Activation of Nicotinic Acetylcholine Receptors Augments Calcium Channel-mediated Exocytosis in Rat Pheochromocytoma (PC12) Cells

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ABSTRACT The functional effect of activating Ca\(^{2+}\)-permeable neuronal nicotinic acetylcholine receptors (nAChRs) on vesicle secretion was studied in PC12 cells. Single cells were patch-clamped in the whole-cell configuration and stimulated with either brief pulses of nicotine to activate the Ca\(^{2+}\)-permeable nAChRs or with voltage steps to activate voltage-dependent Ca\(^{2+}\) channels. Membrane capacitance was used as a measure of vesicle secretion. Activation of nAChRs by nicotine application to cells voltage clamped at \(-80\) mV evoked secretion. This secretion was completely abolished by nicotinic antagonists. When the cells were voltage clamped at \(+20\) mV in the presence of Cd\(^{2+}\) to block voltage-activated Ca\(^{2+}\) channels, nicotine elicited a small amount of secretion. Most interestingly, when the nAChRs were activated coincidentally with voltage-dependent Ca\(^{2+}\) channels, secretion was augmented approximately twofold over the secretion elicited with voltage-dependent Ca\(^{2+}\) channels alone. Our data suggest that Ca\(^{2+}\) influx via nAChRs affects Ca\(^{2+}\)-dependent cellular functions, including vesicle secretion. In addition to the secretion evoked by nAChR activation at hyperpolarized potentials, we demonstrate that even at depolarized potentials, nAChRs provide an important Ca\(^{2+}\) entry pathway underlying Ca\(^{2+}\)-dependent cellular processes such as exocytosis.

KEY WORDS: acetylcholine receptor • exocytosis • Ca\(^{2+}\) channels • nicotine • secretion

INTRODUCTION

Activation of excitatory receptors, such as nicotinic acetylcholine receptors (nAChRs), is known to depolarize cells and activate voltage-gated ion channels. It is widely accepted that when nAChRs are activated, the membrane potential is depolarized, which activates voltage-dependent Ca\(^{2+}\) channels and allows Ca\(^{2+}\) influx. Recent findings indicate that nAChRs themselves serve as Ca\(^{2+}\) entry pathways (Miledi et al., 1980; Zhou and Neher, 1993; Decker and Dani, 1990; and Zhang et al., 1996). The nAChR family includes muscle and neuronal receptor subtypes (for review, see Lindstrom et al., 1990). The neuronal nAChRs are thought to be pentameric complexes composed of different subunit combinations. Eight ligand binding subunits (\(\alpha\)-\(\alpha\)) have been identified in molecular cloning studies, as have three structural subunits (\(\beta\)-\(\beta\)) (for review, see Sargent, 1993). Many of the neuronal nAChRs, unlike the muscle nAChR, have a relatively high permeability to Ca\(^{2+}\) (Decker and Dani, 1990; Muller et al., 1992; Vernino et al., 1992; Vijayaraghavan et al., 1992; Zhou and Neher, 1993; Rathouz and Berg, 1994; Vernino et al., 1994; Zhang et al., 1994; Castro and Albuquerque, 1995). The permeability of these nAChRs to Ca\(^{2+}\) is similar to that of another ligand-gated channel, the N-methyl-d-aspartate (NMDA) receptor (Mayer and Westbrook, 1987). More recently, \(P_{2x}\) purinergic receptors have been shown to be Ca\(^{2+}\)-permeable (Bean, 1992). Thus, different ligand-gated excitatory receptors provide pathways for Ca\(^{2+}\) entry that are independent of voltage-gated Ca\(^{2+}\) channels.

The physiological importance of the nAChRs as a Ca\(^{2+}\) entry pathway is becoming better understood. Recent experiments by McGehee et al. (1995), Alkondon et al. (1992), and Gray et al. (1996) suggest that activation of presynaptic AChRs results in an influx of Ca\(^{2+}\), which increases presynaptic Ca\(^{2+}\) levels and enhances synaptic transmission from central neurons. Furthermore, Mollard et al. (1995) demonstrated that activation of nAChRs in chromaffin cells, which were voltage clamped at negative potentials to avoid activation of voltage-dependent Ca\(^{2+}\) channels, elicited catecholamine secretion. These studies suggested that the Ca\(^{2+}\) influx via nAChRs may play an important role in vesicle release by summatting with Ca\(^{2+}\) influx that occurs under physiological conditions of membrane depolarization.

To study the role of nAChRs in a single cell, we focused on the rat pheochromocytoma cell line (PC12 cells) as a model for Ca\(^{2+}\)-dependent exocytosis. PC12 cells, before differentiation, share many properties with...
adrenal medullary chromaffin cells. After differentiation, PC12 cells acquire many characteristics that resemble sympathetic neurons both morphologically and pharmacologically. For example, they develop flattened, nonspherical cell bodies and extend long, branching neurites (Greene and Tischler, 1976). Their secretory vesicles are redistributed from the somata to the neurites, and they secrete both catecholamines and acetylcholine in a Ca\(^{2+}\)-dependent manner (Greene and Tischler, 1982). Differentiated PC12 cells have nAChRs that have pharmacological and biophysical properties similar to the nAChRs in sympathetic neurons (Sands and Barish, 1991; Ifune and Steinbach, 1992; and Furukawa et al., 1994). These features, as well as the relative homogeneity of the cell population, make PC12 cells an ideal model system to compare Ca\(^{2+}\)-dependent secretion elicited by activation of nAChRs with secretion elicited by activation of voltage-dependent Ca\(^{2+}\) channels.

In this study, we have investigated whether nAChRs can serve as a functional Ca\(^{2+}\) entry pathway to elicit Ca\(^{2+}\)-dependent exocytosis under conditions designed to mimic the physiological effects of activating nAChRs. Brief nicotine applications triggered large amounts of secretion when applied to the cell voltage clamped at negative potentials to preclude activation of voltage-gated Ca\(^{2+}\) channels. Furthermore, when nicotine applications were timed to coincide with depolarization to simultaneously activate both nAChRs and voltage-dependent Ca\(^{2+}\) channels, secretion was dramatically augmented compared with secretion elicited by voltage-dependent Ca\(^{2+}\) channels alone. Overall, the data provide direct evidence that activation of nAChRs functions as an important Ca\(^{2+}\) entry pathway underlying Ca\(^{2+}\)-dependent cellular processes such as exocytosis.

**Materials and Methods**

**Cell Culture**

A rat PC12 cell line (a gift from Dr. Glyn Dawson, The University of Chicago) was maintained in cell culture according to standard methods (Burstein and Greene, 1978). PC12 cells were grown in a culture media that consisted of Dulbecco’s Modified Eagle’s Medium (4,500 mg/liter glucose without sodium pyruvate; HyClone Laboratories, Logan, UT), 10% fetal bovine serum (HyClone Laboratories), 5% heat-inactivated horse serum (HyClone Laboratories, Logan, UT), and 10% fetal bovine serum (HyClone Laboratories, Logan, UT). The cells were kept frozen from passage 6 and grown up to passage 12; every 7 d, the cells were passaged with Ca\(^{2+}\)-free Hank’s balanced salt solution (HyClone Laboratories) and plated onto either T25 flasks for further passages or T25 flasks for differentiation and experimentation. The cells were differentiated with 20 ng/ml of 2.58 grade II nerve growth factor from mouse submaxillary gland (Boehringer Mannheim Biochemicals, Indianapolis, IN) 24 h after they were plated in T25 flasks. PC12 cells that were differentiated between 5 and 12 d were replated on collagen-coated glass coverslips 6 to 18 h before recording was begun. The cell bodies were flattened and well-attached to the glass, and processes had begun to grow, but not as extensively as before replating (described below).

**Figure 1.** The solution changes by the perfusion system do not affect capacitance measurements. (A) A schematic of the recording chamber, perfusion system, and measurement technique is shown. Membrane capacitance and conductance were measured simultaneously from a cell voltage clamped at −80 mV. The cell was perfused continuously with a perfusion system, and the bath volume was kept constant with a peristaltic pump matched to the flow rate of the perfusion system (~70 μl/min). (B) An expanded schematic shows the relationship between a cell and the perfusion system with the bottom of the outlet tube ~250 μm from the cell. In this configuration, the ~30-μm diameter cell is completely bathed with the external solution flowing from the outlet tube (200-μm diameter) of the perfusion system. (C) Membrane capacitance was measured from a cell that was continually voltage clamped at −80 mV, while the valves of the perfusion system were switched between two reservoirs. The two square steps represent the 100-pF calibrations for the capacitance trace, followed by the conductance calibrations. At arrows labeled 1, the cell was exposed to a Na solution flowing from reservoir 1. At arrows labeled 2, the cell was exposed to an identical solution from reservoir 2, indicating that changing solutions between reservoirs had no effect on our capacitance recordings. The valves were then switched to one that contained nicotine (Nic) for almost 2 s of continuous application. The membrane capacitance increased and began to return towards baseline. The valves were then switched back to one that contained the control Na solution (last arrow labeled 1).
Recording Configuration

A single coverslip of cells was removed from nerve growth factor culture media, set into a recording chamber (Fig. 1 A), and perfused with an external Na solution that contained (mM): 135 NaCl, 2 KCl, 1 MgCl₂, 5 CaCl₂, 12 HEPES, and 10 glucose (pH 7.3, osmolality = 295 mOsm).

Electrodes were pulled from microhematocrit capillary tubes (Drummond Scientific Co., Broomall, PA), coated with Sylgard, fire-polished, and filled with an internal solution that contained (mM): 120 CsAsp, 5 MgCl₂, 0.1 EGTA, 40 HEPES, 2 ATP, and 0.3 GTP (pH 7.3, osmolality = 310 mOsm).

The outlet pipe from the perfusion system (ALA Scientific Instruments Inc., Westbury, NY) was directly adjacent (~250 μm) to the cell (Fig. 1 B). The single outlet pipe was fed by six separate inlet pipes that were each joined to a separate reservoir through a computer-controlled valve. The reservoirs were pressurized to ensure equal flow rates, ~70 μl/min, and the output rate was matched with the rate of a peristaltic pump to keep the volume of the bath constant. A constant bath volume reduced changes in capacitance measurements that occurred with changes in fluid level. The reservoirs of the perfusion system were filled with various external solutions, and the cell was continuously bathed by one of these external solutions.

Fig. 1 C illustrates that membrane capacitance was not altered when the perfusion system was switched between two different reservoirs, 1 and 2, that contained identical Na solutions. The arrows labeled 1 and 2 show where the Na solution from reservoir 1 was applied, alternately with the identical solution from reservoir 2. At the arrow labeled Nic, the solution stream was switched to a reservoir that contained nicotine that elicited a large, rapid change in membrane capacitance. At the arrow labeled 1, the solution was switched back to reservoir 1, which contained the Na solution. Fig. 1 C illustrates the sole experiment in this paper in which membrane capacitance was measured while nicotine was applied to the cell. Because nicotine activated a large conductance change via the nAChRs, the amplitude of this capacitance measurement does not accurately record changes in membrane surface area. Thus, in this figure only, we believe that most of the capacitance change recorded is artifactual, due to the large conductance change. Nevertheless, Fig. 1 C does provide information that membrane capacitance was sensitive to the timing of the valve changes between a nicotine solution and a control solution. Although the precise timing of the solution application could not be determined because of variation in the distance between the cell and the outlet pipe of the perfusion system, estimates of the timing were obtained using changes in liquid junction potentials by an electrode and perfusion configuration designed to mimic an experiment. Solution exchange could be as fast as 10 ms and as slow as 150 ms.

Capacitance Measurements

A single PC12 cell was selected with processes that were no longer than twice the cell body diameter to ensure a good space clamp. A cell was patch clamped in the whole-cell configuration (Hamill et al., 1981) with an Axopatch-1C amplifier (Axon Instruments, Foster City, CA) modified for capacitance measurements. Changes in capacitance were measured with the phase-tracking technique (Joshi and Fernandez, 1988) in which a 30-mV sine wave was superimposed on the ~80-mV holding potential (Neher and Marty, 1982). The resulting current was recorded at two orthogonal phase angles relative to the input voltage with a phase-sensitive detector that allows the software to continuously monitor both the membrane conductance and capacitance. The sinusoidal voltage was interrupted to stimulate the cell with either rapid nicotine application or voltage depolarizations, or with simultaneous nicotine application and voltage depolarizations. The data were collected at a 500-μs sampling rate and filtered at 2 kHz (this undersampling was necessitated by the filter requirements of the capacitance sine wave). Due to memory limitations imposed by our operating system (MS-DOS), current records were limited to 1,024 sampling points, which required slow sampling for longer depolarizations. All current records were compensated for series resistance and whole-cell capacitance, and leak subtracted by an average of 10 hyperpolarizing sweeps. Junction potentials of ~−9 mV were not subtracted from the data.

Stimulation Protocol

For rapid application of nicotine (or antagonists) from the perfusion system, the holding potential of the cell was maintained at ~−80 mV, while the solutions were applied for 200 ms (see Fig. 3 B). For step depolarizations, the cell was stimulated with a train of five depolarizations from a ~−80-mV holding potential to +20 mV (unless noted) for a duration of 200 ms and an interpulse interval of 500 ms (for example, see Fig. 2). Trains were applied at 3-min intervals, a duration that limited run down. Longer intertrain intervals lead to greater run down, while shorter intervals did not allow sufficient time for recovery. For simultaneous application of nicotine and depolarizations, both the nicotine application and the depolarizing step duration were 200 ms. The perfusion system’s dead time of 50 ms was compensated by opening the nicotine valve 50 ms before the step depolarization. Because some diffusional exchange occurred in the mixing chamber between the outlet pipe and the other five inlet lines, 30 s before any test application of either nicotine or nicotinic antagonists, the valve that controlled the test reservoir was opened for 50 ms to ensure that the test inlet line was primed to contain 100% of the appropriate solution. This procedure by itself produced a minimal response. Because every train was preceded by the same line flush protocol, we expect that any effect on subsequent secretion would be similar for each train. Both the on and off rates of a test solution were similar, and the slowest solution exchange (150 ms) was faster than the slow deactivation kinetics of the nAChR tail currents.

Nicotine (200 μM; Sigma Chemical Co., St. Louis, MO) was added to the Na solution (described above), or to a Na solution that also contained a cocktail of nicotinic antagonists, including methyllycaconitine (1 μM), mecamylamine (20 μM), and dihydro-β-erythroidine (20 μM) (all from Research Biochemicals, Inc., Natick, MA), which block most muscle and neuronal subtypes of nAChRs (Sorensen and Chiappinelli, 1990; Mulle et al., 1991; Wong and Gallagher, 1991; Alkondon et al., 1992; and McMahon et al., 1994). For one set of experiments, Cd²⁺ was used to block the Ca²⁺ channels at a concentration (100 μM, Sigma Chemical Co.) that does not block nAChRs (Gray et al., 1996). At the end of many experiments, the external solution was switched to one that optimized Ca²⁺ currents and efficiently blocked K and Na currents. This solution is referred to as tetraethylammonium (TEA) solution and contained (mM): 140 TEA-Cl, 0.0001 tetrodotoxin, 5 CaCl₂, 10 HEPES, and 10 glucose (pH 7.3, osmolality = 300 mOsm). All experiments were carried out at room temperature (22–24°C).

Intracellular Ca²⁺ Measurements

Intracellular Ca²⁺ was monitored from small groups of individual cells with a Ga²⁺ imaging system that was operated with software kindly provided by Dr. Eric Gruenstein (University of Cincinnati). The PC12 cells were placed in Hank’s Balanced Salt Solution (HBSS; Life Technologies, Inc.) that contained (mM): 138 NaCl, 5 KCl, 20 HEPES, 5.6 glucose, 0.8 MgSO₄, 1.3 CaCl₂, 0.3
Activated Nicotinic Acetylcholine Receptors Augment Exocytosis

KH$_2$PO$_4$ and 0.3 Na$_2$HPO$_4$ (pH 7.3, osmolality $\approx \sim 300$ mOsm). Cells were incubated at 37°C in HBSS that contained 5 $\mu$M fura-2 (AM ester form; Molecular Probes, Inc., Eugene, OR) for 45 min. The cells were washed in an indicator-free HBSS solution for 30 min at 37°C. The cells were transferred to a recording chamber where image pairs at wavelengths of 340 and 380 nm were collected every 10 s. 16 frames were averaged at each wavelength, after which the background was subtracted for each wavelength. The ratio of the 340- to 380-nm wavelengths was calibrated with an in vitro fura-2 calibration curve estimated from 50 $\mu$M fura-2 (free acid; Molecular Probes, Inc.) solutions that contained known concentrations of Ca$^{2+}$ that ranged from 0 to 2,000 nM. HBSS was superfused over the cells for several minutes and the ratio of 340- to 380-nm wavelengths were collected before changing to a 0 Ca$^{2+}$ solution that contained (mM): 150 NaCl, 2 KCl, 1 MgCl$_2$, 1 EGTA, 12 HEPES, and 10 glucose (pH 7.3, osmolality $\approx \sim 315$ mOsm). Nicotine (200 $\mu$M), acetylcholine (100 $\mu$M, Sigma Chemical Co.), caffeine (0.5 mM, Sigma Chemical Co.), thapsigargin (1 $\mu$M, Calbiochem Corp., La Jolla, CA), bradykinin (1 $\mu$M, Sigma Chemical Co.), and histamine (10 $\mu$M, Research Biochemicals Inc.) were all mixed in the 0-Ca$^{2+}$ solution.

**Results**

**Data Analysis**

To compare the changes in capacitance between different cells, the data were normalized to the initial change in capacitance observed in response to five depolarizations. Statistical analysis of the data are expressed as mean (X) ± SEM, and an independent Student’s t test (Microcal Origin, Northampton, MA) was performed to test statistical significance ($P < 0.05$).

**Results**

Activation of Ca$^{2+}$ channels by depolarization evoked release that could be assayed by measurement of whole cell capacitance. Two capacitance traces from the same cell are shown in Fig. 2. The first capacitance trace was recorded in a Na solution that had 5 mM external Ca$^{2+}$, without K or Na channel blockers (Fig. 2 A). Fig. 2 B shows a trace that was recorded in a TEA solution (5 mM Ca$^{2+}$, 1 $\mu$M tetrodotoxin) that effectively suppressed the K and Na channels. In each solution, the cell was depolarized five times for 200 ms (500-ms interpulse interval) from a −80-mV holding potential to +20 mV. Upon stimulation of the cell, membrane capacitance increased rapidly. At the peak, the capacitance increased by 380 fF in the Na solution (Fig. 2 A) and 395 fF in the TEA solution (Fig. 2 B). The breaks in the capacitance trace depict the time the depolarizations were applied. The ensemble of current traces are shown below each of their respective capacitance traces; the order in which they were obtained is indicated in the figure. In the Na solution, the early current had a rapid Na component that was absent in the TEA solution, and the depolarization activated a large outward conductance (most likely a Ca$^{2+}$-activated K current), which increased with subsequent stimulation (Fig. 2 C). The TEA solution, which allows for the isolation of the Ca$^{2+}$ currents, suppressed the outward current (Latorre and Miller, 1983) (Fig. 2 D). The five sequential Ca$^{2+}$ currents recorded in the TEA solution exhibited a rapid reduction in amplitude due to inactivation (Fig. 2 D). This reduction in amplitude was also present in the tail currents that provide a separate assay of Ca$^{2+}$ current amplitude (data not shown). Experiments like those shown in Fig. 2 allowed us to examine the Ca$^{2+}$ currents that underlie secretion in isolation; however, because external TEA has antagonist effects on AChRs (Adler et al., 1979; Wall and Dale, 1994), all of the subsequent experiments were performed in an external Na solution.

Change in membrane capacitance can be correlated with an estimate of the number of vesicles released from the cell (see Fig. 2 B). PC12 cells have two types of vesicles, synaptic-like microvesicles that store ACh and have diameters of $\sim$50 nm, and large dense-core vesicles that store catecholamines and have diameters of $\sim$100 nm (Bauerfeind et al., 1993; Thomas-Reetz and De Camilli, 1994). Assuming that both vesicle types are released in equal proportions and a specific capacitance of 1 $\mu$F/cm$^2$, we calculated an average vesicle ra-
dius of 39.5 nm (square root of the mean radii squared). Thus, a change in membrane capacitance of 1 fF would correspond to the release of approximately five of these average vesicles (or ~13 of the 50-nm diameter vesicles, or ~3 of the 100-nm diameter vesicles). In Fig. 2 B, the peak change of capacitance in the TEA solution, ~400 fF, corresponds to the release of ~2,000 average vesicles. This is probably an underestimate of the number of released vesicles since, at the peak of the capacitance change, both exocytosis and endocytosis overlap. It is possible that the two populations of vesicles are released via different pathways with different rates of exocytosis and endocytosis, which could further complicate the interpretation of the peak of the capacitance change.

Activation of nAChRs Evokes Secretion

In cells voltage clamped at −80 mV, 200 μM nicotine activated large inward currents that triggered secretion (Fig. 1 C). With our perfusion system, a rapid switch between different solutions did not by itself alter the membrane capacitance or current and did not interrupt the flow of solution (see METHODS). Although we do not know how much of the inward current activated by nicotine was carried by Ca\(^{2+}\) in the differentiated PC12 cells, we do know that neuronal nAChRs have a relatively high permeability to Ca\(^{2+}\). Cultured rat sympathetic neurons have nicotinic receptors with a Ca\(^{2+}\) to Na\(^{+}\) permeability ratio between 2.6 and 3.8 (Trouslard et al., 1993). Cloned a7 nicotinic subunits expressed in oocytes are ~20-fold more permeable to Ca\(^{2+}\) than Na\(^{+}\) (Seguela et al., 1993). These experiments suggest that a substantial fraction of the nAChR current in the PC12 cells is carried by Ca\(^{2+}\). The response of the cells to nicotine varied widely, and to routinely elicit measurable responses, we chose to test the effects of five 200-ms applications of nicotine (500-ms interpulse interval) on exocytosis.

Nicotine application triggered secretion in the ab-

![Figure 3](image-url)
crease in membrane capacitance recovered to 50% to allow sufficient Ca\(^{2+}\) permeation through nAChRs, which was reported in adrenal medullary chromaffin cells (Mollard et al., 1995), where activation of nAChRs was reported 10% of control. These results are similar to those reported in Fig. 3 C). Because the conductance of the membrane is large and changing even after the nicotine application is terminated, the capacitance trace immediately after nicotine application is quite noisy and not a reliable indicator of secretion. Several hundred milliseconds after application of nicotine, the conductance of the membrane returns to resting conditions, which allows an accurate assessment of membrane capacitance. We measured capacitance after these conductance changes stabilized; the upper dashed line in Fig. 3 A indicates the peak amplitude of the measured capacitance change, 540 fF, corresponding to \(\sim 2,700\) average vesicles released. To show that the nicotine response is mediated by nAChRs, we tested the effects of perfusing the cell with a cocktail of nicotinic receptor antagonists (see METHODS). After 2 min of application, the antagonists effectively blocked the ability of nicotine to evoke secretion (Fig. 3 B).

The cocktail of nicotinic antagonists also inhibited the nicotine-induced whole cell current. Fig. 3 C shows a control application of 200 mM nicotine for 200 ms. Within 2 min of the antagonist cocktail application, the nicotine-activated inward current was abolished (Fig. 3 D). The effects of the antagonist cocktail were partially reversed after 5 min of washout (Fig. 3 E). In the presence of the antagonist cocktail, five depolarizing steps to +20 mV stimulated increases in membrane capacitance, presumably by activating voltage-gated Ca\(^{2+}\) channels. Voltage-dependent membrane currents were unaffected by the presence of the nicotinic antagonists (both the voltage-dependent Ca\(^{2+}\) current and membrane capacitance changes were similar to those of Fig. 2, A and C). These data support the idea that nicotine by itself elicits changes in secretion through activation of nAChRs, and that application of nicotinic antagonists could abolish this secretion. The pooled capacitance and current data are shown in Fig. 3 F. The nicotine-activated current was almost completely abolished to 3% ± 2% of control \((n = 7, P < 0.001)\), resulting in the complete inhibition of any changes in membrane capacitance (five cells). After 5–15 min washout of the antagonists, the nicotine-induced current recovered to 51% ± 11% of control and the nicotine-induced increase in membrane capacitance recovered to 50% ± 10% of control. These results are similar to those reported in adrenal medullary chromaffin cells (Mollard et al., 1995), where activation of nAChRs was reported to allow sufficient Ca\(^{2+}\) to enter the cell to evoke a secretory response.

Nicotine potently stimulates secretion when the cell membrane potential is −80 mV, but it was important to determine whether nicotinic receptors could evoke secretion at the depolarized potentials likely to be reached after nAChR activation. Under these conditions, Ca\(^{2+}\) permeation through nAChRs might not be sufficient to induce secretion since the driving force on the Ca\(^{2+}\) ions is greatly reduced. To test this, we first voltage clamped the cell at −80 mV, and then applied 100 mM Cd\(^{2+}\), a concentration that rapidly and reversibly blocked all of the voltage-activated Ca\(^{2+}\) channels (Fig. 4 A). This Cd\(^{2+}\) concentration does not interfere with activation of the nicotinic receptor (Gray et al., 1996). After 1 min of Cd\(^{2+}\) treatment, all of the Ca\(^{2+}\) channels were blocked and the holding potential of the cell was

![Figure 4](image-url)

**Figure 4.** Sufficient Ca\(^{2+}\) enters the cell through nAChRs to evoke a small increase in secretion even at +20 mV. (A) 100 mM Cd\(^{2+}\) completely blocks the Ca\(^{2+}\) channel current. (left) The inward Ca\(^{2+}\) current in response to a step depolarization to +20 mV from a −80-mV holding potential \((V_H)\) for 200 ms. (middle) The inhibited current response after the same depolarization step after 20-s application of 100 mM Cd\(^{2+}\). (right) The current response after a 20-s wash with control Na solution. (B) The two current traces are in response to a 780-ms continuous application of nicotine (arrow), while the cell was bathed in an external solution of 100 mM Gd\(^{3+}\) and voltage clamped at +20 mV (left) and −80 mV (right). (C) The pooled capacitance data are graphed for five cells stimulated with a train of nicotine applications, each of 200 ms in duration either at a holding potential of +20 or −80 mV in the presence of 100 mM Cd\(^{2+}\).
changed to +20 mV. After 3–5 min at +20 mV, the cell was stimulated with nicotine, and membrane capacitance and nicotinic-activated currents were measured. Then the cell was repolarized to −80 mV, allowed to recover for a few minutes, and stimulated with a second application of nicotine in the continued presence of Cd²⁺.

Fig. 4 B illustrates the single cell in which one long application of nicotine (780 ms) was applied to the cell while voltage clamped at a holding potential of +20 mV (left) or −80 mV (right) in the presence of Cd²⁺. At +20 mV, there is a small outward current and, at −80 mV, there is a large inward current that is similar to the large inward current activated by nicotine in the absence of Cd²⁺ (data not shown).

Two sets of cells were stimulated with a train of five applications of nicotine. One set of cells was stimulated with nicotine applications 200 ms in duration, while the other set was stimulated with nicotine applications 400 ms in duration. In the set of cells stimulated with the train of applications lasting 200 ms, at a holding potential of +20 mV, four of the five cells did not display measurable inward or outward current, which indicates that +20 mV is near or at the reversal potential of the nAChRs under these conditions. One of the five cells had a small outward current (peak, 141 pA). For the five cells tested, only one cell had evoked secretion at +20-mV holding potential; it responded to nicotine with a very small increase in capacitance of ~35 fF (on average 7 ± 7 fF). These results indicate that although some Ca²⁺ is likely to flux through the receptor at this potential, the levels of intracellular Ca²⁺ were insufficient to support a large amount of secretion. At −80 mV, all of the cells responded to nicotine with large inward currents of 800–1,500 pA and increases in capacitance that averaged 170 ± 74 fF. The pooled capacitance data for this set of five cells are graphed in Fig. 4 C for the nicotinic-activated secretion at both +20- and −80-mV holding potentials in the presence of Cd²⁺.

To more thoroughly examine the possibility of nAChR-mediated Ca²⁺ entry at positive potentials, a second set of cells was stimulated with a train of five nicotine applications 400 ms in duration while voltage-clamped at +20 mV in the presence of Cd²⁺ (n = 8). The average increase in capacitance was 23 ± 14 fF, with an average outward current of 46 ± 26 pA. These data indicate that sufficient Ca²⁺ can enter the cell through the nicotinic receptor at positive membrane potentials to trigger small levels of secretion.

**Simultaneous Activation of nAChRs and Ca²⁺ Channels Augments Secretion**

Under normal physiological conditions, activation of nAChRs leads to depolarization and activation of voltage-dependent Ca²⁺ channels. Thus, we wanted to determine whether the simultaneous activation of both the nAChRs and the voltage-dependent Ca²⁺ channels would stimulate a larger secretory response than from activation of either of these Ca²⁺ entry pathways alone. Initially, each cell was stimulated with five step depolarizations to +20 mV (the same protocol as shown in Fig. 2) and examples of the capacitance and current responses are shown in Fig. 5, A and D, respectively. With activation of voltage-dependent Ca²⁺ channels, mem-

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brane capacitance increased rapidly (Fig. 5 A). Like the initial current in Fig. 2 B, the inward current has a large Ca\(^{2+}\) component (Fig. 5 D), and subsequent currents showed an outward K current (subsequent currents not shown; for example, see Fig. 2 C). After a 3-min recovery period, the cell was stimulated with the same depolarization protocol coupled with simultaneous nicotine applications during each depolarizing step. The combined application of nicotine with depolarizations resulted in a marked increase in the amplitude of the capacitance response that was 128% of the control response with activation of Ca\(^{2+}\) channels alone (Fig. 5 B). The corresponding current trace (Fig. 5 E) was similar to the Ca\(^{2+}\) current alone except that it has a large tail current that is likely due to current flow through nAChRs, which are still active after the end of the nicotine application. After a 3-min recovery period, the cell was stimulated a third time with depolarizations only (Fig. 5 C and F). The capacitance response was 57% of control, which may be due to a change in the secretory machinery, since the current was remarkably similar to the control current. The secretory response did not recover to control levels within the length of the experiment (~30 min). The pooled data shown in Fig. 5 G for 14 cells stimulated with this protocol indicate that simultaneous activation of both the nAChRs and the voltage-dependent Ca\(^{2+}\) channels augmented secretion 176% ± 23% relative to control (P < 0.005). The third stimulation with depolarization alone elicited a capacitance change that was 32% ± 9% (P < 0.001) of the initial capacitance change. These data show that simultaneous application of nicotine and depolarizations augment secretion in PC12 cells.

A voltage step to +20 mV elicits the largest Ca\(^{2+}\) current measured in the Na solution (data not shown). By moving to a less depolarized step potential, we reasoned that the resultant increase in the driving force for Ca\(^{2+}\) through the nicotinic receptors would offset the decrease in the voltage-dependent Ca\(^{2+}\) current. We then tested whether a weaker depolarization, when coupled with nicotine application, would give a greater augmentation of secretion. The stimulation protocol of Fig. 5 was repeated, except that the depolarizing steps were to 0 mV rather than +20 mV. Fig. 6 A shows the control capacitance trace obtained in response to depolarization, followed by the change in capacitance with simultaneous application of nicotine during the depolarizing steps (Fig. 6 B). For this cell, capacitance increased to 207% of control. The capacitance change was greatly reduced (31%) for the third stimulation (Fig. 6 C), using only depolarizations to 0 mV (no nicotine) compared with control. The cell was stimulated a fourth time with step depolarizations to +20 mV rather than 0 mV, without nicotine, which resulted in a capacitance increase of 233% (Fig. 6 D) compared with control. The increase in capacitance with depolarization to +20 mV most likely results from the increased Ca\(^{2+}\) influx into the cell, but at present we cannot rule out changes in the secretory machinery as well. As in the previous figures, the peak of the capacitance change is an underestimate since at the peak, both exocytosis and endocytosis can overlap. Fig. 6 E graphs the pooled

![Figure 6](image_url)
The secretion augmented by activation of the nAChRs is not dependent on the stimulation protocol. Membrane capacitance is shown for a series of stimulations with a train of five depolarizations to +20 mV (200-ms duration, 500-ms interpulse). The capacitance response is shown for (A) a first and (B) a second stimulation with depolarizations only. (C) The capacitance was measured in response to a third stimulation with simultaneous application of nicotine. (D) The capacitance response is shown after a fourth stimulation with depolarizations alone. Each stimulation occurred 3 min apart. (E) Normalized capacitance data for 15 cells are graphed for each of the stimulations: a control depolarization to +20 mV, a second depolarization to +20 mV, simultaneous stimulation with depolarizations and nicotine, a fourth depolarization to +20 mV, and a fifth stimulation with nicotine alone.

Data from five cells. Similar to the results obtained with +20-mV depolarizations (Fig. 5), simultaneous application of nicotine and step depolarizations to 0 mV resulted in an increase in capacitance to 193% ± 47% of control. The third stimulation produced a capacitance change 46% ± 16% (P < 0.01) of the control capacitance change. Even after three stimulations, a fourth stimulation to +20 mV rather than 0 mV increased the change in capacitance to 344% ± 100% (P < 0.05) of control.

Figs. 5 and 6 show that secretion is augmented when Ca^{2+} enters the cell via both nAChRs and voltage-dependent Ca^{2+} channels. We were concerned that the augmentation of secretion may be an artifactual occurrence due to a “priming” effect of the first stimulation.

On the second stimulation. To determine whether the first stimulation caused a second stimulation to be larger, the cell was stimulated with two trains of depolarizing steps to +20 mV delivered 3-min apart, both in the absence of nicotine. If there was a priming effect, the second stimulation would be increased over that of the first stimulation. Fig. 7, A and B show the capacitance changes for a first and second stimulation to +20 mV, respectively, in which the second stimulation was 83% of the first. For the 13 cells tested with this protocol, all exhibited reduced capacitance for the second stimulation. Thus, it is unlikely that the augmented secretion is due to any priming of the release mechanism. Rather, the opposite appears to be true. Instead of priming the subsequent secretory responses, stimulations cause a rundown of secretion. When the cell was stimulated a third time (Fig. 7 C), the capacitance change with simultaneous application of nicotine and depolarization was 308% of the first depolarization. A fourth stimulation with depolarizations only (Fig. 7 D) caused capacitance to increase, but only to 44% of the initial control capacitance change. The pooled data are graphed in Fig. 7 E for 13 cells. The second stimulation gives a capacitance change that on average is 55% ± 6% (P < 0.001) of control. The capacitance change for the third stimulation with simultaneous application of nicotine increased to 143% ± 18% (P < 0.05) of control. This change in capacitance is slightly less than when the cell was stimulated with only two sets of depolarizations rather than three. This result was expected, as we have shown that the secretory response runs down. The rundown would tend to minimize the effects of the nicotine augmentation. A fourth stimulation with depolarization alone resulted in a capacitance change only 29% ± 9% (P < 0.001) of control and a fifth stimulation with nicotine only (no depolarizations) also results in secretion of 46% ± 8% (P < 0.001) of control.

Activation of nAChRs Does Not Release Intracellular Ca^{2+} Stores

Although the results with nicotinic antagonists shown in Fig. 3 suggested that nicotine exerted its effect primarily through Ca^{2+} influx via nAChRs rather than through the release of Ca^{2+} from intracellular stores, we tested this hypothesis directly with a Ca^{2+} imaging system under conditions designed to promote the release of Ca^{2+} from intracellular stores. Individual cells pre-loaded with fura-2 were imaged and the ratio of 340- to 380-nm wavelengths emitted from fura-2 were collected throughout the duration of the experiment. The PC12 cells were first bathed for 180 s in HBSS (1.3 mM Ca^{2+}) external solution, and then bathed with a 0-Ca^{2+} solution for either 30 or 120 s. The cells were exposed for 100 s to nicotine (200 μM) mixed in the
0-Ca\textsuperscript{2+} solution. None of the cells (n = 62) responded to nicotine, which indicates that nicotine does not release Ca\textsuperscript{2+} from the cytoplasmic stores. The cells were then washed with the 0-Ca\textsuperscript{2+} solution for 1 min, and then returned to the HBSS solution for at least 5 min. The cells were washed a second time with the 0-Ca\textsuperscript{2+} solution for 30 or 120 s before being exposed to agents known to release Ca\textsuperscript{2+} from cytoplasmic stores, either acetylcholine (100 \textmu M, to activate muscarinic receptors), caffeine (0.5 mM), thapsigargin (1 \textmu M), bradykinin (1 \textmu M), or histamine (10 \textmu M) mixed in the 0-Ca\textsuperscript{2+} solution. With exposure to each of these agents, 92% of the cells did not show a detectable increase in intracellular Ca\textsuperscript{2+}, and the other 8% of the cells showed only modest increases in intracellular Ca\textsuperscript{2+}. All 15 cells tested responded to 200 \textmu M nicotine in HBSS (1.3 mM Ca\textsuperscript{2+}) with a large increase in intracellular Ca\textsuperscript{2+}, presumably from activation of the nAChRs and from the resulting depolarization that activates voltage-dependent Ca\textsuperscript{2+} channels. These results indicate that Ca\textsuperscript{2+} release from cytoplasmic stores probably plays little or no role in the augmentation of secretion elicited by nicotine.

**Discussion**

PC12 cells are known to express voltage-dependent Ca\textsuperscript{2+} channels (Plummer et al., 1989) and nAChRs (Ifune and Steinbach, 1992; Furukawa et al., 1994), and release catecholamines and acetylcholine in a Ca\textsuperscript{2+}-dependent manner (Greene and Tischler, 1982). We have measured Ca\textsuperscript{2+}-dependent vesicular release from individual voltage-clamped PC12 cells using membrane capacitance as an assay of release. Our results show that activation of nAChRs in cells voltage-clamped at −80 mV elicited large currents and large amounts of secretion, a result similar to that obtained in chromaffin cells (Mollard et al., 1995). Secretion evoked by nicotinic receptor activation was completely abolished by nAChR antagonists. In the presence of the antagonists, activation of Ca\textsuperscript{2+} channels still evoked secretion when Ca\textsuperscript{2+} channels were activated, which indicates that the antagonists blocked nAChRs but had no effect on the secretory machinery.

In addition to showing that nicotine elicited secretion in PC12 cells voltage clamped at −80 mV, a result identical to that obtained by Mollard et al. (1995) in chromaffin cells, our primary goal in these studies was to examine the physiologically relevant secretion elicited by the activation of nAChRs and Ca\textsuperscript{2+} channels simultaneously. We chose to model our studies on well known chromaffin cell physiology, where ACh release triggered by splanchnic nerve activation activates nAChRs, which in turn depolarizes chromaffin cells and initiates action potentials. Thus, both nAChRs and Ca\textsuperscript{2+} channels are activated simultaneously. Chromaffin cells fire action potentials and the membrane potential traverses a range from −60 to +25 mV and repolarizes with a prominent shoulder near −10 mV. We chose to explore the effects of nAChR activation at potentials near the peak of the action potential (+20 mV), near the shoulder of the action potential (0 mV), and near the resting potential (−80 mV). Please note that 0 mV was chosen for the shoulder potential, rather than −10 mV, to optimize Ca\textsuperscript{2+} influx via Ca\textsuperscript{2+} channels. At −80 mV, activation of nAChRs could by themselves elicit secretion, while at +20 mV activation of nAChRs elicited little secretion, but rather augmented the secretion elicited by activation of Ca\textsuperscript{2+} channels. Because of the complicated nature of action potentials, in vivo activation of nAChRs may elicit some secretion at hyperpolarized potentials, as shown in this paper and previously by Mollard et al. (1995), as well as augment Ca\textsuperscript{2+} influx via Ca\textsuperscript{2+} channels at more depolarized potentials. The optimal approach to study the contribution of the two channel types would involve the use of action potential waveforms to coincide with extremely brief (2–3-ms) applications of nicotine. In addition, ACh will activate muscarinic receptors, which, in chromaffin cells, will trigger the release of Ca\textsuperscript{2+} from the stores.

In the PC12 cell line used in this study, the Ca\textsuperscript{2+} current recorded in the external Na solution was maximal at +20 mV (our unpublished data). At +20-mV step potential, the driving force on Ca\textsuperscript{2+} ions was greatly reduced relative to −80 mV (Ifune and Steinbach, 1992; Mulle et al., 1992; Vernino et al., 1992). However, as pointed out by Decker and Dani (1990), even when the receptor is near its reversal potential there can be a substantial Ca\textsuperscript{2+} influx. Our results indicate that Ca\textsuperscript{2+} influx occurred through the nAChR at positive membrane potentials, +20 mV, and that the influx of Ca\textsuperscript{2+} was sufficient to elicit a small amount of evoked secretion, although much less than that observed at −80 mV.

More interestingly, when brief pulses of nicotine were timed to coincide with activation of Ca\textsuperscript{2+} channels, thereby activating Ca\textsuperscript{2+} channel currents and nAChRs simultaneously, secretion was augmented to 176% of that observed with activation for Ca\textsuperscript{2+} channels alone. Our data provide an underestimation of the augmentation of secretion since the magnitude of secretion is diminished by rundown of the secretory machinery and the overlap between exocytosis and endocytosis. If the effects of rundown are taken into account, an upper estimate of augmented release would be 320% rather than 176% (see Fig. 7). Although the rate of endocytosis was variable, in many cells it was fast enough to affect the measured exocytotic peak. We did not observe any clear differences in endocytosis when either the nicotinic receptors or the voltage-activated Ca\textsuperscript{2+} channels were activated, but if there were differ-
ences, they would complicate the measurement of exocytosis. Similarly, because PC12 cells release two different populations of vesicles, it is possible that there are two pathways for release that vary in their rates of exocytosis and endocytosis, which would further complicate the measurement of exocytosis.

The slow deactivation of nicotinic receptors, even after the agonist was removed, is likely to contribute to the augmentation of secretion since large inward currents remained after a step depolarization. Continued activation of the nicotinic receptors would allow Ca\(^{2+}\) to enter the cell after repolarization. Augmentation of release was observed at different step potentials. Weaker depolarizations to 0 mV with simultaneous activation of the nAChRs augmented secretion 193\% over that with depolarization alone. Because only two potentials were used, and because the differences in augmentation at 0 and +20 mV were not significantly different, we do not yet have an appreciation for the relationship between nicotine-induced augmentation and the membrane potential. (We are not certain of the proportion of Ca\(^{2+}\) ions that entered the cells during the depolarizing test pulses relative to the slow tail currents. The slow deactivation rate seems to be a feature of PC12 cell nicotinic receptors, although some small fraction of the response may be due to the perfusion exchange system.) Nonetheless, our results indicate that regardless of whether the Ca\(^{2+}\)-influx occurs during the depolarization or during the slow tail, activation of nAChRs at the same time as Ca\(^{2+}\) channels augments the secretion produced by Ca\(^{2+}\) channels alone.

Because the first stimulus in these experiments always activated Ca\(^{2+}\) channels alone, it was possible that the first stimulus primed the release machinery for the second stimulus. Our results were not consistent with this hypothesis. When a depolarizing stimulus was followed by a second depolarizing stimulus, rundown of secretion was always observed. Augmentation only occurred in those experiments where nicotinic receptors were activated. These results indicate that the nAChRs provide a functional Ca\(^{2+}\) influx pathway. In addition, this Ca\(^{2+}\) entry pathway can function independently of membrane depolarization to evoke release or even more relevantly, can function with depolarization to augment evoked release. Because release is a steep nonlinear function of Ca\(^{2+}\) entry, it is possible that a small increase in Ca\(^{2+}\) concentration provided by the nAChRs may trigger a large change in secretion if a cell is in the steep part of the Ca\(^{2+}\) versus release relationship.

Recently, the Ca\(^{2+}\) permeability of the nAChRs has been investigated by a number of laboratories. Decker and Dani (1990) showed that the nAChR has a high Ca\(^{2+}\) permeability compared with other cations, and they predicted that under physiological conditions, activation of the nicotinic receptors could provide a significant source of Ca\(^{2+}\) influx. From clones of a neuronal and a muscle nicotinic receptor injected into oocytes, Vernino et al. (1992) distinguished the receptor types based on two properties. First, neuronal receptors have a sevenfold higher permeability to Ca\(^{2+}\) than muscle types, and, second, neuronal receptor currents are enhanced in a dose-dependent manner by an increase in external Ca\(^{2+}\) concentrations. The functional aspect of Ca\(^{2+}\)-permeable nicotinic receptors has been considered in a number of different preparations and from a variety of functional perspectives. In particular, in voltage-clamped sympathetic neurons, Trouslard et al. (1993) showed that intracellular Ca\(^{2+}\) was increased in response to nicotinic agonists, giving a Ca\(^{2+}\) permeability between 2.6- and 3.8-fold over that of Na\(^{+}\) permeability. Similarly, under voltage-clamp conditions, Mulle et al. (1992) and Zhou and Neher (1993) showed that activation of nAChRs increased intracellular Ca\(^{2+}\) in rat medial habenular neurons and chromaffin cells, respectively. In the same study, Mulle et al. (1992) demonstrated that Ca\(^{2+}\) entry through the neuronal nAChRs was of the same order of magnitude as Ca\(^{2+}\) entry through voltage-dependent Ca\(^{2+}\) channels. Furthermore, they showed that the influx of Ca\(^{2+}\) directly activated a Cl\(^{-}\) conductance and interacted with GAB\(\alpha\) receptors.

It is possible that in the adrenal gland, ACh may be released tonically in small amounts rather than as a large bolus. Our results suggest that under these conditions, the nAChRs expressed by the chromaffin cells would provide sufficient Ca\(^{2+}\) entry to trigger a small amount of catecholamine secretion. However, recordings from animals suggest that the splanchnic nerve is not silent, but instead fires action potentials even at rest, which will trigger ACh release, and then postsynaptic action potentials and catecholamine release (Wakade, 1988). But if small amounts of ACh are released by the splanchnic nerve, this may activate the receptors and prime the release machinery by maintaining an elevated concentration of Ca\(^{2+}\), or it may even allow enough Ca\(^{2+}\) into the cell to sustain release in the absence of action potentials. Recent experiments by McGehee et al. (1995), Alkondon et al. (1996), and Gray et al. (1996) suggest that activation of presynaptic AChRs results in an influx of Ca\(^{2+}\) to increase presynaptic Ca\(^{2+}\) concentrations and enhance synaptic transmission from central neurons. On the other hand, Vernino et al. (1994) simultaneously measured membrane current and intracellular Ca\(^{2+}\) with fura-2 as a Ca\(^{2+}\) indicator in voltage-clamped BC3H1 cells (a muscle cell line) and chromaffin cells, and showed that, under physiological conditions, Ca\(^{2+}\) carries only 2\% of the inward current through muscle-type nicotinic receptors in the BC3H1 cells and 4\% of the inward current
through neuronal-type receptors in the chromaffin cells.

The augmentation of evoked release at holding potentials of either −80 or +20 mV may represent the limits for the range of the effects on the physiological evoked response. Since there was secretion in a few cells when nAChRs were activated and while voltage clamped at +20 mV, in the presence of Cd2+, enough Ca2+ entered the cell via the nAChRs, even with the reduced driving force, to evoke secretion. Because an action potential has a long and slow repolarization phase, it is not similar to an application of nicotine at a holding potential of −80 mV, which would overestimate the physiological response, or to a step potential to +20 mV, which probably underestimates the physiological response. Therefore, the stimulation protocol used in these experiments is likely to represent the range of nAChR-mediated augmentation of Ca2+-evoked release. Further, during long test depolarizations, there may be sufficient Ca2+ entry via Ca2+ channels to saturate the secretory machinery. However, the extremely brief nature of action potentials is likely to limit the Ca2+ entry via voltage-gated channels, making Ca2+ entry via AChRs even more important in augmenting release.

PC12 cells are an excellent cell system in which to study ligand-gated channels such as nAChRs and voltage-activated Ca2+ channels. Because secretion in PC12 cells is triggered by activation of either Ca2+ channels or nAChRs, they provide a good model of release for both chromaffin cells and neurons. However, there are a few important caveats to PC12 cells that should be mentioned. From our unpublished results with several subclones of the PC12 cell line, we found that the cell lines were not equivalent. Not all of the cell lines exhibited secretion in response to depolarization. PC12 cells expressed different AChRs as determined by the varied receptor kinetics observed in different cell lines. Furthermore, different lines of PC12 cells expressed an assortment of Ca2+ channels before and after differentiation, which gave rise to different densities of Ca2+ currents. Nonetheless, the PC12 cell line used for these studies exhibited robust nACh and Ca2+ currents.

In recent years, it has become increasingly clear that ligand-gated channels can function as Ca2+ channels. Ca2+-dependent processes like secretion may be regulated by all Ca2+ influx pathways including ligand-gated channels. We still need to determine what differences, if any, exist in secretion elicited by different Ca2+ influx pathways. Low levels of ACh will activate only a few nAChRs, insufficient for large membrane depolarization, but which may provide a localized source of Ca2+ entry. It may be advantageous for a cell to release vesicles from a localized region without the need for depolarizing the entire cell. Low levels of Ca2+ influx may also, for instance, serve to mobilize vesicles from their cytoskeletal attachments and keep the secretory machinery primed. Because action potentials are significantly different in both duration and magnitude in comparison with step depolarizations, the importance of the ligand-gated channels as Ca2+ influx pathways may be magnified under physiological conditions. Our results suggest that at all membrane potentials Ca2+ influx through nAChRs can affect secretion in PC12 cells.

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