Activation of nicotinic receptors triggers exocytosis from bovine chromaffin cells in the absence of membrane depolarization
(stimulus–secretion coupling/calium stores/calium entry/capacitance detection)

PATRICE MOLLARD*, ELIZABETH P. SEWARD†, AND MARTHA C. NOWICKY‡
Department of Anatomy and Neurobiology, Medical College of Pennsylvania, 3200 Henry Avenue, Philadelphia, PA 19129

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ABSTRACT The traditional function of neurotransmitter-gated ion channels is to induce rapid changes in electrical activity. Channels that are Ca²⁺-permeable, such as N-methyl-D-aspartate receptors at depolarized membrane potentials, can have a broader repertoire of consequences, including changes in synaptic efficacy, developmental plasticity, and excitotoxicity. Neuronal nicotinic receptors for acetylcholine (nAChRs) are usually less Ca²⁺-permeable than N-methyl-D-aspartate receptors but have a significant Ca²⁺ permeability, which is greater at negative potentials. Here we report that in neuroendocrine cells, activation of nAChRs can trigger exocytosis at hyperpolarized potentials. We used whole-cell patch-clamp recordings to record currents and the capacitance detector technique to monitor Ca²⁺ exocytosis in isolated bovine chromaffin cells. Stimulation of nAChRs at hyperpolarized potentials (−60 or −90 mV) evokes a large current and a maximal capacitance increase corresponding to the fusion of ~200 large dense-core vesicles. The amount of exocytosis is controlled both by the Ca²⁺ influx through nAChRs and by a contribution from thapsigargin-sensitive Ca²⁺ sequestering stores. This is a form of neurotransmitter action in which activation of nAChRs triggers secretion through an additional coupling pathway that coexists with classical voltage-dependent Ca²⁺ entry.

Catecholamine secretion from chromaffin cells is evoked by acetylcholine (ACh) released from sympathetic preganglionic fibers. In bovine chromaffin cells, ACh elicits a nonspecific current change through neuronal-type nicotinic acetylcholine receptors (nAChRs) and consequently depolarizes the plasma membrane (1). Action potentials triggered on ACh stimulation are sustained by both Na⁺ and Ca²⁺ influx (2) and are assumed to be the main source of the rise in cytosolic Ca²⁺ that results in exocytosis of large dense-core vesicles which contain catecholamines. However, other sources of Ca²⁺ have been implicated in the secretory response, including the nAChR itself (3) and, more recently, contributions from intracellular stores (4), which are abundant and diverse in bovine chromaffin cells (5–7).

Single-cell studies using whole-cell and single-channel patch-clamp techniques have established that Ca²⁺ entry related to action potentials corresponds to the opening of at least three distinct types of voltage-gated Ca²⁺ channels (8). Interestingly, combination of Ca²⁺ channel current recordings with detection of single-cell secretion by capacitance measurements has indicated a preferential link between specific Ca²⁺ channel types and the exocytotic machinery. Ca²⁺ entry through dihydropyridine-sensitive facilitation channels (L-type) is five-fold more effective in triggering exocytosis than entry through P- and N-type channels (9). Such a highly efficient relationship between a given Ca²⁺ source and exocytosis has not yet been reported for other Ca²⁺-regulating mechanisms.

Chromaffin cell nAChRs belong to a ligand-gated ion channel family with a modest, but significant, Ca²⁺ permeability (10–12). nAChRs, unlike N-methyl-D-aspartate (NMDA) receptors, which are Ca²⁺-permeable only during concomitant depolarization, are possible sources of Ca²⁺ entry at hyperpolarized membrane potentials. Since Ca²⁺ entry via nAChRs has been shown to have physiological consequences (10, 13), we investigated whether the nicotinic current can support catecholamine release in the absence of additional depolarization. Using capacitance measurements performed with the patch-clamp technique, we examined the link between nAChR activation and its influence on exocytosis by voltage clamping cells at polarized membrane potentials below Ca²⁺ channel thresholds. In some experiments, capacitance measurements were combined with whole-cell cytosolic Ca²⁺ recording.

METHODS

Cell Preparation. Bovine adrenal glands were obtained locally and chromaffin cells were prepared by standard methods (14). Cells were used after 2–3 days in culture.

Recording Conditions. Whole-cell patch-clamp recordings were performed with a List EPC-7. Internal solution was 145 mM cesium glutamate/10 mM cesium-Hepes/9.5 mM NaCl/0.3 mM bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), sodium salt/2 MgATP, pH 7.2, unless otherwise stated. External solution was 130 mM NaCl/1 mM MgCl₂/2 mM KCl/10 mM glucose/10 mM sodium Hepes/2 mM CaCl₂, pH 7.2. Recordings were performed with cells held at ~90 mV, except for Fig. 1 a–c. For capacitance detection, a 30-mV (root-mean-square) sine wave was added to the holding potential. In independent recordings, we determined that with our recording conditions, no voltage-gated channels were opened until positive to ~20 mV. Membrane capacitance (C_m) sampling was calculated every 22 msec from an average of 10 sine waves, 1.2-KHz frequency. Nicotine (50–200 μM) was diluted in the extracellular solution, loaded in an unpolished patch pipette, and placed 50–60 μm from the cell. The drug was ejected by application of 10–15 psi pressure (1 psi = 6.89 kDa) for 200 msec except where noted, under computer control through a General Systems Picospritzer.

Measurement of Intracellular Ca²⁺ Concentration ([Ca²⁺])j. Fluorescent Ca²⁺ probes (Iluo-3 or Fura Red; Molecular Probes) were used to monitor changes in cytosolic

Abbreviations: ACh, acetylcholine; nAChR, nicotinic ACh receptor; BAPTA, bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; [Ca²⁺]j and [Na⁺]j, intracellular (cytosolic) concentrations of Ca²⁺ and Na⁺; NMDA, N-methyl-D-aspartate; TG, thapsigargin.

*Present address: Institut National de la Santé et de la Recherche Médicale, rue de la Cardonille, 34094 Montpellier, Cedex 5, France.
†Present address: Glaxo Institute for Molecular Biology, 14 chemin des Aulx, Case Postale 674, 1228 Plan-les-Ouates, Geneva, Switzerland.
‡To whom reprint requests should be addressed.

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3065
Ca\textsuperscript{2+}. Fluo-3 (0.2 mM) was balanced with 0.1 mM BAPTA in the intracellular solution. Equilibration with the cell interior occurred within a few minutes. Fluo-3 was excited through a 450- to 490-nm-band-pass filter and emission was collected through a long-pass filter at 520 nm. Fura Red (0.3 mM) was excited through either a 440- or 490-nm-band-pass filter (Oriel) and emission was collected through a long-pass filter at 580 nm (15). The 440-nm Ca\textsuperscript{2+}-insensitive wavelength recording was done just before stimulation and recording at the 490-nm Ca\textsuperscript{2+}-sensitive wavelength. Fluorescence was recorded by a Gen-III CCD camera and stored on a U-matic recorder (Sony). Fluorescence traces were analyzed off-line, measured with a photodiode (Hamamatsu, Middlesex, NJ), and digitized with Axobasic software at the same sampling rate as the C\textsubscript{m} traces. Student’s t test and the Mann–Whitney U test were used when appropriate.

RESULTS

C\textsubscript{m} Responses Are Proportional to the Magnitude of the Nicotinic Current (\textit{I\textsubscript{Nic}}). The ability of \textit{nAChRs} to regulate exocytosis in the absence of Ca\textsuperscript{2+} entry through voltage-gated Ca\textsuperscript{2+} channels was investigated in bovine chromaffin cells voltage-clamped to −60 mV, below the threshold for channel activation. Extracellular Ca\textsuperscript{2+} was maintained at a physiological level (2 mM). The capacitance detection technique (16–18) was used to monitor changes in membrane surface area (ΔC\textsubscript{m}) presumably due to exocytosis of catecholamine-containing granules (19). Nicotine, applied from a puffer pipette, triggered a large inward current (\textit{I\textsubscript{Nic}}) which was followed by a sustained increase in C\textsubscript{m}, but no change in conductance (\textit{G}) (Fig. 1a). Both the inward current and the ΔC\textsubscript{m} were abolished when 1 μM (+)-tubocurarine was present, indicating that the responses resulted from activation of nicotinic \textit{AChRs} (Fig. 1b).

The amount of C\textsubscript{m} increase was related to the amplitude of \textit{I\textsubscript{Nic}}. Individual cells responded several times to successive applications of nicotine. In Fig. 1c, three traces from a single cell are superimposed. Increasing the driving force for external cations by changing the holding potential from −60 to −90 mV increased both the inward current and amplitude of ΔC\textsubscript{m}. On subsequent return to −60 mV, the C\textsubscript{m} response was smaller than after the first application, because of secretory “run-down.” The exocytotic response was also larger when the nicotinic current was increased by prolonging the puff duration from 50 msec to 200 msec or by increasing puff pressure (data not shown). The efficiency of \textit{I\textsubscript{Nic}} in causing ΔC\textsubscript{m} is summarized in Fig. 1d. Data are binned for the integral of \textit{I\textsubscript{Nic}} (expressed in nanocoulombs) and amplitude of C\textsubscript{m} jumps (in femtofarads). The secretory response increases for larger total charge and eventually saturates.

Ca\textsuperscript{2+}, Not Na\textsuperscript{+}, Is Responsible for the C\textsubscript{m} Increase. The neuronal \textit{nAChR} family has a variable permeability for Ca\textsuperscript{2+} (10–12, 20–22) which is generally low compared with the permeability for monovalent cations. To determine the importance of Na\textsuperscript{+} vs. Ca\textsuperscript{2+} entry in triggering C\textsubscript{m} increases, we manipulated internal concentrations of these ions. In eight of nine cells loaded with 10 mM BAPTA, a Ca\textsuperscript{2+}-chelating agent with a fast on-rate (23), \textit{Cm} increases were blocked despite normal \textit{I\textsubscript{Nic}} (data not shown). By contrast, at normal experimental chelator concentrations, but with a reduced driving force for Na\textsuperscript{+} ions ([Na\textsuperscript{+}] increased from 9.5 mM to 19 mM), nicotine elicited typical ΔC\textsubscript{m} responses and the total C\textsubscript{m} of cells did not increase in the absence of stimulation (\textit{n} = 3). Thus, it appears that a rise in Ca\textsuperscript{2+} rather than Na\textsuperscript{+} underlies the nicotinic-induced C\textsubscript{m} increase.

To determine the relationship between changes in averaged [Ca\textsuperscript{2+}] and \textit{Cm} increases, we combined whole cell recordings with simultaneous measurement of fluorescent Ca\textsuperscript{2+} indicators. A typical response to nicotine is shown in Fig. 2 Left. The Ca\textsuperscript{2+} signal peaked within 1–3 sec of the puff. The first calculation of ΔC\textsubscript{m}, 5 sec later, represents the maximal detected exocytotic increase with no further change in C\textsubscript{m}, although cytosolic [Ca\textsuperscript{2+}]\textsubscript{i} remained elevated for many seconds. The limited response indicates the presence of a limited releasable pool of vesicles—which is estimated at about 200 vesicles, or 400 pF, in chromaffin cells (24, 25)—and/or that a transient [Ca\textsuperscript{2+}]\textsubscript{i} rise over a threshold rather than steady-state elevation is the trigger for the C\textsubscript{m} increase. In the same cell, Ca\textsuperscript{2+} entry through voltage-gated Ca\textsuperscript{2+} channels elicited a larger [Ca\textsuperscript{2+}]\textsubscript{i} response with a more rapid rate of rise and decay (Fig. 2 Right), accompanied with a larger ΔC\textsubscript{m}. This favors the second proposal, although we do not rule out a combination of both factors. This also demonstrates that two distinct pathways of Ca\textsuperscript{2+} entry with different kinetics can trigger exocytosis in a single cell.
Thapsigargin (TG) Pretreatment Decreases the Nicotinic Response. Neuroendocrine cells contain active Ca\(^{2+}\) stores that can act as both Ca\(^{2+}\) sources and Ca\(^{2+}\) sinks (26). We investigated their potential roles by pretreating cells with TG, which blocks Ca\(^{2+}\) uptake by microsomal Ca\(^{2+}\)-ATPases (5) and depletes nonmitochondrial Ca\(^{2+}\) stores. TG treatment (6) for 30 min before recordings caused a significant decrease in ΔC\(_m\) but not in I\(_{\text{nic}}\) amplitude (Fig. 3 a–c). It appears that Ca\(^{2+}\) stores play a prominent role in nicotinic triggered exocytosis by acting as a Ca\(^{2+}\) source.

Surprisingly, TG did not detectably change the nicotinic-induced Ca\(^{2+}\) signals (Fig. 3 a, b, and d). In Fig. 3d, the peak amplitude of the Ca\(^{2+}\) signal is plotted, normalized to integrated I\(_{\text{nic}}\). We also compared rates of rise and found no difference. ΔC\(_m\) is expressed relative to changes in [Ca\(^{2+}\)]\(_i\); in Fig. 3e. The efficacy of changes in [Ca\(^{2+}\)]\(_i\) in eliciting ΔC\(_m\) was strongly diminished by TG treatment when cells were stimulated with nicotine (NicC vs. NicTG). By contrast, TG had no effect on the coupling for voltage-activated Ca\(^{2+}\) entry (StC vs. StTG). Parallel experiments during which 1 μM TG was directly applied onto cells voltage-clamped at −90 mV showed that TG application alone did not cause C\(_m\) changes. TG pretreatment also did not alter the basal [Ca\(^{2+}\)]\(_i\) level, since the ratio of fluorescence at 440 nm to that at 490 nm was 1.21 ± 0.25 (n = 8) in control cells and 1.09 ± 0.19 (n = 6) in TG-treated cells (means ± SD).

**DISCUSSION**

Here we demonstrate that activation of a ligand-gated ion channel (nAChR) is able to trigger exocytosis of large dense-core vesicles from neuroendocrine cells independent of depolarization and opening of voltage-gated Ca\(^{2+}\) channels. Thus, chromaffin cells possess at least two Ca\(^{2+}\) entry pathways that can initiate exocytosis: the well-established voltage-dependent Ca\(^{2+}\) pathway and neurotransmitter-gated receptor Ca\(^{2+}\) entry. These two pathways could act synergistically or independently. In the case of nAChR activation by ligand, the amount of Ca\(^{2+}\) entering the channel increases upon membrane hyperpolarization. Inhibitory factors, which decrease voltage-gated Ca\(^{2+}\) channel activities and/or strongly hyperpolarize the membrane, can therefore leave the nAChR Ca\(^{2+}\) entry unchanged or potentiated.

A direct role for nAChR activation in secretion was suggested as early as 1963 by Douglas and Rubin (3), who speculated that acetylcholine might promote Ca\(^{2+}\) entry either by depolarizing chromaffin cells and generating action-potential activity or through some other action independent of changes in membrane potential. More recent studies with Ca\(^{2+}\) channel antagonists have concluded that secretion may result from a combination of these two pathways, with the dominant entry occurring through voltage-gated Ca\(^{2+}\) channels (27). Direct functional actions of Ca\(^{2+}\) entry through nAChRs have been demonstrated for ion channel activation (13, 28) and protein phosphorylation (29), but not for stimulus–secretion coupling.

A major difference between the ligand-gated and voltage-gated pathways is the requirement for additional sources of Ca\(^{2+}\). For voltage-gated Ca\(^{2+}\) channels, it seems likely that only Ca\(^{2+}\) coming from open channels is essential for secretion. No participation of any additional Ca\(^{2+}\) mechanism has been proven (30, 31). Here we show that the situation is markedly different when the exocytosis is due to nicotinic currents at hyperpolarized potentials. The secretory response is determined both by the Ca\(^{2+}\) permeability of nAChR and by an intracellular mechanism which is sensitive to TG. TG is generally accepted as a specific inhibitor of the family of sarcoplasmic or endoplasmic reticulum Ca\(^{2+}\)-ATPases (5). We cannot exclude the possibility that TG could have effects other than blocking microsomal Ca\(^{2+}\) uptake, since higher concentrations inhibited L-type Ca\(^{2+}\) channels in GH3 pituitary cells (32). However, our results strongly suggest that TG does not perturb the exocytotic machinery and does not deplete secretory vesicles, since neither basal release nor exocytosis due to activation of voltage-gated Ca\(^{2+}\) channels is affected by TG pretreatment. We have also not detected changes in Ca\(^{2+}\) currents or disassembly of the submembranous F-actin network (33). Therefore it seems likely that nAChR stimulation recruits TG-sensitive Ca\(^{2+}\) stores.

Further work will be required to determine the nature of the TG-sensitive stores and the mechanism of coupling to nAChR activation. Bovine chromaffin cells possess a wide repertoire of nonmitochondrial Ca\(^{2+}\) stores which include not only separate inositol 1,4,5-trisphosphate-sensitive and ryanodine-sensitive (Ca\(^{2+}\)-induced Ca\(^{2+}\) release) stores, but also overlapping (both inositol trisphosphate- and ryanodine-sensitive) and as yet undefined stores (34, 35). Ca\(^{2+}\) could be released from ryanodine-sensitive stores as a result of Ca\(^{2+}\) entry through nAChRs. Alternatively, nAChR stimulation may act through other intermediaries, such as increased inositol trisphosphate turnover, which can occur in the absence of extracellular Ca\(^{2+}\) (36).

An intriguing but puzzling observation is the apparent uncoupling between averaged cytosolic Ca\(^{2+}\) and nicotine-
elicted $\Delta C_m$ changes on TG pretreatment (Fig. 3e). This suggests that the observed increase in $[\text{Ca}^{2+}]_i$ arises mostly from $\text{Ca}^{2+}$ entry through nAChRs, while the contribution from TG-sensitive stores is critical but highly localized so that it is not reflected in averaged $[\text{Ca}^{2+}]_i$. This contrasts with the prominent reductions of $\text{Ca}^{2+}$ signals caused by TG pretreatment following activation of metabotropic G-protein-coupled receptors in chromaffin cells (7), as well as activation of NMDA receptors and voltage-gated Ca$^{2+}$ channels in neuronal cells (37, 38). These findings add to the growing list in which local, rather than global, $\text{Ca}^{2+}$ increases seems to be the privileged activator of many cellular events. These include not only secretion at fast synapses (39) and in neuroendocrine cells (9), but also ion channel (40) and enzyme (41) activities. Interestingly, activation of inositol trisphosphate receptors contributes highly localized $\text{Ca}^{2+}$ increases for nuclear vesicle fusion (42).

In summary, this form of neurotransmitter action extends the repertoire by which a cell or a synapse can respond to nAChR stimulation. The response to nicotinic activation will depend on the cell’s complement of intracellular $\text{Ca}^{2+}$ stores and voltage-gated channels and the concurrent level of electrical activity. For instance, nAChR stimulation could operate even in the absence of action-potential activity, as during strong inhibitory input or in developing cells lacking excitable mechanisms. These observations indicate an unsuspected level of versatility in the mode of function of the nAChR.

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