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Activation of nicotinic receptors triggers exocytosis from bovine chromaffin cells in the absence of membrane depolarization

(stimulus–secretion coupling/calcium stores/calcium entry/capacitance detection)

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ABSTRACT **The traditional function of neurotransmitter-gated ion channels is to induce rapid changes in electrical activity. Channels that are Ca^{2+}-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 Ca^{2+}-permeable than *N*-methyl-D-aspartate receptors but have a significant Ca^{2+} 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 detection technique to monitor 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 Ca^{2+} influx through nAChRs and by a contribution from thapsigargin-sensitive Ca^{2+} 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 Ca^{2+} entry.**

Catecholamine secretion from chromaffin cells is evoked by acetylcholine (ACh) released from sympathetic preganglionic fibers. In bovine chromaffin cells, ACh elicits a nonspecific cation current 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 Ca^{2+} influx (2) and are assumed to be the main source of the rise in cytosolic Ca^{2+} that results in exocytosis of large dense-core vesicles which contain catecholamines. However, other sources of Ca^{2+} 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 Ca^{2+} entry related to action potentials corresponds to the opening of at least three distinct types of voltage-gated Ca^{2+} channels (8). Interestingly, combination of Ca^{2+} channel current recordings with detection of single-cell secretion by capacitance measurements has indicated a preferential link between specific Ca^{2+} channel types and the exocytotic machinery. Ca^{2+} 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 Ca^{2+} source and exocytosis has not yet been reported for other Ca^{2+}-regulating mechanisms.

Chromaffin cell nAChRs belong to a ligand-gated ion channel family with a modest, but significant, Ca^{2+} permeability (10–12). nAChRs, unlike *N*-methyl-D-aspartate (NMDA) receptors, which are Ca^{2+}-permeable only during concomitant depolarization, are possible sources of Ca^{2+} entry at hyperpolarized membrane potentials. Since Ca^{2+} 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 Ca^{2+} channel thresholds. In some experiments, capacitance measurements were combined with whole-cell cytosolic Ca^{2+} 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 $MgCl_2$/2 mM KCl/10 mM glucose/10 mM sodium Hepes/2 mM $CaCl_2$, 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- to 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 Ca^{2+} Concentration ($[Ca^{2+}]_i$). Fluorescent Ca^{2+} probes (fluo-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; $[Ca^{2+}]_i$ and $[Na^+]_i$, intracellular (cytosolic) concentrations of Ca^{2+} and Na^+; NMDA, *N*-methyl-D-aspartate; TG, thapsigargin.

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Ca^{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^{2+}-insensitive wavelength recording was done just before stimulation and recording at the 490-nm Ca^{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_m traces. Student's *t* test and the Mann–Whitney *U* test were used when appropriate.

RESULTS

C_m Responses Are Proportional to the Magnitude of the Nicotinic Current (I_{Nic}). The ability of nAChRs to regulate exocytosis in the absence of Ca^{2+} entry through voltage-gated Ca^{2+} channels was investigated in bovine chromaffin cells voltage-clamped to −60 mV, below the threshold for channel activation. Extracellular Ca^{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_m) presumably due to exocytosis of catecholamine-containing granules (19). Nicotine, applied from a puffer pipette, triggered a large inward current (I_{Nic}) which was followed by a sustained increase in C_m, but no change in conductance (G) (Fig. 1*a*). Both the inward current and the ΔC_m were abolished when 1 μM (+)-tubocurarine was present, indicating that the responses resulted from activation of nicotinic AChRs (Fig. 1*b*).

The amount of C_m increase was related to the amplitude of I_{Nic}. Individual cells responded several times to successive applications of nicotine. In Fig. 1*c*, 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_m. On subsequent return to −60 mV, the C_m response was smaller than after the first application, because of secretory "rundown." 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 I_{Nic} in causing ΔC_m is summarized in Fig. 1*d*. Data are binned for the integral of I_{Nic} (expressed in nanocoulombs) and amplitude of C_m jumps (in femtofarads). The secretory response increases for larger total charge and eventually saturates.

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

To determine the relationship between changes in averaged $[Ca^{2+}]_i$ and C_m increases, we combined whole cell recordings with simultaneous measurement of fluorescent Ca^{2+} indicators. A typical response to nicotine is shown in Fig. 2 *Left*. The

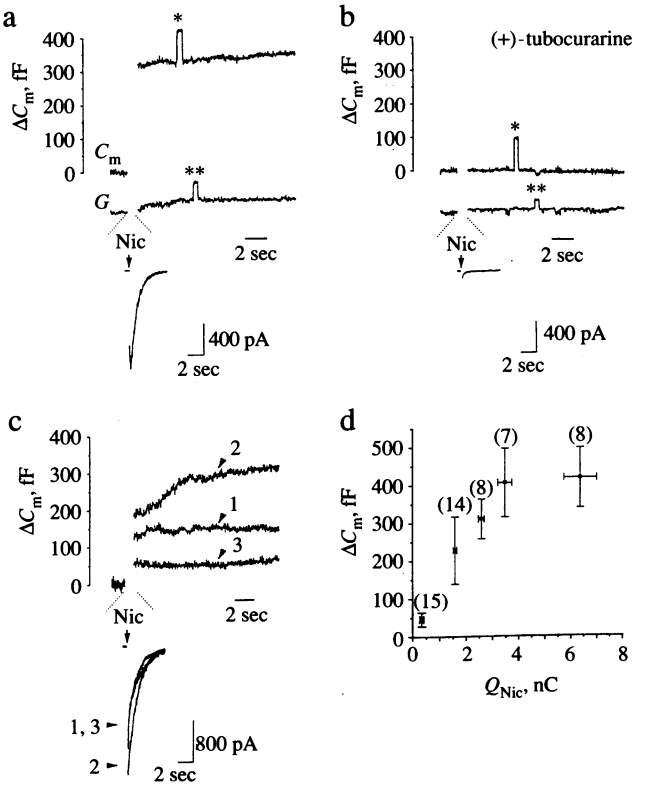

FIG. 1. Nicotine application elicits increases of C_m that are proportional to the integral of I_{Nic} and are blocked by (+)-tubocurarine. (*a*) C_m trace. A 1.5-sec baseline segment is followed by a break for the nicotine puff (Nic, 50 μM, 200 msec) and a 5-sec period during which I_{Nic} was recorded (lowest trace). After the break (which is not to scale, lasting a total of 5.7 sec), C_m was increased by ≈320 fF, while the conductance component (G, middle trace) is unchanged. The square pulses in the C_m (*) and G (**) traces represent calibrations of 100 fF and 10 nS, respectively, achieved by unbalancing the C_{slow} and G_{series} compensations of the patch clamp. (*b*) An experiment similar to that in *a*, but with 1 μM (+)-tubocurarine in the bath. There is no change in the C_m (top) or G (middle) traces. I_{Nic} is almost completely eliminated (bottom trace). (*c*) Three superimposed C_m traces obtained sequentially from one cell, held at −60 mV (traces 1 and 3) or −90 mV (trace 2). The third C_m trace illustrates partial "rundown" of the secretory response, but not of I_{Nic}. The nicotine puffs were applied at ≈5-min intervals. (*d*) Binned data (means ± SEM) illustrating the correlation between the integral of I_{Nic} (charge, Q, expressed in nanocoulombs) and amplitude of the C_m jumps. Various I_{Nic} integrals were obtained because of cell-to-cell variations, different puff durations and puff pressures, and different nicotine concentrations (50–200 μM). The number above each point is the number of individual C_m traces. Responses were binned for 1-nC intervals except above 4 nC, where all eight cases were pooled.

Ca^{2+} signal peaked within 1–3 sec of the puff. The first calculation of ΔC_m, 5 sec later, represents the maximal detected exocytotic increase with no further change in C_m, although cytosolic $[Ca^{2+}]_i$ 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 fF, in chromaffin cells (24, 25)—and/or that a transient $[Ca^{2+}]_i$ rise over a threshold rather than steady-state elevation is the trigger for the C_m increase. In the same cell, Ca^{2+} entry through voltage-gated Ca^{2+} channels elicited a larger $[Ca^{2+}]_i$ response with a more rapid rate of rise and decay (Fig. 2 *Right*), accompanied with a larger ΔC_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^{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_{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. 3*d*, the peak amplitude of the Ca^{2+} signal is plotted, normalized to integrated I_{Nic}. We also compared rates of rise and found no difference. ΔC_m is expressed relative to changes in $[Ca^{2+}]_i$ in Fig. 3*e*. The efficacy of changes in $[Ca^{2+}]$ 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).

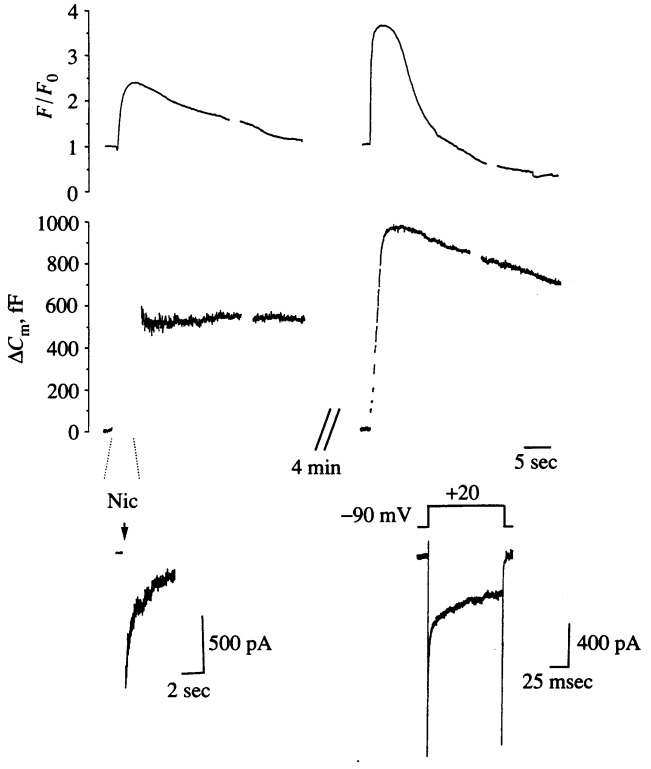

FIG. 2. Changes in $[Ca^{2+}]_i$ and C_m in response to activation of nAChRs and to voltage-gated Ca^{2+} entry. (*Left*) The cell was voltage-clamped to −90 mV and a 200-msec puff of nicotine was applied (Nic). The change in $[Ca^{2+}]_i$ is normalized to the pre-puff fluorescence (F_o) (first 2 sec of trace). The duration of the break in the C_m trace is plotted to scale. The late breaks in the C_m and $[Ca^{2+}]_i$ traces are due to interruption of sampling in order to check the accuracy of phase alignment and store the data. The first 5 sec of C_m is noisier due to the small, slowly decaying conductance change due to I_{Nic}. (*Right*) Four minutes after the end of the recording on the left, the cell was stimulated with a train of 10 depolarizing pulses, from −90 to +20 mV. The first Ca^{2+} current of the train is shown at bottom right. The early large spike reflects the voltage-gated Na^+ current. Averaged $[Ca^{2+}]_i$ is much larger and more transient than that elicited by I_{Nic}, and the C_m response is about 2-fold larger. The decay of C_m presumably represents endocytosis and was always more pronounced after activation of Ca^{2+} currents. The $[Ca^{2+}]_i$ trace occasionally decayed below baseline following large amounts of Ca^{2+} entry. Fluorescent dye was fluo-3.

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 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-

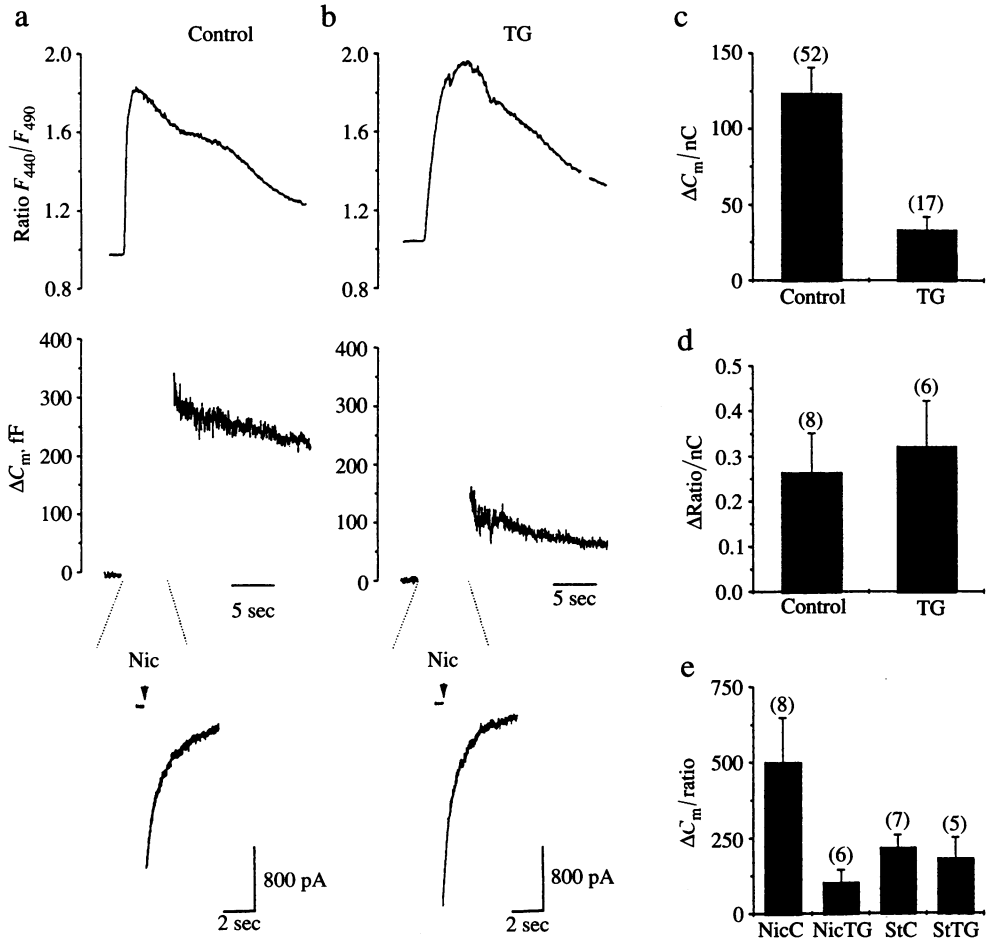

FIG. 3. TG pretreatment decreases the efficacy of coupling between I_{Nic} and ΔC_m but does not alter the response to Ca^{2+} entry through voltage-gated channels. (*a*) $[Ca^{2+}]_i$ [ratio of fluorescence at 440 nm to that at 490 nm (F_{440}/F_{490})], ΔC_m, and I_{Nic} responses to puff application of nicotine (Nic) under control conditions. (*b*) Similar experiment, but following pretreatment with TG (30 min, 1 μM, bath applied). There were no large differences between I_{Nic} (3.1 nC vs. 3.82 nC) or $\Delta[Ca^{2+}]_i$ for TG vs. control conditions. The C_m response, however, was much smaller. (*c*) Comparison of averaged ΔC_m responses for control and TG conditions, normalized to the integral of I_{Nic} (means ± SEM). The two values are statistically different ($P < 0.05$). Numbers in parentheses indicate the numbers of individual traces. (*d*) The ratio F_{440}/F_{490} is normalized to the integral of I_{Nic}. Data are from a subset of *c*. There is no significant difference in $[Ca^{2+}]_i$ changes after TG pretreatment. (*e*) Comparison of the efficacy of changes in $[Ca^{2+}]_i$ in eliciting C_m increases. Comparisons are made for I_{Nic} induced C_m changes in control (NicC) and TG pretreated (NicTG) cells, and for step depolarizations in control (StC) and TG-pretreated cells (StTG). NicC and NicTG are significantly different ($P < 0.05$). Fluorescent dye was Fura Red.

elicited C_m changes on TG pretreatment (Fig. 3*e*). This suggests that the observed increase in $[Ca^{2+}]_i$ arises mostly from 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 $[Ca^{2+}]_i$. This contrasts with the prominent reductions of 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, Ca^{2+} increases seems to be the priviliged 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 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 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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1. Douglas, W. W., Kanno, T. & Sampson, S. R. (1967) *J. Physiol. (London)* **191,** 107–121.
2. Brandt, B. L., Hagiwara, S., Kidokoro, Y. & Miyazaki, S. (1976) *J. Physiol. (London)* **263,** 417–439.
3. Douglas, W. W. & Rubin, R. (1963) *J. Physiol. (London)* **167,** 288–310.
4. Burgoyne, R. D., Cheek, T. R., Morgan, A., O'Sullivan, A. J., Moreton, R. B., Berridge, M. J., Mata, A. M., Colyer, J., Lee, A. G. & East, J. M. (1989) *Nature (London)* **342,** 72–74.
5. Thastrup, O., Cullen, P. J., Drobak, B. K., Hanley, M. R. & Dawson, A. P. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 2466–2470.
6. Cheek, T. R. & Thastrup, O. (1989) *Cell Calcium* **10,** 213–221.

7. Robinson, I. A. & Burgoyne, R. D. (1991) *J. Neurochem.* **56,** 1587–1593.
8. Artalejo, C. R., Mogul, D. J., Perlman, R. L. & Fox, A. P. (1991) *J. Physiol. (London)* **444,** 213–240.
9. Artalejo, C. R., Adams, M. E. & Fox, A. P. (1993) *Nature (London)* **367,** 72–75.
10. Vernino, S., Amador, M., Luetje, C. W., Patrick, J. & Dani, J. A. (1992) *Neuron* **8,** 127–134.
11. Zhou, Z. & Neher, E. (1993) *Pflügers Arch.* **425,** 511–517.
12. Vernino, S., Rogers, M., Radcliffe, K. A. & Dani, J. A. (1994) *J. Neurosci.* **14,** 5514–5524.
13. Mulle, C., Choquet, D., Korn, H. & Changeux, J. P. (1992) *Neuron* **8,** 135–143.
14. Vitale, M. L., del Castillo, A. R., Tchakarov, L. & Trifaro, J.-M. (1991) *J. Cell Biol.* **113,** 1057–1067.
15. Kurebayashi, N., Harkins, A. B. & Baylor, S. M. (1993) *Biophys. J.* **64,** 1934–1960.
16. Neher, E. & Marty, A. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 6712–6716.
17. Joshi, C. & Fernandez, J. M. (1988) *Biophys. J.* **53,** 889–892.
18. Fidler, N. & Fernandez, J. M. (1989) *Biophys. J.* **56,** 1153–1162.
19. Chow, R. H., von Ruden, L. & Neher, E. (1992) *Nature (London)* **356,** 60–63.
20. Role, L. W. (1992) *Curr. Opin. Neurobiol.* **2,** 256–262.
21. Trouslard, J., Marsh, S. J. & Brown, D. A. (1993) *J. Physiol. (London)* **468,** 53–71.
22. Seguela, P., Waidiche, J., Dineley-Miller, K., Dani, J. A. & Patrick, J. W. (1993) *J. Neurosci.* **13,** 596–604.
23. Tsien, R. Y. (1980) *Biochemistry* **19,** 2396–2404.
24. Neher, E. & Zucker, R. S. (1993) *Neuron* **10,** 21–30.
25. Heinemann, C., von Ruden, L., Chow, R. H. & Neher, E. (1993) *Pflügers Arch.* **424,** 105–112.
26. Barry, V. A. & Cheek, T. R. (1994) *Biochem. J.* **300,** 589–597.
27. Gandia, L., Casado, L.-F., Lopez, M. G. & Garcia, A. G. (1991) *Br. J. Pharmacol.* **103,** 3068–3072.
28. Vernino, S., Amador, M., Luetje, C. W., Patrick, J. & Dani, J. A. (1992) *Neuron* **8,** 127–135.
29. Messing, R. O., Stevens, A. M., Kiyasu, E. & Sneade, A. B. (1989) *J. Neurosci.* **9,** 507–512.
30. O'Sullivan, A. J., Cheek, T. R., Moreton, R. B., Berridge, M. J. & Burgoyne, R. D. (1989) *EMBO J.* **8,** 401–411.
31. Augustine, G. & Neher, E. (1992) *J. Physiol. (London)* **450,** 247–271.
32. Nelson, E. J., Li, C. C. R., Bangalore, R., Benson, T., Kass, R. S. & Hinkle, P. M. (1994) *Biochem. J.* **302,** 147–154.
33. Seward, E. P., Mollard, P. & Nowycky, M. C. (1993) *Mol. Biol. Cell* **4,** 257 (abstr.).
34. Stauderman, K. A., McKinney, R. A. & Murawsky, M. M. (1991) *Biochem. J.* **278,** 643–650.
35. Robinson, I. M., Cheek, T. R. & Burgoyne, R. D. (1992) *Biochem. J.* **288,** 457–463.
36. Grassi, F., Giovannelli, A., Fucile, S. & Eusebi, F. (1993) *Pflügers Arch.* **422,** 591–598.
37. Lei, S. Z., Zhang, D. & Lipton, S. A. (1992) *Neurosci. Abstr.* **18,** 256.
38. Alford, S., Frenguelli, B. G., Schofield, J. G. & Collingridge, G. L. (1993) *J. Physiol. (London)* **496,** 693–716.
39. Adler, E. M., Augsutine, G. J., Duffy, S. N. & Charlton, M. P. (1991) *J. Neurosci.* **11,** 1496–1507.
40. Roberts, W. M., Jacobs, R. A. & Hudspeth, A. J. (1990) *J. Neurosci.* **10,** 3664–3684.
41. Sihra, T. S., Bogonez, E. & Nicholls, D. G. (1992) *J. Biol. Chem.* **267,** 1983–1989.
42. Sullivan, K. M. C., Buss, W. B. & Wilson, K. L. (1993) *Cell* **73,** 1411–1422.