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European Journal of Pharmacology 506 (2004) 37–46

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Nicotine and epibatidine triggered prolonged rise in calcium and TH gene transcription in PC12 cells

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Received 24 June 2004; received in revised form 5 October 2004; accepted 20 October 2004
Available online 14 November 2004

Abstract

The effect of epibatidine on regulation of $[Ca^{2+}]_i$ and tyrosine hydroxylase (TH) transcription was examined. Epibatidine triggers a biphasic rise in $[Ca^{2+}]_i$ in PC12 cells similar to that observed with nicotine. There was an immediate transient increase in $[Ca^{2+}]_i$ and a subsequent sustained second elevation. In contrast to nicotine, the epibatidine-triggered increase in $[Ca^{2+}]_i$ was independent of activation of α7 nicotinic acetylcholine receptors, as it was not altered by either methyllycaconitine or α-bungarotoxin. The second $[Ca^{2+}]_i$ elevation involves calcium release from intracellular stores and is inhibited by dantrolene or xestospongin C. Epibatidine, like nicotine, elevated TH promoter driven reporter transcription, mostly mediated by the cyclic-AMP responsive motifs. Elevation in TH promoter activity requires Ca^{2+} and cAMP since it is inhibited by 1,2-bis(*o*-Aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic Acid Tetra (acetoxymethyl ester) (BAPTA-AM) or 2′,5′-dideoxyadenosine (DDA). The results reveal that epibatidine can elevate $[Ca^{2+}]_i$ in an α7 independent manner and nevertheless induce TH transcription.

Keywords: Nicotine; Epibatidine; Tyrosine hydroxylase; Calcium; Transcription

1. Introduction

Nicotine, a naturally occurring alkaloid among the thousands of compounds in cigarette smoke, is considered responsible for its major pharmacological effects (reviewed by Benowitz, 1996). Nicotine is a potent sympathomimetic agent, and its administration, in doses similar to those obtained in smoking, increases heart rate, systolic and diastolic blood pressure in humans and many animal species. These cardiovascular effects are largely attributed to the direct stimulation of catecholamine release from peripheral sympathetic nerve endings and the adrenal medulla (Haass and Kubler, 1997). Release of catecholamines, especially dopamine, plays a crucial role in the rewarding properties of nicotine in the central nervous system (Balfour et al., 1998).

Other nicotinic acetylcholine receptor agonists such as epibatadine can elicit similar responses. Originally isolated from the skin of an Ecuadorian tree frog, *Epipedobates tricolor* (Spande et al., 1992), and shown to display a strong analgesic effect, epibatidine triggers the release of catecholamines from bovine chromaffin cells and also releases dopamine from rat striatum and norepinephrine from hippocampal slices (Lloyd et al., 1998).

In addition to catecholaminergic stimulation, nicotine and some nicotinic acetylcholine receptors agonists can trigger neuroprotective actions in vivo and in vitro and alterations in gene expression (Dani, 2001). The gene for tyrosine hydroxylase (TH), the first and major rate limiting enzyme in catecholamine biosynthesis (Nagatsu et al., 1964), is an important target for stimulation by nicotine. An excellent model system to study the mechanism of induction of catecholamine biosynthetic enzymes gene expression by nicotine are cells of adrenomedullary origin, such as chromaffin and PC12 cells (Craviso et al., 1995; Gueorguiev

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doi:10.1016/j.ejphar.2004.10.045

et al., 1999; Hiremagalur et al., 1993; Tang et al., 1996). Exposure of PC12 cells to nicotine has been shown to lead to elevation of TH and dopamine β-hydroxylase (DBH) mRNA, which is dependent on activation of adenylyl cyclase and elevation of $[Ca^{2+}]_i$ (Hiremagalur et al., 1993; Gueorguiev et al., 1999). Nicotine was found to trigger a rapid, but transient rise in $[Ca^{2+}]_i$, mostly dependent on activation of voltage operated calcium channels (VOCCs). With further exposure to nicotine, there was a second sustained rise in $[Ca^{2+}]_i$, lasting up to 6 h (Gueorguiev et al., 1999), which has been shown to involve release of calcium from internal stores and was inhibited by the IP_3 receptor antagonist, xestospongin C (Gueorguiev et al., 2000).

Our experiments indicate that α7 nicotinic acetylcholine receptors are important for nicotine-elicited activation of TH and DBH gene expression. The activation of α7 nicotinic acetylcholine receptors is sufficient for this induction and specific α7 nicotinic acetylcholine receptors agonists, 3-(2,4-dimethoxybenzilidene)anabaseine (DMXB) and E,E-3-(cinnamylidene)anabaseine (3-CA), were effective in increasing TH and DBH gene expression (Gueorguiev et al., 2000). It is unclear whether activation of other nicotinic acetylcholine receptor subtypes can also trigger similar changes in gene expression.

While the regulatory role of nicotine on elevation of $[Ca^{2+}]_i$ and gene expression for catecholamine biosynthetic enzymes in cultured cells has been extensively studied, the effect of epibatidine has not been previously investigated. Here we examined the effect of epibatadine, a very potent nicotinic acetylcholine receptor agonist, which can activate a number of nicotinic acetylcholine receptor subtypes (Lloyd et al., 1998; Whiteaker et al., 1998), on regulation of $[Ca^{2+}]_i$ and TH gene expression in PC12 cells. The results reveal that epibatidine can elevate $[Ca^{2+}]_i$ in an α7 nicotinic receptor independent manner and nevertheless potently induce TH transcription.

2. Materials and methods

Materials were obtained as follows: Dulbecco's Modified Eagle's Medium (DMEM), streptomycin and penicillin from Gibco BRL (Gaithersburg, MD, USA); tissue culture dishes from Falcon (Lincoln Park, NJ, USA); fura-PE3 (acetoxymethyl ester) from Texas Fluorescence Labs (Austin, TX, USA); fetal bovine serum and donor horse serum from Gemini Bio-Products (Calabasas, CA, USA); 2′,5′-dideoxyadenosine (DDA) from Calbiochem (San Diego, CA); 1,2-bis(*o*-Aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic Acid Tetra (acetoxymethyl ester) (BAPTA-AM) from Molecular Probes (Eugene, OR); Super Fect™ Transfection Reagent from Qiagen (Valencia, CA). All other reagents were purchased from Sigma (St. Louis, MO, USA).

2.1. Treatment of cells

PC12 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 5% heat-inactivated donor horse serum, 50 μg/ml streptomycin and 50 IU/ml penicillin in a humidified atmosphere at 37 °C and 7% CO_2 as described previously (Gueorguiev et al., 1999). Cells were maintained at a medium density (~3×10^5/cm^2). For nicotine treatment, nicotine ditartrate dissolved in sterile water was added to obtain 200 μM final concentration. Stock solutions of 1 mM epibatidine hydrochloride in dimethyl sulfoxide (DMSO) were diluted in sterile water before addition to cell culture. In some experiments, cells were treated with methyllycaconitine (10 nM), mecamylamine (10 μM), α-bungarotoxin (25 or 100 nM), 2′,5′-dideoxyadenosine (100 μM), xestospongin C (5 μM), BAPTA-AM (5 μM) or dantrolene (100 μM).

2.2. Intracellular calcium measurements

PC12 cells were grown on two-well glass coverslip chambers (Nunc) previously coated with collagen. Fura-PE3 (acetoxymethyl ester) was added to the cell culture to a final concentration of 5 μM, and cells were exposed for 30 min at 37 °C in CO_2 incubator. Alterations in intracellular calcium levels were measured by analyzing the ratio of Fura-PE3 fluorescence (>480 nm) excited at 340 and 380 nm as previously described (Gueorguiev et al., 1999). Fluorescent images of Fura-PE3-loaded PC12 cells were captured with a Zeiss Axiovert S100 fluorescence microscope equipped with a CCD camera and Northern Eclipse 6.0 image analyzing software (Empix, North Tonawanda, NY). The $[Ca^{2+}]_i$ of individual cells was calculated after obtaining the average values of pixels overlying each cell in ratioed (340/380 nm) images (Grynkiewicz et al., 1985). A K_D of 224 nM was used as the dissociation constant of Fura-PE3-Ca^{2+}. The values from at least 15 cells within the field from each separate chamber were analyzed. All experiments were repeated three times.

2.3. Measurement of TH promoter driven reporter activity

The preparation of the constructs with the first 272 nucleotides of the rat TH promoter driving the expression of a firefly luciferase gene [p5′TH-Luc (−272/+27)] has been previously described (Nakashima et al., 2003). The mutated construct was generated by deletion of six nucleotides from −44 to −39 in the major perfect consensus cAMP/calcium response element (CRE/CaRE) (TGACGTCA, at −45 to −38) motif and mutation of CRE 2 (−97 to −90) (Best et al., 1995) from 5′-GGCGTG-3′ to 5′-TCTAGA-3′ at −96 to −91 in p5′TH-Luc (−272/+27) using the QuickChange Site Directed Mutagenesis kit (Stratagene, Cedar Creek, TX). The corresponding plasmid was mixed with Super Fect™ in ratio 5 μg to 10 μl according to the manufacturer's instructions (Qiagen), and then the mixtures were added to PC12 cells grown on six-well plates in 0.6 ml of serum-free DMEM. The cells were incubated with the transfection mixtures for 3 h at 37 °C in humidified air containing 5% CO_2. The transfection mixtures were removed, and the cells

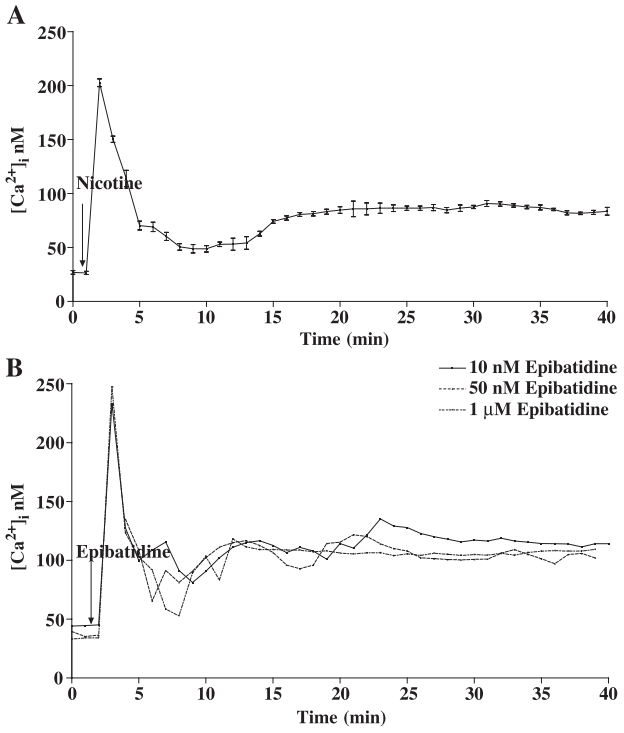

Fig. 1. Changes in $[Ca^{2+}]_i$ with continual exposure to nicotine or epibatidine. The level of $[Ca^{2+}]_i$ was measured at 1-min time interval in PC12 cells treated with 200 μM nicotine (A) or different concentrations of epibatidine (B). Each time point is the mean±S.E.M. from at least 15 cells in each individual field.

were washed twice with phospho-buffered saline (PBS), which was then replaced with 1 ml of DMEM containing 10% horse serum and 5% FBS and the cells were incubated for an additional 24 h at 37 °C in humidified air containing 7% CO_2 and then treated with different concentrations of nicotine or epibatidine for another 16 h. The cells were harvested in 1 ml of PBS and collected by centrifugation.

Firefly luciferase activity was determined using Luciferase Reporter Assay System from Promega (Madison, WI). The PC12 cells were homogenized with 30 μl passive lysis buffer and 4 μl of the lysate was added to 20 μl of the luciferase substrate. Luminescence was measured immediately with Luminometer, model TD-20/20 (Turner, Sunnyvale, CA, USA). Luciferase activity was normalized to protein concentration of the samples, determined with the Bio-Rad Protein Assay system using the Bradford method. At least three or four duplicate cell culture plates were used for each treatment. All experiments were performed at least twice.

2.4. Statistical analysis

Statistical significance was determined by Student's *t* test for experiments with two groups or by performing an analysis of variance (ANOVA) followed by Fisher's Least Significant Difference Test for experiments with more than two groups. A level of $p<0.05$ was considered statistically significant.

3. Results

PC12 cells were treated with several concentrations of epibatidine and $[Ca^{2+}]_i$ was monitored for up to 40 min and compared to the changes observed with nicotine (Fig. 1). As previously reported (Gueorguiev et al., 1999), 200 μM nicotine elicited a rapid increase in $[Ca^{2+}]_i$ which declined towards basal levels and was followed by a sustained second elevation (Fig. 1A). Exposure to epibatidine also elicited a relatively similar profile in $[Ca^{2+}]_i$ with a sharp initial rise followed by a smaller prolonged elevation (Fig. 1B). A very similar pattern was observed with 10 nM, 50 nM or 1 μM epibatidine.

The specificity of this response was studied with nicotinic acetylcholine receptor antagonists. We examined the effect of the selective α7 nicotinic acetylcholine receptor antagonist methyllycaconitine, which we previously found to inhibit the nicotine triggered changes in $[Ca^{2+}]_i$ (Gueorguiev et al., 2000). In contrast to the results with nicotine, pretreatment with methyllycaconitine did not inhibit the epibatidine-triggered rise in $[Ca^{2+}]_i$ (Fig. 2). These results suggest that the epibatidine-triggered elevation in $[Ca^{2+}]_i$ is occurring by an α7 nicotinic acetylcholine receptors independent mechanism. To further confirm these results, the cells were pretreated with α-bungarotoxin, an irreversible nicotinic acetylcholine receptors inhibitor, which is well known to selectively inhibit receptor subtypes α7, α8, α9 and α10. Similar to our previous results (Gueorguiev et al., 2000), pretreatment with 25 nM α-bungarotoxin markedly reduced the initial response in $[Ca^{2+}]_i$ and completely prevented the second sustained rise induced by nicotine (Fig. 3A). However, pretreatment of the cells with either 25 or 100 M α-bungarotoxin, did not alter the epibatidine elicited changes in $[Ca^{2+}]_i$ (Fig. 3B); both the initial and sustained response to epibatidine were unaffected by α-bungarotoxin.

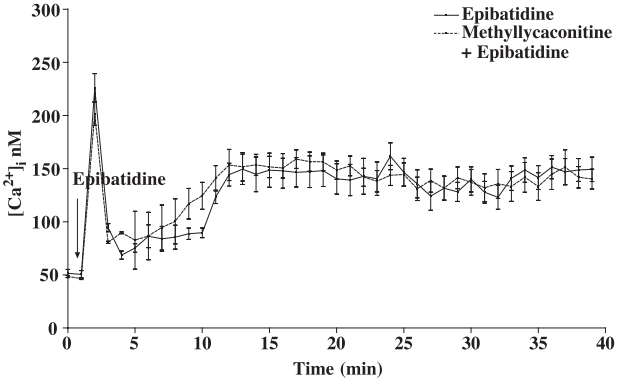

Fig. 2. Effect of methyllycaconitine on changes in $[Ca^{2+}]_i$ with epibatidine. PC12 cells were pretreated for 15 min with 10 nM methyllycaconitine before addition of 50 nM epibatidine and changes in $[Ca^{2+}]_i$ was measured at 1-min intervals and compared to parallel cell cultures without pretreatment with methyllycaconitine. Each time point is the mean±S.E.M. from at least 15 cells in each individual field.

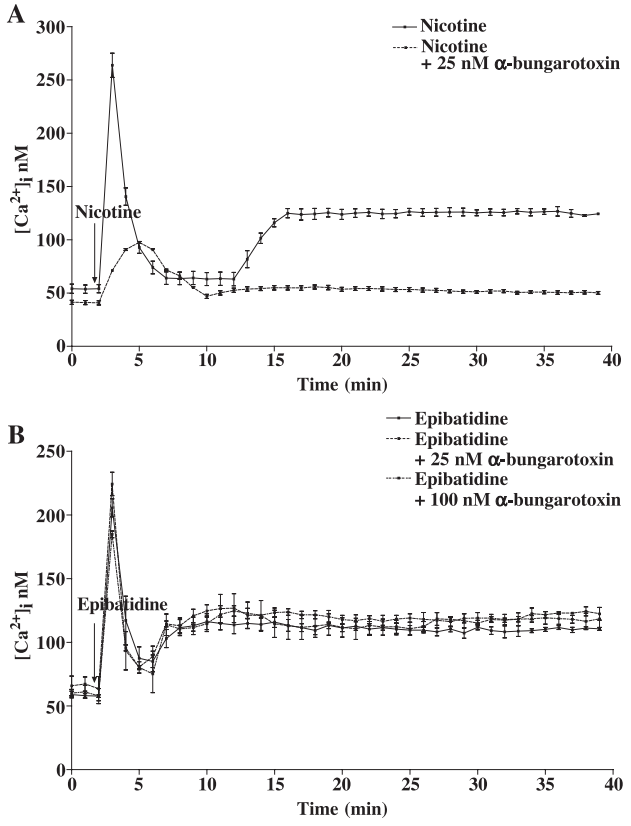

Fig. 3. Effect of α7 nicotinic receptor antagonist α-bungarotoxin on nicotine or epibatidine triggered elevation of $[Ca^{2+}]_i$. PC12 cells untreated or pretreated for 15 min with the indicated concentrations of α-bungarotoxin before the addition of 200 μM nicotine (A) or 50 nM epibatidine (B). Each time point is the mean±S.E.M. from at least 15 cells in each individual field.

Since the epibatidine-triggered rise in $[Ca^{2+}]_i$ was not affected by the specific α7 nicotinic acetylcholine receptor antagonists, methyllycaconitine or α-bungarotoxin, we examined the involvement of nicotinic acetylcholine receptors in the first as well as the sustained elevation of $[Ca^{2+}]_i$ with mecamylamine, an inhibitor for several nicotinic acetylcholine receptor subtypes including $\alpha_3\beta_4$. Pretreatment for 15 min with 10 μM mecamylamine prevented the rapid initial rise in $[Ca^{2+}]_i$ in response to either nicotine (Fig. 4A) or epibatidine (Fig. 4B). A slow and prolonged elevation in $[Ca^{2+}]_i$ was observed from about 10 min and afterwards.

We have previously shown that the initial elevation in $[Ca^{2+}]_i$ in response to nicotine is inhibited by L- and N-type VOCC blockers, and that the sustained second rise is inhibited by store operated calcium channel blockers and the IP_3 receptor antagonist, xestospongin C (Gueorguiev et al., 1999). Pretreatment with 500 nM of the L-type VOCC blocker calciseptine also inhibited the initial rise in $[Ca^{2+}]_i$ in response to epibatidine (data not shown). The involvement of ryanodine and/or IP_3 receptors expressed on the intracellular stores was examined by using specific inhibitors. Once the second elevation was attained with nicotine or epibatidine treatment, 100 μM dantrolene or 5 μM xestospongin C was added to the cell cultures. Dantrolene immediately abolished the rise in $[Ca^{2+}]_i$ triggered by prolonged treatment with nicotine (Fig. 5A) or epibatidine (Fig. 5B) and reduced $[Ca^{2+}]_i$ to below basal levels. Addition of xestospongin C during the sustained second elevation also diminished $[Ca^{2+}]_i$, but to a lesser extent than observed with dantrolene, and this reduction appears to be temporary. The findings reveal that epibatidine elicits prolonged elevation of calcium resulting from intracellular store release.

We found previously, that activation of α7 nicotinic acetylcholine receptors with specific agonist DMXB and 3-CA, mediated release of intracellular calcium and increased TH transcription (Gueorguiev et al., 2000). Epibatidine enabled us to test whether prolonged release of intracellular calcium, without activation of α7 nicotinic acethylcholine receptors, might be sufficient to trigger regulation of TH transcription. Therefore we examined whether epibatidine is capable of activating TH gene expression. Cells were transfected with reporter constructs in which luciferase activity was controlled by the first 272 nucleotides of the rat TH promoter [p5′TH/Luc (−272/+27)]. Following incubation with 200 μM nicotine or 10 nM, 50 nM or 1 μM epibatidine, there was a significant increase in TH promoter activity (Fig. 6).

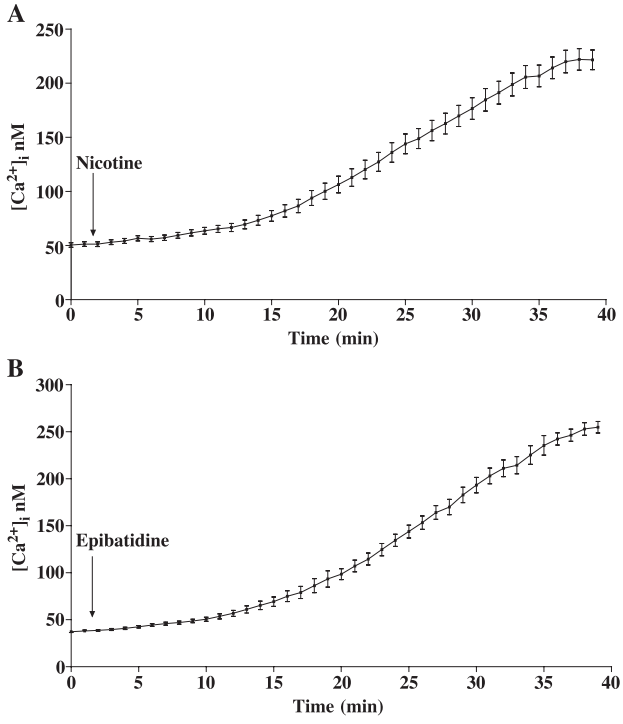

Fig. 4. Mecamylamine inhibits the first peak of $[Ca^{2+}]_i$ induced by nicotine or epibatidine. PC12 cells, preincubated for 15 min with 100 μM mecamylamine, were treated with 200 μM nicotine (A) or 50 nM epibatidine (B). Each time point is the mean±S.E.M. from at least 15 cells in each individual field.

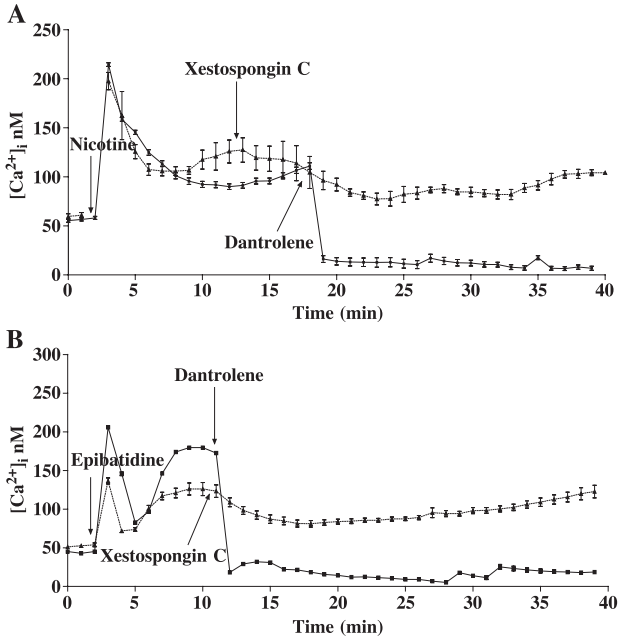

Fig. 5. Dantrolene or xestospongin C altered the second sustained elevation of $[Ca^{2+}]_i$ induced by nicotine or epibatidine. PC12 cells were treated with 200 μM nicotine (A) or 50 nM epibatidine (B) and 100 μM dantrolene or 5 μM xestospongin C was added at the indicated time points. Each time point is the mean±S.E.M. from at least 15 cells in each individual field.

To ascertain whether or not the increased $[Ca^{2+}]_i$ is required for the epibatidine-triggered elevation of TH promoter driven reporter activity, we used BAPTA-AM, a specific cell permeable calcium chelator. Cells were pre-treated with 5 μM BAPTA-AM for 15 min, and then epibatidine was added. As expected, these conditions prevented the elevation of $[Ca^{2+}]_i$ induced by all the concentrations (10 nM to 1 μM) of epibatidine (Fig. 7A). Next, the effect of intracellular Ca^{2+} chelation on TH promoter activity was examined. In the presence of BAPTA-AM, both nicotine and epibatidine were unable to induce TH promoter driven reporter activity (Fig. 7B). These results indicate that the rise of $[Ca^{2+}]_i$ is necessary for the induction of TH promoter activity in response to nicotine and to epibatidine.

The TH promoter construct used contains several putative and confirmed *cis*-acting regulatory elements such as hypoxia response element 1 (HRE1), AP-1 and AP-2, Egr1/Sp1, octamer/heptamer, Sp1, partial dyad element and cAMP regulatory motifs, including perfect consensus cAMP/calcium response element (TGACGTCA, at −45 to −38) necessary for the response to nicotine and a second CRE element (−97 to −90), which has a modulatory role (Best and Tank, 1998; Fung et al., 1992; Hiremagalur et al., 1993; Kim et al., 1994; Yang et al., 1998). To investigate

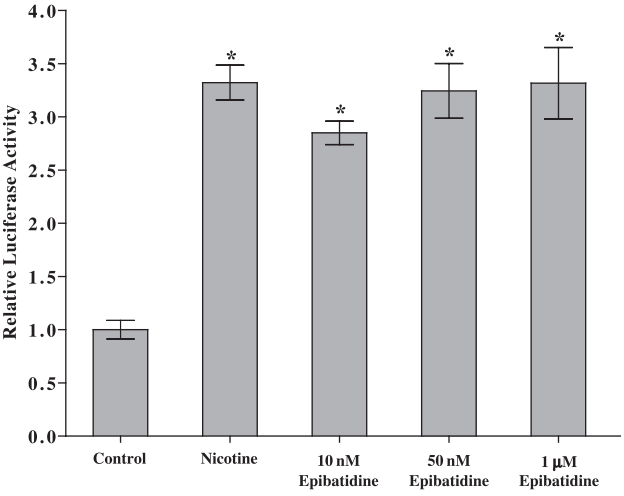

Fig. 6. Effect of different concentrations of epibatidine or nicotine on TH promoter driven reporter activity. PC12 cells, transiently transfected with p5′TH-Luc (−272/+27) promoter construct, were treated for 16 h with either 200 μM nicotine, 10 nM, 50 nM or 1 μM epibatidine and compared to untreated cells (Control). Cell lysates were assayed for firefly luciferase activity, and normalized to protein concentration as described in the Materials and methods. Promoter activity is shown relative to vehicle control taken as 1.0. Values represent the mean±S.E.M. *p<0.01 compared with control.

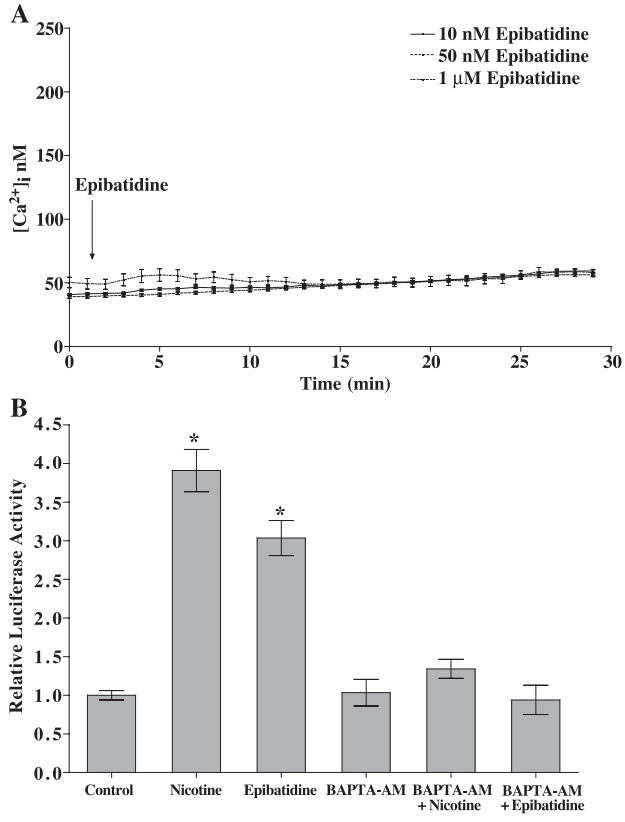

Fig. 7. Reduction in intracellular Ca^{2+} with BAPTA-AM prevents nicotine or epibatidine-induced elevation in intracellular Ca^{2+} concentration and TH promoter driven reporter activity. (A) PC12 cells were pretreated for 15 min with 5 μM BAPTA-AM before the addition of 10 nM, 50 nM or 1 μM epibatidine. $[Ca^{2+}]_i$ was then measured at 1-min intervals after addition of different concentrations of epibatidine. (B) PC-12 cells were incubated for 16 h in the presence of 200 μM nicotine or 50 nM epibatidine alone or under conditions that reduce intracellular Ca^{2+}. Cells were untreated (Control) or incubated in presence of 5 μM BAPTA-AM alone, or treated with nicotine and epibatidine with or without 5 μM BAPTA-AM added 15 min before treatment. Data are means±S.E.M. *P<0.01 compared with control group taken as 1.0.

whether transcriptional induction of TH by nicotine and epibatidine utilizes similar promoter motifs, PC12 cells were transfected with the wild type TH promoter reporter construct or a mutated version in which cAMP response elements are deleted (δ CRE) (Fig. 8A). Incubation with 200 μM nicotine induced a robust increase in TH promoter driven luciferase activity. Treatment with 50 nM or 1 μM epibatidine similarly elevated TH promoter activity. Mutation of CRE sites reduced the response to nicotine or epibatidine by about 90%. These results demonstrate that epibatidine is as effective as nicotine in increasing TH reporter driven activity and the regulation requires the cAMP promoter regulatory elements. Therefore, we investigated the involvement of cAMP mediated pathways. Cells transfected with p5′TH/Luc (−272/+27) reporter construct were incubated with 100 μM DDA, an adenylyl cyclase inhibitor, before addition of nicotine or epibatidine. As shown in Fig. 8B, DDA prevented the epibatidine or nicotine-induced rise in TH promoter driven luciferase activity. These results indicate that cAMP is critically involved in the induction of TH gene expression not only by nicotine, but also by epibatidine.

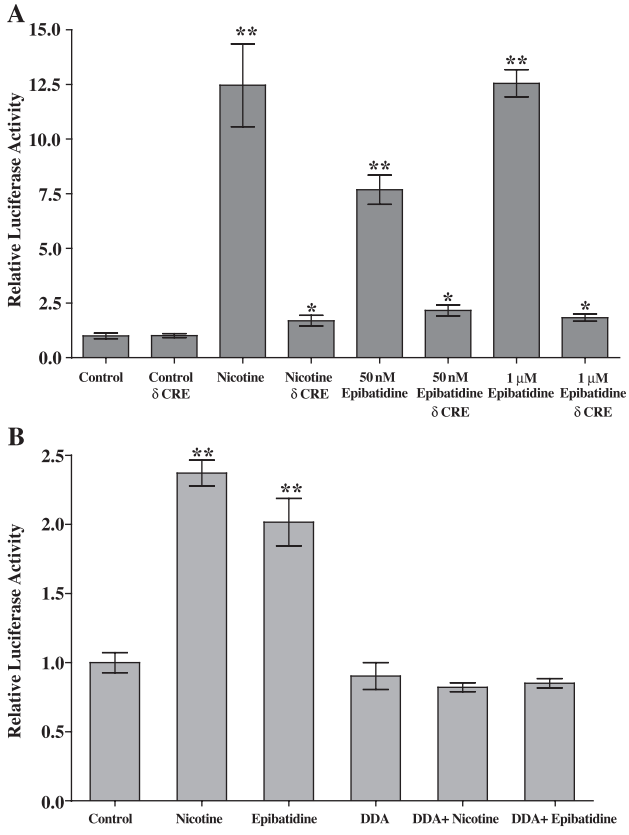

Fig. 8. Effect of epibatidine or nicotine on TH promoter driven reporter activity. (A) PC12 cells, transiently transfected with p5′TH-Luc (−272/+27), or the construct with mutant CREs, (δ CRE), were treated for 16 h with either 200 μM nicotine, 50 nM or 1 μM epibatidine and compared to untreated cells (Control). Cell lysates were assayed for firefly luciferase activity, and normalized to protein concentration as described in the Materials and methods. (B) Inhibition of adenylyl cyclase prevents the induction of TH promoter driven reporter activity induced by nicotine or epibatidine. PC12 cells were untreated (Control), or treated for 16 h with nicotine or epibatidine alone, or were pretreated with 100 μM 2′,5′-dideoxyadenosine (DDA) for 15 min before addition of 200 μM nicotine or 50 nM epibatidine. Promoter activity is shown relative to vehicle control taken as 1.0. Values represent the mean±S.E.M. *p<0.05, **p<0.01 compared to the control.

4. Discussion

This study is the first to examine the relative effects of epibatidine compared to nicotine on eliciting changes in $[Ca^{2+}]_i$, and activating TH promoter driven transcription in PC12 cells. Epibatidine was found to elicit a biphasic elevation of $[Ca^{2+}]_i$ in an α7 nicotinic acetylcholine receptors independent manner. The sustained elevation in $[Ca^{2+}]_i$ appears to be mediated by release from intracellular calcium stores. Epibatidine was similar to nicotine in triggering induction of TH promoter activity, through cAMP by a mechanism requiring both calcium and cAMP.

4.1. Changes in intracellular calcium

The pattern of changes in $[Ca^{2+}]_i$ with epibatidine was similar to that observed with nicotine; there was not only an initial rise in $[Ca^{2+}]_i$, but also a sustained second elevation. After the first rise, $[Ca^{2+}]_i$ rapidly declined toward basal levels, presumably due to desensitization of nicotinic acetylcholine receptors. Upon continued incubation with nicotine or epibatidine a second sustained elevation in $[Ca^{2+}]_i$ was observed. The first large and sharp elevation in response to nicotine was previously shown to be inhibited by pretreatment with VOCC blockers, especially L- or N-type and less by P/Q- or T-type channel blockers (Gueorguiev et al., 1999). In this study, we found that calciseptine, an L-type channel blocker, also inhibited the epibatidine-induced rise in $[Ca^{2+}]_i$.

Antagonists of α7 nicotinic acetylcholine receptors had a marked effect on the nicotine-triggered $[Ca^{2+}]_i$ response. Pretreatment with α-bungarotoxin or methyllycaconitine reduced the first peak and prevented the prolonged rise in $[Ca^{2+}]_i$ with nicotine similar to previous studies (Gueorguiev et al., 2000; Oshikawa et al., 2003). The α7 nicotinic acetylcholine receptors have high calcium conductance which may directly affect intracellular signaling (Seguela et al., 1993). Interestingly, methyllycaconitine was also found to inhibit elevation of TH mRNA in dopaminergic cell bodies of the substantia nigra and ventral tegmental area in rats injected with nicotine (Serova and Sabban, 2002).

In contrast to their effect with nicotine, the selective α7 nicotinic acetylcholine receptor antagonists methyllycaconitine or α-bungarotoxin did not alter the epibatidine-induced rise in $[Ca^{2+}]_i$. These results indicate that epibatidine can elicit alterations in $[Ca^{2+}]_i$ by an α7 nicotinic acetylcholine receptor independent mechanism. This finding was unexpected since epibatidine can stimulate human α7 nicotinic acetylcholine receptors expressed in transfected

Xenopus oocytes (Briggs et al., 1995) and was shown to trigger elevations of $[Ca^{2+}]_i$ in the human embryonic kidney (HEK) 293 cell line stably expressing transfected human α7 nicotinic acetylcholine receptors (Delbono et al., 1997). However, in these studies, the EC50 for epibatidine is quite high—1.3 μM (Briggs et al., 1995) and 3.5 μM (Delbono et al., 1997), respectively, and other studies found that epibatidine is not very selective for α7 nicotinic acetylcholine receptors (Sullivan et al., 1994). Epibatidine was about 5000-fold less potent in the displacement of [^{125}I] α-bungarotoxin binding in rat brain membranes compared to displacement of cytosine binding to the $\alpha_4\beta_2$ nicotinic acetylcholine receptors subtype. The discrepancies regarding activation of α7 nicotinic acetylcholine receptors by epibatidine may stem from the different systems used. The artificial system of transfected cells with human α7 nicotinic acetylcholine receptors, are likely to express homomeric α7 nicotinic acetylcholine receptors. In this regard, there is an inconsistency between the rapid densensitization of homomeric α7 nicotinic acetylcholine receptors in *Xenopus* oocytes and the sustained responses to activation of α7 nicotinic acetylcholine receptors observed in many physiological systems, such as ganglionic neurons and PC12 cells (Berg and Conroy, 2002; Gueorguiev et al., 2000; Li et al., 1999). In addition, α7 nicotinic acetylcholine receptors were shown to be localized to the lipid rafts in PC12 cells (Oshikawa et al., 2003) which might alter their physiological properties compared to the receptors over-expressed in transfected cells.

The inhibition of epibatidine-triggered elevation in $[Ca^{2+}]_i$ by mecamylamine, but not by methyllycaconitine or α-bungarotoxin, demonstrates involvement of non α7 nicotinic acetylcholine receptors. The PC12 cells express several neuronal nicotinic acetylcholine receptor subtypes, including α3, α5, α7 and β2–β4 (Ishiguro et al., 1997; Rogers and Wonnacott, 1997). It remains to be determined which nicotinic acetylcholine receptor subtypes are critical for the observed response to epibatidine. Epibatidine is reported to bind heterologously expressed $\alpha_3\beta_4$ nicotinic acetylcholine receptors with potency 500-fold higher than nicotine (Meyer et al., 2001). The nicotinic acetylcholine receptors containing $\alpha_3\beta_4$ is believed to be one of the major nicotinic acetylcholine receptors in some sympathetic ganglia, sensory ganglia, adrenal gland as well as in several important regions of the CNS (Campos-Caro et al., 1997; Conroy and Berg, 1995; Zoli et al., 1998).

In the presence of mecamylamine, both agonists elicited a very slow and delayed elevation of $[Ca^{2+}]_i$ which likely involves release of intracellular calcium and might indicate that mecamylamine is no longer effective at the later times. In this regard, it has been found that inhibition of $\alpha_3\beta_2$ and $\alpha_4\beta_2$ receptors results in a slowed recovery of their acetylcholine response 10–30 min after exposure to mecamylamine (Papke et al., 2001). Physiologically, mecamylamine prevents the antinociceptive effects of epibatidine in rodents (Badio and Daly, 1994), antagonizes epibatidine-induced muscle contraction and blood pressure elevation (Lembeck, 1999; Yokotani et al., 2002).

The findings suggest that the long-term elevation in $[Ca^{2+}]_i$, induced by nicotine or by epibatidine, results mainly from the release of calcium from intracellular stores. The addition of xestospongin C, or the ryanodine receptor inhibitor dantrolene, during the second elevation of $[Ca^{2+}]_i$ rapidly reduces $[Ca^{2+}]_i$, demonstrating that the ryanodine or IP_3 sensitive calcium stores are needed for the sustained $[Ca^{2+}]_i$ elevation induced by epibatidine or nicotine. Dantrolene led to a long lasting decline in $[Ca^{2+}]_i$ to below basal levels, while xestosponginic C induced a more modest and transient inhibition. The more modest and transient nature of the response to xestospongin C, could reflect a smaller contribution of IP_3 receptor mediated calcium induced calcium release, or it may reflect instability and only partial inhibition by xestospongin C. The robust response to dantrolene may indicate a major contribution of ryanodine sensitive calcium stores to the second elevation in $[Ca^{2+}]_i$. Studies on intracellular calcium stores in PC12 cells revealed that ryanodine and IP_3 sensitive receptors are co-localized (Zacchetti et al., 1991). However, part of the response to dantrolene, especially the lowering of basal levels, may be an artifact due to a decreased Fura fluorescence and increased cell autofluorescence, which has been proposed to lead to an additional false decrease in basal calcium levels (Nohmi et al., 1991).

The importance of intracellular calcium has also been recognized in other systems as well. Intracellular calcium stores have been shown to contribute to the sustained Ca^{2+} signals activated by nicotinic acetylcholine receptors stimulation in chick cilliary ganglion neurons (Shoop et al., 2001), human neuroblastoma cells (Dajas-Bailador et al., 2002) and substantia nigra brain slices (Tsuneki et al., 2000).

4.2. Regulation of TH transcription

Several studies have shown that nicotine increases TH gene expression and protein in vivo, as well as TH activity in the rat adrenal medulla and several brain catecholaminergic areas (Fossom et al., 1991; Hiremagalur and Sabban, 1995; Jahng et al., 1997; Mitchell et al., 1993; Serova et al., 1999; Slotkin and Seidler, 1975; Sun et al., 2003). Transcriptional activation has been reported to play an important role in the induction of TH gene expression by nicotine. Chronic injections of nicotine elicited sustained increases in TH gene transcription in rat adrenal medulla which persisted up to 7 days after the final injection (Sun et al., 2003). Administration of epibatidine to rats increased expression of c-Fos as well as FGF-2 mRNA and protein levels in rat brain, including a number of TH-expressing cell types (Belluardo et al., 1998; Cucchiaro and Commons, 2003; Watanabe et al., 1998).

Our study revealed that prolonged treatment with either epibatidine or nicotine triggers a similar large induction in TH promoter driven reporter activity. A substantial elevation

in luciferase activity was elicited by 10 nM, 50 nM or 1 μM epibatidine. In addition, the changes in $[Ca^{2+}]_i$ were similar with these concentrations of epibatidine. These concentrations were chosen since they inhibit release of norepinephrine or dopamine from brain slices completely (1 μM epibatidine) or by 50% (50 nM epibatidine) (Rao et al., 2003). The findings indicate that the epibatidine-triggered elevations in $[Ca^{2+}]_i$ and induction of TH transcription in the PC12 cells are maximal with very low concentrations of epibatidine.

The elevation of $[Ca^{2+}]_i$ appears to be a necessary event for the induction of TH promoter activity by epibatidine and nicotine, since it was inhibited by intracellular calcium chelator BAPTA-AM. In this regard, experiments with bradykinin also suggested that release of intracellular calcium from IP_3 sensitive intracellular calcium stores can be sufficient to induce TH gene expression (Menezes et al., 1996).

The CRE motif at −45 to −38 in the TH promoter was previously shown to be required for its transcriptional activation by nicotine in PC12 cells (Hiremagalur et al., 1993). In addition to nicotine, this element was also shown to be involved in the activation of TH reporter driven transcription by other treatments which increase $[Ca^{2+}]_i$ such as depolarization with elevated K^+, veratridine or calcium ionophores (Kilbourne et al., 1992; Lewis-Tuffin et al., 2004; Nagamoto-Combs et al., 1997; Nankova et al., 1996; Sabban, 1997). The present study also showed that mutation of CREs reduced by over 90% the elevation of TH promoter driven reporter activity induced by either epibatidine or nicotine. There was still a small increase in activity in response to stimulation by epibatidine or nicotine, which suggests that alternative promoter sites, such as AP1 may also have a regulatory role. Further experiments are needed to ascertain whether phosphorylation of CREB is directly involved in epibatidine- or nicotine-triggered induction of TH gene transcription.

The crucial involvement of the PKA pathway in the elevation of TH promoter activity was further supported by our results. DDA inhibition of the rise in TH promoter activity 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 (Anderson et al., 1992). Pretreatment with the adenylyl cyclase inhibitor DDA prevented both the epibatidine- and nicotine-elicited induction of TH promoter activity. We found previously (Gueorguiev et al., 1999) that the inhibition of TH gene expression was effective when DDA was added 15 min, but not 60 min, after nicotine. We speculate that the activation of adenylyl cyclase by epibatidine or nicotine may be evoked 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 (Taussig and Gilman, 1995). There are several isoforms of adenylyl cyclases, and some of them are reported to be Ca^{2+} sensitive (Taussig and Gilman, 1995). The type I adenylyl cyclase is a neural-specific, Ca^{2+}-stimulated enzyme that couples $[Ca^{2+}]_i$ to cAMP increases, and could be involved in the observed responses.

Overall, the findings demonstrate that the elevation of intracellular calcium is needed to activate gene expression induced by nicotine or epibatidine. The results suggest that sustained release of $[Ca^{2+}]_i$ from intracellular calcium stores, regardless of the initiating event, may be sufficient to stimulate TH transcription.

Acknowledgements

We gratefully acknowledge the support by a grant from Philip Morris and NIH grant NS28869, and thank Shreekrishna Maharjan for preparation of the mutant TH promoter construct.

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