydropyridine-sensitive Ca\(^{2+}\) influx (2). Calmodulin (CaM) kinase (activated by elevation of [Ca\(^{2+}\)]) and PKA can both phosphorylate CREB, which as a homodimer or heterodimer with other transcription factors [such as activating transcription factor-1 (ATF-1) or Jun] can transactivate TH transcription at its cAMP/Ca\(^{2+}\) response element (CRE/CaRE) site. After desensitization of nicotinic receptors, [Ca\(^{2+}\)] attains a smaller but steady elevation in which contribution of store-operated Ca\(^{2+}\) channels is greater, whereas that of PKA and L-type channels is diminished. AP1, activator protein-1.
rectly involved in nicotine-triggered induction of TH gene transcription. We observed that the TH CRE/ CaRE also forms complexes in PC-12 cells with other transcription factors, such as activating transcription factor-1 and Jun (31, 32).

The findings of this study indicate that there are several temporal phases involved in the induction of TH mRNA levels by nicotine, each with different requirements for cAMP and Ca\(^{2+}\). There is an early phase of cAMP formation, and there is a late phase that is not inhibited by DDA but requires Ca\(^{2+}\) in order to lead to elevated TH mRNA levels. In this regard, studies with DMPP stimulation in bovine chromaffin cells distinguished an early phase of transcriptional activation that peaked at ~30 min and then declined, although mRNA levels continued to accumulate and were maximal at 8–18 h (4). One explanation for these differing temporal requirements for cAMP and Ca\(^{2+}\) in the present study is that adenylyl cyclase may only be required for an early transcriptional phase. Alternatively, different intracellular sites containing elevated Ca\(^{2+}\) may be involved in promoting CREB phosphorylation and perhaps activation of other transcription factors involved in CRE-dependent gene expression. The role of different intracellular sites of elevated Ca\(^{2+}\) in activation of gene expression has been shown in hippocampal neurons, where BAPTA, which is selective compared with EGTA for submembranous microdomains, blocks the phosphorylation of nuclear CREB after N-methyl-D-aspartate receptor activation (7). In these hippocampal cells, calmodulin that is in close proximity to the postsynaptic Ca\(^{2+}\) channels is thought to be responsible for calmodulin kinase-induced CREB phosphorylation. In contrast, it was found that elevated nuclear, but not cytosolic, Ca\(^{2+}\) is required for CRE-dependent gene expression by depolarization in AtT-20 cells, a pituitary cell line (14).

In the case of nicotine-induced TH gene expression, elevations in [Ca\(^{2+}\)], at several subcellular locations (Fig. 11) may be required at different times. For example, after receptor desensitization to nicotine, [Ca\(^{2+}\)], falls nearly to basal levels. Under these conditions, submembranous microdomains containing both concentrated amounts of Ca\(^{2+}\) as well as Ca\(^{2+}\)/calmodulin-dependent adenylyl cyclase may lead to activation of PKA and CREB phosphorylation. Later, when Ca\(^{2+}\) levels rise again, the activation of nuclear calmodulin kinase(s) may occur, thereby modulating TH gene expression. Consistent with this scheme, we have shown that the induction of TH mRNA was blocked by chelating intracellular Ca\(^{2+}\) with BAPTA, as well as with EGTA, which allows microdomains of highly concentrated elevated Ca\(^{2+}\) to persist within the cell due to slower binding kinetics. This biphasic mechanism would occur if nicotine-induced TH gene expression required localized (both nuclear and cytosolic) increases in Ca\(^{2+}\) levels. A more detailed study of the time course of alterations in Ca\(^{2+}\) levels in different subcellular cytoplasmic and nuclear locations is required for further elucidation of the diverse mechanisms by which neuronal activation leads to Ca\(^{2+}\)-mediated gene expression.

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