Nicotine Increases $[Ca^{2+}]_i$ in Rat Sublingual Mucous Acini by Stimulating Neurotransmitter Release from Presynaptic Terminals (43819)

GUO H. ZHANG*,[1] AND JAMES E. MELVIN*,†

Departments of Dental Research and of Neurobiology & Anatomy,† University of Rochester, Rochester, New York 14642*

Abstract. **The effects of nicotine on the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) were examined using the Ca^{2+}-sensitive fluorescent dyes indo-1 and fura-2 in isolated rat sublingual mucous acini. Nicotine induced a dose-dependent increase in $[Ca^{2+}]_i$. In contrast to the muscarinic agonist carbachol-induced rise in $[Ca^{2+}]_i$, the nicotine-stimulated increase was abolished in a Ca^{2+}-free medium, in the presence of L-type Ca^{2+} channel blockers (diltiazem and D888), by depolarization (high extracellular K^+), and if the intracellular Ca^{2+} pool was first depleted with thapsigargin, an endoplasmic Ca^{2+}-ATPase inhibitor. Furthermore, inhibitors of the nicotine acetylcholine receptor (mecamylamine, decamethonium, hexamethonium, tubocurarine, and α-bungarotoxin) blocked the nicotine-stimulated increase in $[Ca^{2+}]_i$ without affecting the muscarinic-stimulated $[Ca^{2+}]_i$ increase, whereas, muscarinic antagonists (atropine, pirenzepine and 4-diphenylacetoxy-N-methylpiperidine methiodide [4-DAMP]) inhibited both the nicotine- and carbachol-induced $[Ca^{2+}]_i$ increases. Nicotine stimulation increased inositol 1,4,5-trisphosphate (IP_3) content by 50%. Inhibition of the IP_3-sensitive intracellular Ca^{2+} release pathway with 8-(diethylamino)-octyl-3,4,5-trimethoxybenzoate (TMB-8) prevented the nicotine-induced increase in $[Ca^{2+}]_i$. Confocal imaging of $[Ca^{2+}]_i$ indicated that the nicotine-induced and the carbachol-induced increases in $[Ca^{2+}]_i$ occurred in the same cells within an acinus. However, in single sublingual acinar cells nicotine did not increase $[Ca^{2+}]_i$, whereas, carbachol did. Taken together, these results suggest that nicotine first triggers the release of acetylcholine from presynaptic nerve terminals associated with the dispersed sublingual acini which then activates muscarinic receptors.** [P.S.E.B.M. 1994, Vol 207]

An increase in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) regulates the secretagogue-evoked secretory response in salivary glands by regulating ion permeabilities, intracellular pH and cell volume (1–7). Current evidence suggests that the increase in $[Ca^{2+}]_i$ is generated by Ca^{2+} release from intracellular stores and by an enhanced Ca^{2+} influx across the plasma membrane (7–10). The production of inositol 1,4,5-trisphosphate (IP_3) initiates the discharge of the intracellular Ca^{2+} store by binding to IP_3 receptors located at the endoplasmic reticulum membrane (11). After the initial peak, a sustained phase of elevated $[Ca^{2+}]_i$ appears which is produced by Ca^{2+} influx from the extracellular environment (9, 10). Depletion of the IP_3-releasable Ca^{2+} store activates the Ca^{2+} entry pathway by an unknown mechanism (5, 12–14). The Ca^{2+} store-regulated Ca^{2+} influx mechanism in salivary acinar cells is not inhibited by blockers of voltage-sensitive, L-type Ca^{2+} channels (5).

Nicotine, an agonist of nicotinic acetylcholine receptors (nAchR), stimulates salivary gland secretion.

[1] To whom requests for reprints should be addressed at Department of Dental Research, University of Rochester, Box 611, Rochester, NY 14642.

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Intravenous injection of nicotine increased the salivary flow rate from rabbit parotid gland, while hexamethonium, an inhibitor of the nAchR, blocked the nicotine-induced secretion (15). The resting salivary flow from human parotid gland is 1.5–2.9 times greater during smoking (16), most likely due to the nicotine found in tobacco products. Similarly, it was found that cigarette smoking significantly increased parotid salivary secretion in both smokers and nonsmokers (17). The mechanism by which nicotine increases salivary secretions is unknown. Nicotine may activate receptors located on the surface of the salivary acinar cells, although nAchR have not been described in nonexcitable tissues. A second possibility is that nicotine stimulates fluid secretion by activating peripheral ganglia or by acting on nerve terminals located within the gland. In excitable cells, such as neurons, nicotine initiates Ca^{2+} mobilization (18–20) and influx (21, 22). Furthermore, nicotine is reported to induce catecholamine secretion in the adrenals mediated by Ca^{2+} entry (23, 24). Other studies (25–27) have observed that nicotine causes a contraction of ventricular muscle, and enhances the uptake and release of $^{45}Ca^{2+}$.

The present studies were designed to determine whether nicotine-induced salivary secretion is related to an increase in $[Ca^{2+}]_i$ in sublingual acinar cells and, if so, to characterize the mechanism responsible for the rise in $[Ca^{2+}]_i$. Our results suggest the sequential activation of two types of cholinergic receptors. Nicotine initially acts on presynaptic nerve terminals located within the gland to stimulate acetylcholine release, which, in turn, activates the $[Ca^{2+}]_i$ increase by binding to muscarinic receptors located on the surface of sublingual acinar cells.

Materials and Methods

Materials. Male Wistar strain rats (150–250 g; Charles River, Kingston facility, NY) were used in all experiments. Fura-2/acetoxymethyl ester (fura-2/AM), indo-1/AM, α-bungarotoxin, and ionomycin were from Molecular Probes (Eugene, OR). Earle's minimal essential medium was purchased from Biofluids (Rockville, MD), and collagenase (type CLSPA) was from Worthington Biomedical (Malvern, PA). Hyaluronidase (type I-S), carbamyl choline (carbachol), decamethonium bromide, hexamethonium chloride, diltiazem hydrochloride, [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]-substance P, and bovine serum albumin (BSA, Type V) were from Sigma Chemical Co. (St. Louis, MO). Nicotine and TMB-8 (8-[diethylamino]-octyl-3,4,5-trimethoxybenzoate) were from Aldrich Chem, Milwaukee, WI. Tubocurarine chloride and mecamylamine hydrochloride were from Research Biochemicals Incorporated (Natick, MA). Pirenzepine dihydrochloride and 4-DAMP (4-diphenylacetoxy-N-methylpiperidine methiodide) were from RBI Research Biomedical Inc. (Natick, MA). The D-*myo*-inositol 1,4,5-trisphosphate [^{3}H]assay system and D888 were from Amersham (Arlington Heights, IL). All other chemicals were of the highest grade available.

The digestion medium consisted of Earle's minimal essential medium, 1% bovine serum albumin, 50 units/ml collagenase, and 0.02 mg/ml hyaluronidase. Unless otherwise stated, the experiments were conducted in a Modified Earle's-Hanks' solution (MEH) containing (m*M*): 110 NaCl, 25 $NaHCO_3$, 20 Hepes, 10 glucose, 5.4 KCl, 1.2 $CaCl_2$, 0.8 $MgSO_4$, 0.4 KH_2PO_4, 0.33 NaH_2PO_4 adjusted to pH 7.4 at 23°C with NaOH after bubbling with 95% O_2/5% CO_2. The high potassium medium contained (m*M*): 115.4 KCl, 25 $NaHCO_3$, 20 Hepes, 10 glucose, 1.2 $CaCl_2$, 0.8 $MgSO_4$, 0.4 KH_2PO_4, and 0.33 NaH_2PO_4, and the pH was adjusted to 7.4 with Tris base after gassing with 95% O_2/5% CO_2. For the Ca^{2+}-free MEH, 3-m*M* EGTA was added and the pH adjusted to 7.4.

Isolation of Sublingual Acini. Rat sublingual mucous acini were isolated and enzymatically dispersed as previously described (5, 7). Briefly, three rats were sacrificed by exsanguination following exposure to CO_2. The sublingual glands were removed and finely minced in 2 ml of the ice-cold digestion medium. The minced glands were suspended in an additional 8 ml of digestion medium and incubated at 37°C in a Dubnoff shaker with continuous gassing with 95% O_2 and 5% CO_2 (humidified) and agitation at 80 cycles/min. The mince was dispersed by gently pipetting 10 times with a 10-ml plastic pipette at 15-min intervals. After 45 min of digestion, the digestion medium was replaced with fresh medium by centrifugation at 400*g* for 30 sec. The mince was digested for another 45 min. After a total of 1.5 hr of digestion, the preparation was washed three times with MEH containing 0.01% bovine serum albumin. The resulting preparation was centrifuged at 400*g* for 1 min and the pellet resuspended in MEH containing 0.01% bovine serum albumin.

Isolation of Single Acinar Cells. Rat sublingual glands were isolated and minced as above. The mince was then transferred into a siliconized 125-ml flask containing 32 ml Earle's mineral essential medium with 2.5 mg/ml collagenase/dispase (B. Mannheim, #1097113) and 2% BSA, and incubated in a 37°C shaking water bath with gassing (95% O_2/5% CO_2). The mince was dispersed by pipeting 10 times with a 1-ml pipette at 15-min intervals. The medium was replaced with fresh medium at 40-min intervals by centrifugation at 300*g* for 40 sec. After 120 min of digestion, the preparation was centrifuged at 300*g* for 40 sec. The supernatant was saved and the pellet resuspended in

the same digestion medium and incubated for another 20 min. After the additional 20 min of digestion, the preparation was centrifuged as above. The supernatants were pooled and centrifuged at 200*g* for 5 min, and the pellets (single cells) resuspended in MEH with 0.01% BSA and kept at 23°C with gassing and shaking until use.

Intracellular $[Ca^{2+}]_i$ Measurement with Fura-2. The intracellular free Ca^{2+} concentration was determined by using the Ca^{2+}-sensitive fluorescent indicator fura-2 as described previously (5). Dispersed sublingual acini and single acinar cells were loaded with fura-2 by incubating in 2 μM fura-2/AM for 30 min at 23°C. The fura-2-loaded acini and single cells were rinsed twice with MEH containing 0.01% bovine serum albumin, and resuspended in the same medium. A 0.5-ml aliquot of the fura-2-loaded acini or single acinar cells was centrifuged at 400*g* for 15 sec. The pellet was resuspended in bovine serum albumin-free MEH to promote attachment to a coverslip mounted to the bottom of a perfusion chamber on the stage of a Nikon inverted microscope interfaced to a SPEX AR-CM fluorometer (Edison, NJ). A Nikon fluor X40 1.3-NA oil immersion objective was used to isolate 5–8 acinar cells using a pinhole turret. The solutions were continuously gassed with 95% O_2 and 5% CO_2 at 23°C. The superfusion rate was 4 ml/min, and turnover time was less than 5 sec. Fluorescence ratios, obtained by using 340- and 380-nm excitation filters and a 505-nm emission filter, were converted to $[Ca^{2+}]_i$ by *in situ* calibration as described previously (3, 5). The acini or single acinar cells were superfused with Ca^{2+}-free MEH containing 3 mM EGTA and 5 μM ionomycin to obtain R_{min}, and then superfused with MEH containing 5 μM ionomycin to obtain R_{max}. The $[Ca^{2+}]_i$ was calculated according to Grynkiewicz *et al.* (28) using 224 nM was the K_d of fura-2 for Ca^{2+}.

Confocal Imaging of $[Ca^{2+}]_i$ Using Indo-1. For confocal imaging of the $[Ca^{2+}]_i$, salivary acini were treated as above except indo-1 was substituted for fura-2. Sublingual acini were loaded with 2 μM indo-1/AM for 30 min at 23°C and then rinsed twice. Indo-1-loaded acini attached to glass coverslips mounted in a perfusion chamber were monitored using an Olympus 100X 1.3-NA oil immersion objective and 225-μm pinhole on the ACAS 570 Laser Cytometer (Meridian Instr., Okemos, MI). With this objective and pinhole setting, 1-μm thick optical sections of cells were produced. Indo-1 fluorescence was collected by exciting with the UV band of a 5 W argon laser (351–363 nm) and simultaneously measuring the 405- and 485-nm emissions. The scan area was 40 × 60 μm using a scan step size of 0.2 μm. To examine whether the same population of sublingual acinar cells responded to both nicotine and carbachol, sublingual acini were first exposed to 10 μM nicotine and then 10 μM carbachol.

Determination of Inositol 1,4,5-trisphosphate Content. Cellular inositol 1,4,5-trisphosphate content was determined using a radioligand assay kit (Amersham, Arlington Heights, IL). Sublingual acini were stimulated with 10 μM nicotine or water (control) for 30 sec, then mixed with an equal volume of 1 M ice-cold trichloroacetic acid to stop the reaction. Samples were left on ice for ~15 min and then centrifuged at 2000 rpm in a microfuge at 4°C for 5 min. The supernatant was vortexed with an equal volume of diethyl ether and the ether phase discarded. This step was repeated three times. Samples were then neutralized to pH 7.0 with 0.5 M $NaHCO_3$ prior to assaying inositol 1,4,5-trisphosphate content. Protein content of samples were determined as described by Smith *et al.* (29) using bovine serum albumin as standard.

Statistics. All results are presented as means ± SEM. Comparisons were made using the unpaired Student's t test.

Results

Nicotine-Induced Increase in $[Ca^{2+}]_i$. The basal $[Ca^{2+}]_i$ in rat sublingual acini was 57.8 ± 3.5 nM (n = 19), comparable to the previously reported level (5, 13). Stimulation of fura-2-loaded acini with nicotine (10 μM) resulted in a 2.7-fold initial (155.7 ± 12.6 nM, $t_{1/2}$ = 33.4 ± 3.0 sec; n = 19) and 1.7-fold (100.8 ± 10.6 nM, n = 19) sustained increase in $[Ca^{2+}]_i$ (Fig. 1A). The biphasic nature of these results are similar, but of a lesser magnitude, to earlier results for muscarinic stimulation in these cells (5, 13). The muscarinic agonist carbachol (10 μM) induced a 4.6-fold initial elevation ($t_{1/2}$ = 4.9 ± 1.1 sec) and 3.5-fold sustained increase in $[Ca^{2+}]_i$ (n = 8) (Fig. 1A). The $[Ca^{2+}]_i$ increase was nicotine concentration-dependent (initial, K_d = 4.5 ± 1.5 μM; sustained, K_d = 0.03 ± 0.01 μM; n = 4; Fig. 1B). When the nicotine concentration was higher than 2 μM, the $[Ca^{2+}]_i$ response consisted of a large transient increase followed by a sustained elevation of lesser magnitude (Fig. 1B). Confocal imaging of indo-1-loaded acini demonstrated that nicotine and carbachol increased $[Ca^{2+}]_i$ uniformly within sublingual acini (data not shown), suggesting that the same cells within an acinus respond to both agonists, and, thus, that a subpopulation of cells is not responsible for the response to nicotine.

In an attempt to differentiate the potential mechanisms involved in the nicotine-induced increase in $[Ca^{2+}]_i$, isolated single acinar cells were sequentially stimulated with nicotine and carbachol. The resting level of $[Ca^{2+}]_i$ in isolated single acinar cells was 56.3 ± 9.6 nM (n = 10). Nicotine (10 μM) did not alter $[Ca^{2+}]_i$ (55.3 ± 9.4 nM, n = 10). However, stimulation of the same cells with carbachol (10 μM) induced a 4.4-fold increase in $[Ca^{2+}]_i$ (245.4 ± 24.6 nM, n = 10) (Fig. 2), similar to the results in intact acini (Fig. 1A).

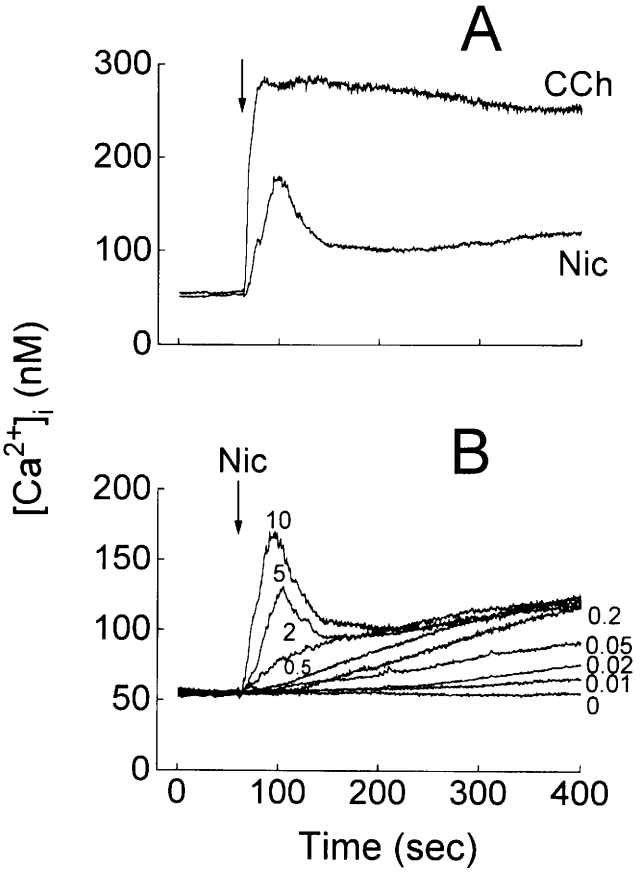

Figure 1. Effects of nicotine and carbachol on $[Ca^{2+}]_i$ in isolated rat sublingual acini. Fura-2-loaded acini were attached to coverslips and superfused with MEH. At the time indicated by the arrows, acini were stimulated with carbachol (CCh, 10 μM) or different concentrations of nicotine. (A) Comparison between the $[Ca^{2+}]_i$ responses induced by carbachol (CCh, 10 μM) and by nicotine (Nic, 10 μM). Each trace is representative (CCh, n = 8; Nic, n = 19). (B) Nicotine concentration dependence of the $[Ca^{2+}]_i$ response. The percentage increase of $[Ca^{2+}]_i$ was determined at 5 min for the sustained increase and at 30 sec for the initial increase. The K_d was determined using a nonlinear regression analysis program (K.cat; BioMetallics, Princeton, NJ). Each trace is representative of separate experiments using different acinar preparations (n = 6).

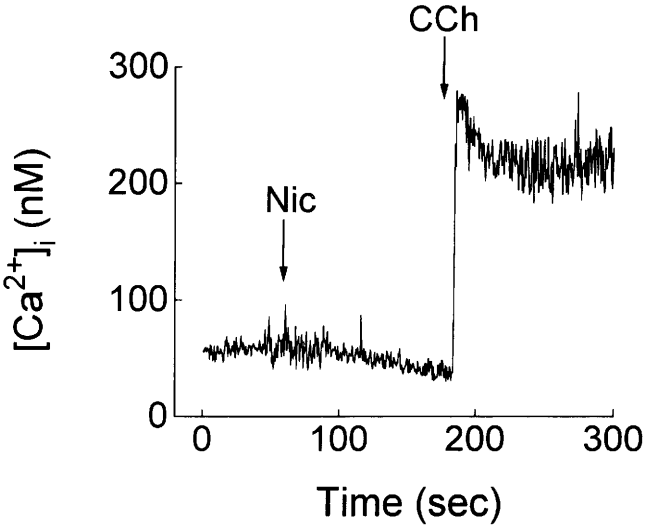

Figure 2. Nicotine- and carbachol-induced $[Ca^{2+}]_i$ increase in single sublingual acinar cells. Single sublingual acinar cells were isolated and loaded with fura-2 as described in Materials and Methods. Fura-2-loaded single cells were attached to a glass coverslip, and $[Ca^{2+}]_i$ was monitored as described in Figure 1. At the time indicated by the arrows, 10 μM nicotine (Nic) and 10 μM carbachol (CCh) were added. The trace is representative of 10 separate experiments.

These results suggest that the sublingual acinar cells do not directly respond to nicotine stimulation.

Role of Ca^{2+} Influx in the Nicotine-Induced $[Ca^{2+}]_i$ Increase. Secretagogues, including substance P and the muscarinic agonist carbachol, increase $[Ca^{2+}]_i$ in salivary acini by releasing an intracellular Ca^{2+} store, depletion of which activates Ca^{2+} uptake (5, 12, 13, 30). When EGTA was added to the perfusion medium to chelate extracellular Ca^{2+}, the magnitude of the muscarinic-stimulated transient Ca^{2+} increase did not change, but the sustained increase was abolished (Fig. 3A). In contrast, both the initial and the sustained nicotine-induced $[Ca^{2+}]_i$ increase were prevented in the absence of extracellular Ca^{2+} (Fig. 3A). Nicotine pretreatment did not influence the $[Ca^{2+}]_i$ response to carbachol in a Ca^{2+}-free medium (Fig. 3B). These results may suggest that extracellular Ca^{2+} is the source of the nicotine-induced $[Ca^{2+}]_i$ elevation in acini and therefore depletion of the intracellular Ca^{2+} store is not required. However, as shown in Figure 2, nicotine did not induce an increase in $[Ca^{2+}]_i$ in single acinar cells in the presence of 1.2-mM external Ca^{2+}.

A second possibility is that extracellular Ca^{2+} influx is necessary to trigger neurotransmitter release from presynaptic nerve terminals (23, 24). The nicotine-induced $[Ca^{2+}]_i$ increase in heart muscle cells is inhibitable by L-type Ca^{2+} channel blockers (25, 26). As shown in Figure 4, diltiazem (10 μM) and D888 (10 μM), blockers of voltage-gated Ca^{2+} channels, inhibited 94.7% ± 3.8% (n = 6) and 87.1% ± 5.3% (n = 5) of the nicotine-induced $[Ca^{2+}]_i$ increase, respectively (Fig. 4). In contrast, the muscarinic-induced increase in $[Ca^{2+}]_i$ is insensitive to L-type Ca^{2+} channel blockers (5). It should be noted that although the inhibition of the effect of nicotine by the various blockers is dramatic, a small and slow increase in $[Ca^{2+}]_i$ occurs, apparently due to incomplete inhibition. Since voltage-gated Ca^{2+} channels play a critical role in neurotransmitter release from nerve terminals, the Ca^{2+} channel agonist Bay K 8644 (31, 32) was applied to study their potential role in the nicotinic response. Bay K 8644 (100 nM) induced a 41.6% increase in the initial and a 70% increase in the sustained $[Ca^{2+}]_i$ (n = 6) (Fig. 5) suggesting the involvement of nerve terminals.

Furthermore, membrane depolarization (high extracellular K^+) prevented the nicotine-stimulated increase in $[Ca^{2+}]_i$; conversely, as previously reported (33), the initial carbachol-stimulated increase in

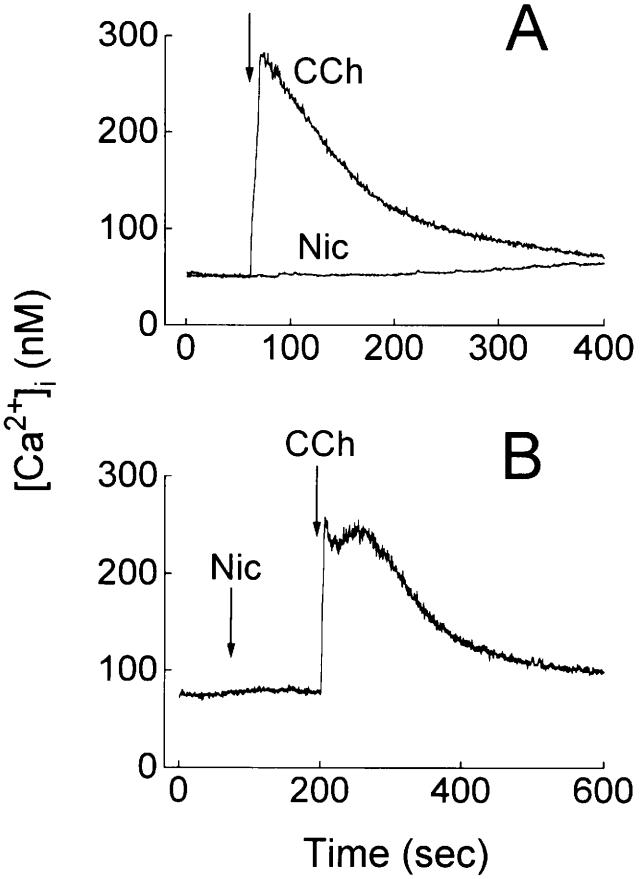

Figure 3. Dependence of the nicotine- and carbachol-induced $[Ca^{2+}]_i$ increases on extracellular Ca^{2+}. (A) Fura-2-loaded acini were superfused with MEH containing 3 m*M* EGTA. Carbachol (CCh, 10 μ*M*) or nicotine (Nic, 10 μ*M*) was added at the time indicated by the arrow. The trace is representative of separate experiments using different acinar preparations (CCh, $n = 8$; Nic, $n = 7$). (B) Fura-2-loaded acini were stimulated in a Ca^{2+}-free solution with nicotine (Nic, 10 μ*M*) and carbachol (CCh, 10 μ*M*) at the times indicated by the arrows. The trace is representative of five separate experiments using different acinar preparations.

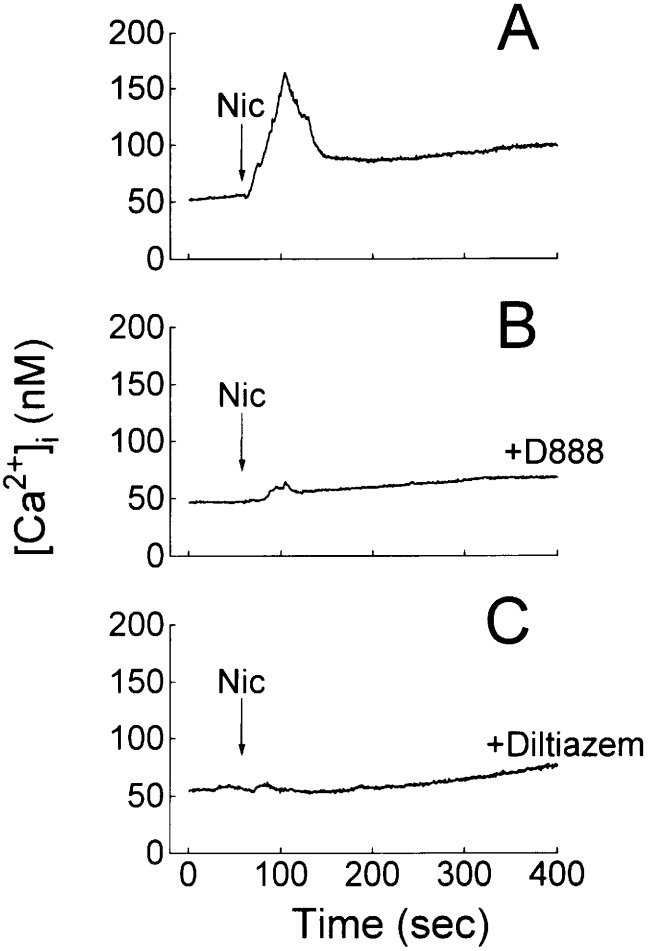

Figure 4. Inhibition of the nicotine-induced $[Ca^{2+}]_i$ increase by voltage-gated Ca^{2+} channel blockers. Fura-2-loaded acini were superfused with MEH. D888 (10 μ*M*) and diltiazem (10 μ*M*) were added approximately 2 min prior to stimulation. Acini were then stimulated with 10 μ*M* nicotine (Nic) at the time indicated by the arrow. Each trace is representative of separate experiments using different acinar preparations (control, $n = 19$; +D888, $n = 5$; +diltiazem, $n = 7$).

$[Ca^{2+}]_i$ was not suppressed by depolarization (Fig. 6A). The above results are consistent with the nature of nicotinic acetylcholine receptor (nAchR) channels (34). Membrane depolarization mimicked the effect of nicotine on $[Ca^{2+}]_i$, inducing a 3-fold initial increase in $[Ca^{2+}]_i$ (from 46.4 ± 5.3 n*M* to 146.4 ± 9.2 n*M*, $n = 14$; Fig. 6B). The muscarinic antagonist atropine (10 μ*M*) prevented the depolarization-induced increase in $[Ca^{2+}]_i$ (from 49.1 ± 3.9 n*M* to 57.1 ± 5.8 n*M*, $n = 6$) (Fig. 6B). This suggests that the effect of membrane depolarization, like nicotine stimulation, activates Ca^{2+} influx in presynaptic nerve terminals, inducing neurotransmitter release and activation of muscarinic receptors on the sublingual acinar cells. Therefore, the blockade of the nicotinic effect by high K^+ is apparently due to the prior release of the acetylcholine pool by depolarization.

Effects of Nicotinic Acetylcholine Receptor Antagonists on the Stimulation-Activated $[Ca^{2+}]_i$ Increase. The mechanism responsible for nicotine-stimulated Ca^{2+} influx in neurons, heart muscle, vascular muscle, and adrenal cells is the nAchR-channel (21, 22, 35, 36). Nicotine may stimulate the $[Ca^{2+}]_i$ increase in sublingual acini by activating the nAchR, and thus opening nAchR channels (35, 37). To test this possibility, inhibitors of the nAchR were used in an attempt to block the nicotine-stimulated $[Ca^{2+}]_i$ increase. Mecamylamine (10 μ*M*), hexamethonium (10 μ*M*), decamethonium (50 μ*M*), tubocurarine (10 μ*M*), and α-bungarotoxin (1 μg/ml), potent inhibitors of the nAchR channel (36), inhibited Ca^{2+} influx by 92.6% ± 2.8%, 89.7% ± 4.3%, 75.8% ± 4.5%, 78.7% ± 3.4%, and 97.9% ± 0.7%, respectively (Table I). In contrast, the carbachol-induced $[Ca^{2+}]_i$ increase was not influ-

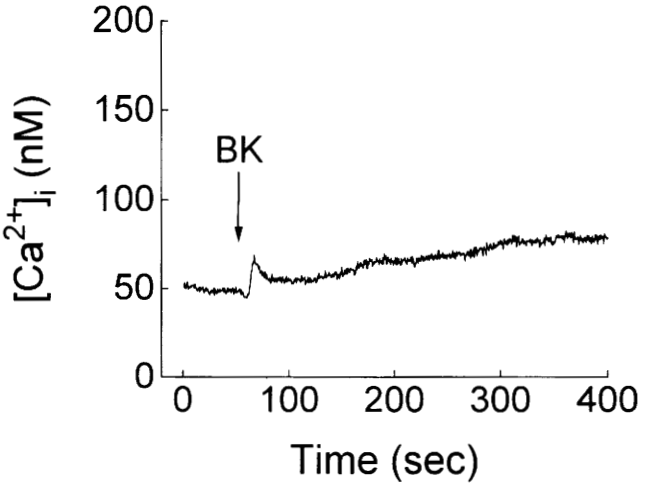

Figure 5. Effect of Bay K 8644 on $[Ca^{2+}]_i$ in isolated sublingual acini. Fura-2-loaded rat sublingual acini were attached to coverslips and superfused with MEH as described in Figure 1. At the time indicated by the arrow, 100 n*M* Bay K 8644 (BK) was added. The trace is representative of separate experiments using different acinar preparations (n = 6).

enced by these antagonists (Table I). These results suggest that the nicotine-induced $[Ca^{2+}]_i$ increase is mediated by activating nAchR channels located on either the sublingual acinar cells or on presynaptic nerve terminals isolated along with the acini.

Effects of Muscarinic Receptor Inhibition on the Nicotine-Induced $[Ca^{2+}]_i$ Increase. The muscarinic receptor antagonists, pirenzepine, 4-DAMP, and atropine blocked the nicotine-induced rise in $[Ca^{2+}]_i$ (Table II), suggesting that the nicotine-induced increase in $[Ca^{2+}]_i$ is mediated by activation of the muscarinic receptors. To examine whether the nicotine-stimulated $[Ca^{2+}]_i$ increase involves the release of a second neurotransmitter, the effect of a substance P (Sub P) receptor antagonist on the nicotine-stimulated $[Ca^{2+}]_i$ increase was assessed. The Sub P receptor inhibitor, [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]-substance P (50 μM), did not influence the nicotine-stimulated $[Ca^{2+}]_i$ increase (data not shown) indicating that substance P is not involved in the nicotine-induced increase in $[Ca^{2+}]_i$.

Role of the Intracellular Ca^{2+} Store in the Nicotine-Induced Increase in $[Ca^{2+}]_i$. TMB-8, an inhibitor of the IP_3-sensitive, intracellular Ca^{2+} release mechanism blocks the muscarinic agonist-stimulated increase in $[Ca^{2+}]_i$ in muscle, colonic, fibroblast, and sublingual cells by inhibiting the release of Ca^{2+} from the sarcoplasmic reticulum and endoplasmic reticulum (39–42). To examine whether Ca^{2+} release is involved in the nicotine-induced increase in $[Ca^{2+}]_i$, the effect of TMB-8 on the nicotine-induced rise in $[Ca^{2+}]_i$ was determined. TMB-8 (50 μM) prevented the nicotine-stimulated increase in $[Ca^{2+}]_i$ (Fig. 7 and Table II), suggesting that nicotine increases $[Ca^{2+}]_i$ not by activating Ca^{2+} uptake but by stimulating the release of acetylcholine from presynaptic nerve terminals and activating muscarinic receptors. Indeed, the nicotine-induced increase in $[Ca^{2+}]_i$ is from the same muscarinic-releasable store since after muscarinic stimulation, nicotine (10 μM) did not induce a further increase in $[Ca^{2+}]_i$ (Fig. 8A). Similarly, nicotine did not induce a further Ca^{2+} release after stimulation with thapsigargin (Fig. 8B), a Ca^{2+}-ATPase inhibitor which empties the IP_3-sensitive Ca^{2+} store (5, 13). These results indicate that the nicotine-induced increase in $[Ca^{2+}]_i$ is mediated by muscarinic stimulated Ca^{2+} release from the IP_3-sensitive Ca^{2+} store.

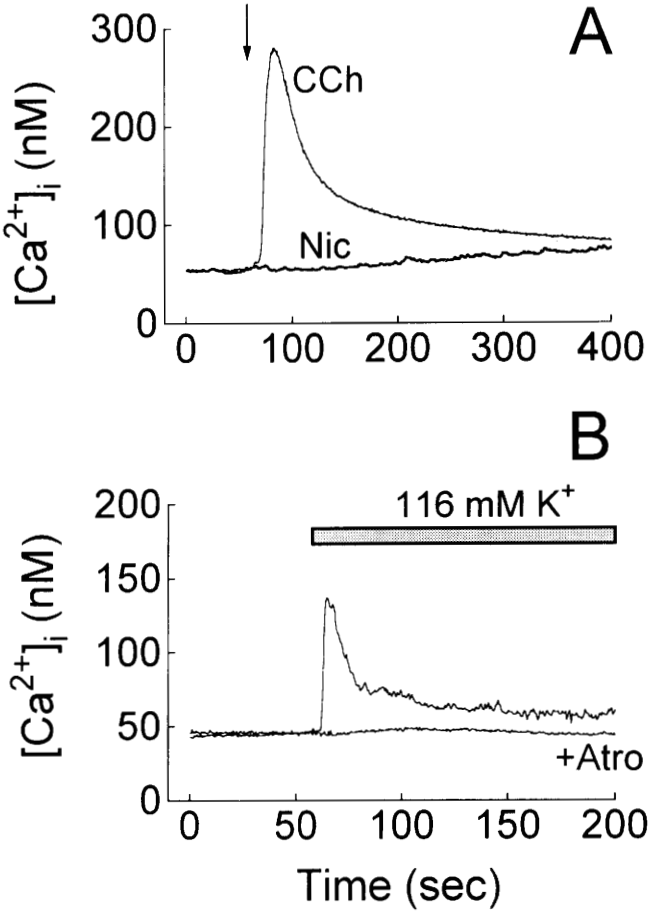

Figure 6. Effects of membrane depolarization in $[Ca^{2+}]_i$ in sublingual acini. (A) Fura-2-loaded sublingual acini were superfused with MEH and the superfusion solution was changed to the high potassium medium approximately 2 min prior to stimulation. Acini were then stimulated with 10 μM carbachol (CCh) or 10 μM nicotine (Nic) at the time indicated by the arrow. Each trace is representative of separate experiments using different acinar preparations (CCh, n = 8; Nic, n = 5). (B) Fura-2-loaded sublingual acini were superfused with MEH or MEH containing 10 μM atropine (+Atro) and the superfusion solution was changed to the high potassium medium at the time indicated by the bar. Traces are representative of separate experiments (control, n = 14; +Atro, n = 6).

Effect of Nicotine on 1,4,5-IP_3 Generation. The muscarinic agonist-induced increase in $[Ca^{2+}]_i$ in sal-

Table I. Effects of Nicotinic Acetylcholine-Receptor Antagonists on Carbachol- and Nicotine-stimulated $[Ca^{2+}]_i$ Increases[a]

Agonist	Antagonist	n[b]	$[Ca^{2+}]_i$ (n*M*)	
			Resting	Initial
Nicotine	Control	9	59.9 ± 3.9	186.0 ± 15.0
	Mecamylamine	4	43.1 ± 9.7	50.4 ± 11.3[c]
	Hexamethonium	7	41.7 ± 4.2	51.0 ± 5.8[c]
	Decamethonium	6	51.5 ± 4.1	79.9 ± 8.4[c]
	Tubocurarine	6	66.5 ± 6.2	96.7 ± 6.8[c]
	α-Bungarotoxin	5	59.5 ± 9.7	62.7 ± 10.8[c]
CCh	Control	8	52.6 ± 6.3	295.1 ± 19.2
	Mecamylamine	4	62.7 ± 3.3	277.9 ± 24.5
	Hexamethonium	5	55.4 ± 11.0	304.4 ± 28.5
	Decamethonium	5	57.3 ± 4.8	327.2 ± 25.4
	Tubocurarine	5	63.2 ± 5.2	236.0 ± 27.3
	α-Bungarotoxin	5	53.1 ± 1.8	257.5 ± 19.9

[a] Fura-2-loaded acini were superfused for approximately 2 min in MEH without (control) or with nicotonic acetylcholine receptor antagonist and then stimulated with 10 μ*M* carbachol (CCh) or 10 μ*M* nicotine. The antagonist concentrations used were: 10 μ*M* mecamylamine, 10 μ*M* hexamethonium, 50 μ*M* decamethonium, 10 μ*M* tubocurarine and 1 μg/ml α-bungarotoxin. The initial agonist-induced $[Ca^{2+}]_i$ increase was measured approximately 30 sec after stimulation.
[b] The number of experiments in each condition.
[c] Values are significantly different from control, $P < 0.001$.

Table II. Effects of Muscarinic Receptor Antagonists and TMB-8 on Nicotine- and CCh-stimulated $[Ca^{2+}]_i$ Increases[a]

Agonist	Antagonist	n[b]	$[Ca^{2+}]_i$ (n*M*)	
			Resting	Initial
Nicotine	Control	19	57.8 ± 3.6	155.7 ± 12.6
	Pirenzepine	7	38.5 ± 4.1	52.7 ± 7.2**
	4-DAMP	6	46.3 ± 5.8	51.3 ± 6.8**
	Atropine	7	56.9 ± 5.2	79.5 ± 6.2*
	TMB-8	8	54.6 ± 4.6	56.6 ± 4.8**
Carbachol	Control	11	50.8 ± 5.8	285.6 ± 13.4
	Pirenzepine	5	57.5 ± 9.3	76.3 ± 7.5**
	4-DAMP	6	74.6 ± 13.8	88.5 ± 10.5**
	Atropine	6	60.3 ± 4.7	89.4 ± 8.9**
	TMB-8	5	56.6 ± 1.8	63.3 ± 4.4**

[a] Fura-2-loaded acini were superfused for approximately 2 min in MEH without (control) or with receptor antagonists or TMB-8 and then stimulated with 10 μ*M* nicotine or 10 μ*M* carbachol. The antagonist concentrations used were: 10 μ*M* pirenzepine, 0.1 μ*M* 4-DAMP, 0.1 μ*M* atropine, and 50 μ*M* TMB-8. The initial agonist-induced $[Ca^{2+}]_i$ increase was measured approximately 30 sec after stimulation.
[b] The number of experiments in each condition.
*,** Values are significantly different from control, $P < 0.002$ and 0.001, respectively.

ivary acinar cells is triggered by an increase in 1,4,5-IP_3 (43, 44). To confirm the involvement of muscarinic receptors in the nicotine-stimulated increase in $[Ca^{2+}]_i$, 1,4,5-IP_3 content was measured. As shown in Figure 9, nicotine increases the 1,4,5-IP_3 content of sublingual acini approximately 50%. This observation is consistent with the hypothesis that acetylcholine is released from presynaptic terminals, activating muscarinic receptors located on the sublingual acinar cells.

Discussion

Nicotine is an agonist of acetylcholine receptors and the addictive substance of tobacco products. Nicotine stimulates saliva secretion in humans and animals (15–17). Infusion of nicotine causes increased salivation in experimental animals (15) and is most likely the agent responsible for the increased salivary flow rates observed in smokers (16, 17). The mechanism by which nicotine increases salivary secretions is unknown. Recent studies indicate that nicotine induces an increase in $[Ca^{2+}]_i$ in excitable tissues, including neurons (18–22), adrenal chromaffin cells (23, 24), vascular smooth muscle cells (25–27), and heart muscle cells (27). Since a rise in $[Ca^{2+}]_i$ is required to stimulate fluid secretion in salivary glands (1–7), it is postulated that the stimulatory effect of nicotine on salivary secretion results from an increase in $[Ca^{2+}]_i$. However, it is unknown whether nicotine induces an

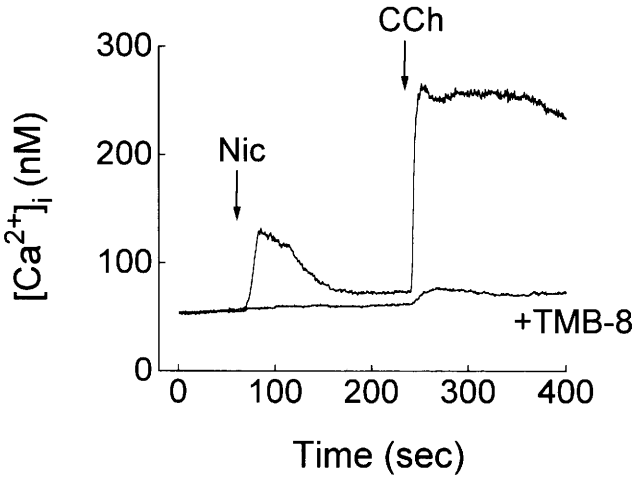

Figure 7. Effect of TMB-8 on the nicotine-induced increase in $[Ca^{2+}]_i$. Fura-2-loaded sublingual acini were superfused with MEH or MEH containing 50 μM TMB-8 (+TMB-8). Nicotine (Nic, 10 μM) and carbachol (CCh, 10 μM) were added at the time indicated by the arrows. The traces are representative of separate experiments (control, n = 10; +TMB-8, n = 8).

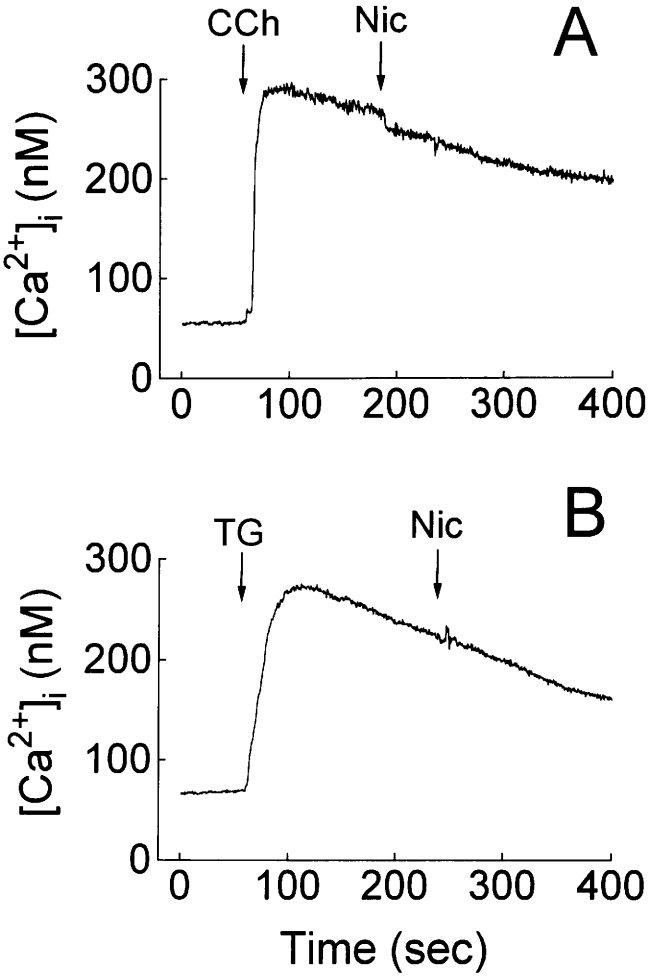

Figure 8. Role of Ca^{2+} release from the IP_3-sensitive Ca^{2+} store in the nicotine-induced increase in $[Ca^{2+}]_i$. (A) Fura-2-loaded sublingual acini were superfused with MEH and stimulated first with carbachol (CCh, 10 μM), then nicotine (Nic, 10 μM) at the time indicated by the arrows (n = 7). (B) Fura-2-loaded sublingual acini were superfused and stimulated first with the endoplasmic Ca^{2+}-ATPase inhibitor thapsigargin (TG, 2 μM), then with nicotine (Nic, 10 μM) at the time indicated by the arrows (n = 7).

increase in $[Ca^{2+}]_i$ in salivary gland acinar cells. The present study clearly demonstrates that nicotine activates a $[Ca^{2+}]_i$ increase in isolated rat sublingual acini. The K_d for the nicotine-stimulated $[Ca^{2+}]_i$ increase is 30 nM, which is in the range of blood nicotine levels in smokers (45).

It is reported that nerve terminals are still present and functioning in dispersed salivary acinar cell preparations (46, 47). Our data suggest that the initial step in the nicotine-induced $[Ca^{2+}]_i$ response is related to activation of nAchR located on presynaptic terminals. First, the nicotine-stimulated $[Ca^{2+}]_i$ increase was inhibited by nicotinic acetylcholine receptor antagonists hexamethonium, decamethonium, tubocurarine, mecamylamine, and α-bungarotoxin (Table I). Second, Figure 3 demonstrates the extracellular Ca^{2+}-dependence of the nicotinic response in sublingual acini. Neurotransmitter release is known to require Ca^{2+} influx (23, 24). Third, the nicotine-induced $[Ca^{2+}]_i$ increase was inhibitable by L-type Ca^{2+} channel blockers (Fig. 4), as previously reported in heart muscle cells (25, 26). Fourth, the nicotine-induced increase in $[Ca^{2+}]_i$ was prevented by membrane depolarization, whereas the initial muscarinic-induced increase from the intracellular Ca^{2+} pool was not. Ca^{2+} influx would be markedly reduced by depolarization due to the decrease in the electrochemical driving force resulting in the prevention of neurotransmitter release. These results strongly suggest that the nicotinic acetylcholine receptor is involved in the nicotine-induced increase in $[Ca^{2+}]_i$.

The increase in $[Ca^{2+}]_i$ observed in the present study is not associated with activation of the postganglionic nerve cell bodies since they are excluded in this preparation. Under these experimental conditions, nicotine must therefore act by stimulating receptors located on either the surface of the acinar cells or on presynaptic nerve terminals isolated in association with the sublingual acini. Single acinar cells did not respond to nicotine, but did respond to muscarinic stimulation (Fig. 2). In contrast, acini responded to both agonists. Nicotinic acetylcholine receptors are therefore not located on the acinar cells.

Consistent with this hypothesis, our data suggest that muscarinic receptors are involved in the nicotine-stimulated increase in $[Ca^{2+}]_i$ and the increase in $[Ca^{2+}]_i$ is produced by Ca^{2+} release from the IP_3-sensitive store. We found that the muscarinic antagonists, atropine, pirenzepine, and 4-DAMP prevent the nicotine-induced increase in $[Ca^{2+}]_i$ (Table II) indicat-

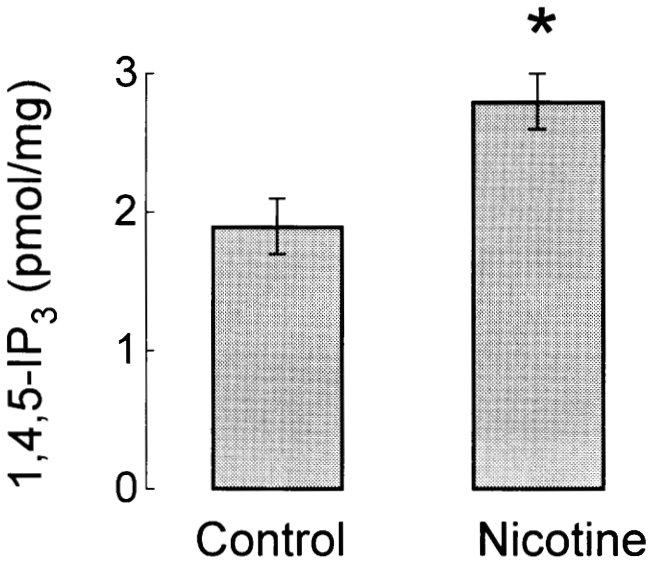

Figure 9. Nicotine-induced increase in 1,4,5-IP_3. Isolated rat sublingual acini were stimulated with 10 μM nicotine for 40 sec. 1,4,5-IP_3 content was then determined using a radioligand assay kit. Each bar illustrates the average response from five separate preparations (mean ± SEM). *Significantly different from control, $P < 0.02$.

ing the involvement of muscarinic receptors. Additionally, emptying of the IP_3-sensitive Ca^{2+} store by the muscarinic agonist carbachol or the endoplasmic Ca^{2+}-ATPase inhibitor thapsigargin completely abolished the nicotine-induced increase in $[Ca^{2+}]_i$, suggesting that the same mechanism is responsible for the nicotine-induced and muscarinic-stimulated increases in $[Ca^{2+}]_i$. Furthermore, nicotine increased 1,4,5-IP_3 production (Fig. 9). Muscarinic receptor-activated increases in $[Ca^{2+}]_i$ require the generation of 1,4,5-IP_3. 1,4,5-IP_3 then activates the release of the intracellular Ca^{2+} store by binding to IP_3-receptors located at the endoplasmic reticulum membrane (11). Finally, inhibition of Ca^{2+} release from the IP_3-sensitive intracellular store by TMB-8 blocked the nicotine-induced increase in $[Ca^{2+}]_i$ (Fig. 7, Table II), suggesting that the nicotine-induced increase in $[Ca^{2+}]_i$ is produced by Ca^{2+} release from the IP_3-sensitive store. It is highly likely that nicotine thus acts by sequentially activating two types of cholinergic receptors. Nicotine initially stimulates the discharge of acetylcholine from presynaptic terminals by activating nicotinic receptors. Acetylcholine then initiates the increase in $[Ca^{2+}]_i$ via muscarinic receptors located on the surface of the sublingual acinar cells. Our data, therefore, indicate that the isolated nerve terminals in this acinar preparation retain the ability to release neurotransmitters in response to nicotine. Parasympathetic nerves may also release substance P, which has been reported to stimulate an increase in $[Ca^{2+}]_i$ (48). The nicotine-induced increase in $[Ca^{2+}]_i$ is not mediated by substance P, since the substance P antagonist [D-Arg^1, D-Phe^5, D-$Trp^{7,9}$, Leu^{11}]-substance P did not influence the effect of nicotine on $[Ca^{2+}]_i$.

In summary, the nicotine-stimulated $[Ca^{2+}]_i$ increase in dispersed sublingual acini is most likely triggered by the activation of the nAchR channel located on presynaptic nerve terminals. The nicotine-induced $[Ca^{2+}]_i$ increase was sensitive to L-type channel blockers, nAchR antagonists, membrane depolarization, and the absence of extracellular Ca^{2+}, whereas, the initial muscarinic agonist-induced $[Ca^{2+}]_i$ increase was insensitive to all of these manipulations. Both nicotine- and carbachol-induced increases in $[Ca^{2+}]_i$ were sensitive to muscarinic antagonists, indicating that the effect of nicotine on the $[Ca^{2+}]_i$ was mediated by releasing acetylcholine from presynaptic nerve terminals and activating muscarinic receptors on the surface of the acini. Consistent with this hypothesis, the nicotine-stimulated $[Ca^{2+}]_i$ increase is associated with an increase in the IP_3 content; is blocked by TMB-8, an inhibitor of the intracellular Ca^{2+} release mechanism, or by prior depletion of the intracellular $[Ca^{2+}]_i$ pool with thapsigargin; and is insensitive to a substance P antagonist. Further experiments to examine the nicotinic response might include parasympathetic denervation or the use of agonists to modify acetylcholine metabolism. Since Ca^{2+} plays a critical role in the regulation of the secretory process in salivary glands (1–7), the effects of nicotine on $[Ca^{2+}]_i$ is of physiological and pathological significance and provides an explanation for the increased salivation observed in smokers.

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