# Calcium Current Activated by Muscarinic Receptors and Thapsigargin in Neuronal Cells

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ABSTRACT The activation of muscarinic receptors in N1E-115 neuroblastoma cells elicits a voltage-independent calcium current. The current turns on slowly, reaches its maximum value ~45 s after applying the agonist, is sustained as long as agonist is present, and recovers by one half in ~10 s after washing the agonist away. The current density is 0.11 ± 0.08 pA/pF (mean ± SD; $n$ = 12). It is absent in zero-$Ca^{++}$ saline and reduced by $Mn^{++}$ and $Ba^{++}$. The $I(V)$ curve characterizing the current has an extrapolated reversal potential > +40 mV. The calcium current is observed in cells heavily loaded with BAPTA indicating that the calcium entry pathway is not directly gated by calcium. In fura-2 experiments, we find that muscarinic activation causes an elevation of intracellular $Ca^{++}$ that is due to both intracellular calcium release and calcium influx. The component of the signal that requires external $Ca^{++}$ has the same time course as the receptor operated calcium current. Calcium influx measured in this way elevates $(Ca^{++})_i$ by 89 ± 41 nM ($n$ = 7). Thapsigargin, an inhibitor of $Ca^{++}$/ATPase associated with the endoplasmic reticulum (ER), activates a calcium current with similar properties. The current density is 0.22 ± 0.20 pA/pF ($n$ = 6). Thapsigargin activated current is reduced by $Mn^{++}$ and $Ba^{++}$ and increased by elevated external $Ca^{++}$. Calcium influx activated by thapsigargin elevates $(Ca^{++})_i$ by 82 ± 35 nM. The $Ca^{++}$ currents due to agonist and due to thapsigargin do not sum, indicating that these procedures activate the same process. Carbachol and thapsigargin both cause calcium release from internal stores and the calcium current bears strong similarity to calcium-release–activated calcium currents in nonexcitable cells (Hoth, M., and R. Penner. 1993. *Journal of Physiology.* 465:359–386; Zweifach, A., and R. S. Lewis, 1993. *Proceedings of the National Academy of Sciences, USA.* 90:6295–6299).

## INTRODUCTION

Muscarinic receptors in N1E-115 neuroblastoma cells activate a signal transduction cascade that leads to an elevation of intracellular calcium. M1 muscarinic receptors stimulate phospholipase C to generate inositol 1,4,5-trisphosphate ($IP_3$) and $IP_3$ causes intracellular calcium release from $Ca^{++}$ storage compartments associated with

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the ER (Berridge and Irvine, 1989; Marty and Tan, 1989; Mathes, Wang, Vargas, and Thompson, 1992). This signal cascade also activates a $Ca^{++}$ current and both calcium release and calcium influx contribute to the increase in $(Ca^{++})_i$.

Indirect evidence suggested that muscarinic agonists activate calcium influx (Study, Breakefield, Bartfai, and Greengard, 1978; El-Fakahany and Richelson, 1980; El-Fakahany and Richelson, 1983; Felder, Poulter, and Wess, 1992; Felder, MacArthur, Ma, Gusovsky, and Kohn, 1993). A voltage-independent calcium current has been described in lymphocytes expressing muscarinic receptors using conventional whole cell voltage clamp (McDonald, Premack, and Gardner, 1993). We report a voltage-independent $Ca^{++}$ current that is activated by muscarinic receptors in mouse N1E-115 neuroblastoma cells. This current is measured in physiological saline, without dialysis and without introducing exogenous buffers, using the nystatin perforated-patch voltage clamp technique (Horn and Marty, 1988). Voltage-gated calcium channels present in these cells (Yoshi, Tsunoo, and Narahashi, 1988) do not contribute to this current. The contribution of $Ca^{++}$ influx to the increase in $(Ca^{++})_i$ was resolved in fura-2 imaging experiments on single cells. Calcium influx activates more slowly than intracellular $Ca^{++}$ release and outlasts release so that it sustains the $Ca^{++}$ response. Calcium influx can also be activated independently of muscarinic receptors with thapsigargin, an inhibitor of the ER $Ca^{++}$/ATPase. In voltage clamp experiments we find that thapsigargin activates a voltage-independent $Ca^{++}$ current with properties very similar to the current activated by muscarinic agonists.

The calcium current reported here is similar in many respects to voltage-independent calcium currents activated by agonists and by depletion of intracellular $Ca^{++}$ stores in nonexcitable cells (Penner, Matthews, and Neher, 1988; Lewis and Cahalan, 1989; Hoth and Penner, 1992; Parekh, Foguet, Lübbert, and Stühmer, 1993; Parekh, Teriau, and Stühmer, 1993; Vaca and Kunze, 1993; Zweifach and Lewis, 1993). This calcium current may represent a general feature of neurons that express muscarinic and other G-protein coupled receptors (Ehrlich, Snider, Kornecki, Garfield, and Lenox, 1988; Reber, Neuhaus, and Reuter, 1992). Preliminary reports of these findings have been published in abstract form (Mathes and Thompson, 1992, 1993).

## METHODS

### *Cell Culture*

Mouse N1E-115 neuroblastoma cells were obtained from the University of California San Francisco Cell Culture Facility and used after 2–8 passages. They were maintained in DMEM with 10% FBS at 37°C with 10% $CO_2$. Cells were plated on glass cover slips and grown to ~80% confluency before differentiation with DMSO for 5 to 21 d (Kimhi, Palfrey, Spector, Barak, and Littauer, 1976). The cells were fed every 2–3 d and were used 1–2 d after feeding.

### *Nystatin Patch Clamp*

The normal external saline contained (in millimolar): 146 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.8 $MgSO_4$, 0.4 $KH_2PO_4$, 0.3 $Na_2HPO_4$, 5 glucose, 20 HEPES, plus 10 μM curare (pH 7.4; 30°C). Zero calcium external saline was the same except $MgCl_2$ was substituted for $CaCl_2$. The bath volume (500 μl) was rapidly exchanged with a gravity perfusion system. Carbachol (Sigma

Chemical Co., St. Louis, MO) was added as a 5-μl droplet (0.1 M) to a final bath concentration of 1 mM; vehicle alone never activated ionic currents (Fig. 4 *A*). The pipette solution contained (in millimolar): 16 KCl or CsCl, 70 $K_2SO_4$ or $Cs_2SO_4$, 5 $MgSO_4$, 10 HEPES, and 100 sucrose to ~320 mosM at pH 7.2. After filtering the pipette solution, 0.05% Pluronic acid F-127 (Molecular Probes Inc., Eugene, OR) was added to increase the solubility of nystatin (Lucero and Pappone, 1990). The calculated reversal potential for $Cl^-$ = −60 mV and for $K^+$ = −85 mV. N1E-115 cells were held at −60 mV, which is near their normal resting potential (Kato, Anwyl, Quandt, and Narahashi, 1983).

Whole cell voltage clamp was done using the nystatin perforated-patch technique (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981; Horn and Marty, 1988; Lucero and Pappone, 1990). Nystatin was added to filtered pipette solution from a DMSO stock (2.5 mg nystatin in 50 μl DMSO) to a final concentration of 100–200 μg/ml. The solution was used within 1 h. Patch electrodes were made from thick-walled Borofilament glass (OD 1.5 mm, ID 0.86 mm; model BF150-86-15, Sutter Instrument Co., Novato, CA). Suction was used to form seals which reached resistances of 1–3 gigaohms. Access resistance was monitored by applying +10 mV voltage pulses from −80 mV and reached 10 to 90 MΩ. Series resistance compensation (up to 50%) was employed. Slow fluctuations in baseline currents are attributed to variations in the seal and access resistances due to nystatin in the pipette (Horn and Marty, 1988). Data acquisition and analysis were done using PCLAMP software. Current records were sampled at 3–5 kHz and low pass filtered at corner frequencies of 1–50 Hz. Junction potentials were −7 to −9 mV and were not corrected.

### *Whole Cell Dialysis with $IP_3$ and EGTA*

Whole cell patch clamp, break-in experiments were done using the same external saline. The pipette solution contained (in millimolar): 145 Cs·Aspartate, 7 NaCl, 1 $MgCl_2$, 0.5 $Na_2$·ATP, 10 HEPES·CsOH, 10 EGTA at pH 7.2. $IP_3$ (10 μM; Sigma Chemical Co.) was added to this solution. The calculated reversal potential for $Cl^-$ = −70 mV, and therefore, the holding potential during these experiments was set to −70 mV to match the nystatin patch conditions. Series resistances were 2–18 MΩ. Junction potentials were −8 to −10 mV and were not corrected.

### *Imaging $(Ca^{++})_i$*

Cells were loaded with fura-2 (5 μM fura-2/AM, Molecular Probes Inc.; 1 hour) and viewed in epifluorescence using a Nikon Diaphot and xenon arc illumination as described previously (Mathes et al., 1992). A computer-controlled filter wheel holding 10-nm bandpass interference filters was used to select excitation wavelengths of 340, 360, and 380 nm. Images were recorded using a SIT camera (Hamamatsu Hamamatsu Photonics K.K., Hamamatsu City, Japan) and VHS tape recorder. Fluorescence signals were analyzed in a region of interest corresponding to the interior of the cell body using a Megavision 1024XM digital image processor. The signals were background subtracted and calibrated to units of $(Ca^{++})_i$ using standard procedures (Grynkiewicz, Poenie, and Tsien, 1985). Values of $\sigma$, $R_{min}$, and $R_{max}$ were 21.0, 0.072, and 2.44 respectively. Calcium calibrations from single wavelength determinations were obtained by extrapolating values for $F_{max}$ and $F_{min}$ at 380 nm and using the formula $(Ca^{++})_i = K_d(F_{380}-F_{min})/(F_{max} - F_{380})$.

## RESULTS

### *Calcium Current in Physiological Saline*

Voltage clamp experiments using the nystatin patch technique show that carbachol activates an inward current in N1E-115 mouse neuroblastoma cells (Fig. 1 *A*). The

response is blocked reversibly by the selective M1 antagonist pirenzepine (1 μM) and it is unaffected by the nicotinic antagonist curare (10 μM) or by TTX (1 μM). When cells are bathed in normal physiological saline, the inward current develops slowly, reaching a peak in 44 ± 9 s (mean ± SD; $n$ = 10). The half-recovery time is 10 ± 16 s when the agonist is washed away. This inward current was observed in 67 of 131 cells (51%). The peak amplitude was 37 ± 42 pA and the current density was 0.11 ± 0.08 pA/pF (Fig. 1 *B*).

Receptor operated inward current is not observed when the cells are bathed in $Ca^{++}$-free saline (Fig. 2 *A*). Addition of $Mn^{++}$ to the external medium rapidly blocks the current (Fig. 2 *B*). The current is also reduced by the addition of $Ba^{++}$ to the bath in the presence of $Ca^{++}$ (Fig. 2 *C*). Chloride currents do not contribute to the response because the holding potential in these experiments, −60 mV, approximates the $Cl^-$ equilibrium potential. Modulation of M-current does not contribute to the

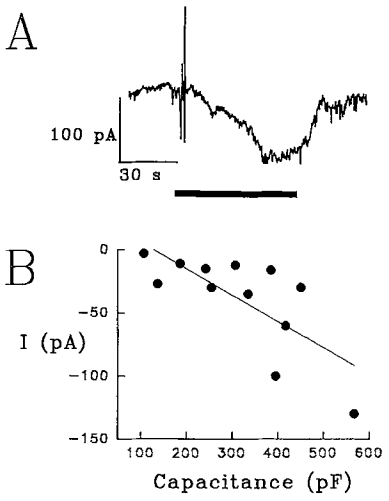


FIGURE 1. Inward current during muscarinic activation in N1E-115 mouse neuroblastoma cells voltage clamped using the nystatin perforated-patch technique. (*A*) Whole cell current at a holding voltage of −60 mV. Carbachol (1 mM) activates an inward current that develops with a delay, reaches a maximum in 45 s, and decays with a half time of 6 s. The transient near the start of the record is a mechanical artifact marking the beginning of carbachol application (*bar*). The whole cell capacitance was 358 pF. (*B*) Current versus whole cell capacitance from 12 cells analyzed in this way. The current amplitude increases as the capacitance increases. The regression coefficient is $r$ = 0.73 and the slope is 0.23 pA/pF. The average whole cell capacitance was 259 ± 150 pF.

response for two reasons: the holding potential is below the activation threshold for M-current (Brown and Adams, 1980) and inward currents are observed when the pipette solution contains $Cs^+$ to block potassium channels. The conclusion we draw is that the inward current that results from muscarinic receptor activation is carried predominantly and perhaps exclusively by calcium.

When the pipette solution contains $K^+$ and in the absence of potassium channel blockers, the response to carbachol is often biphasic, consisting of an early outward current that reaches a peak and then decays into an inward current (Fig. 3 *A* and *C*). Biphasic responses were seen in 59% of the cells (34 of 58). The outward current reverses at −85 mV, the expected equilibrium potential for $K^+$. It is never seen when the pipette solution contains $Cs^+$ or when the cells are heavily loaded with BAPTA (10 μM BAPTA/AM; 1 h). For these reasons, the outward current appears to be a $Ca^{++}$-dependent potassium current, described in this cell line by Leinders and Vijverberg (1992).

Substitution of $Ba^{++}$ for $Ca^{++}$ in the external saline reduces both the inward current and the outward calcium-dependent potassium current. In the example illustrated in Fig. 3 *A*, carbachol elicits a biphasic response when the cell is bathed in external saline containing 1.8 mM $Ca^{++}$ (Fig. 3 *A*). The saline was exchanged for one in which $Ba^{++}$ was substituted for $Ca^{++}$ and after a 4-minute rest it was found that both the inward and the outward components of the carbachol response were

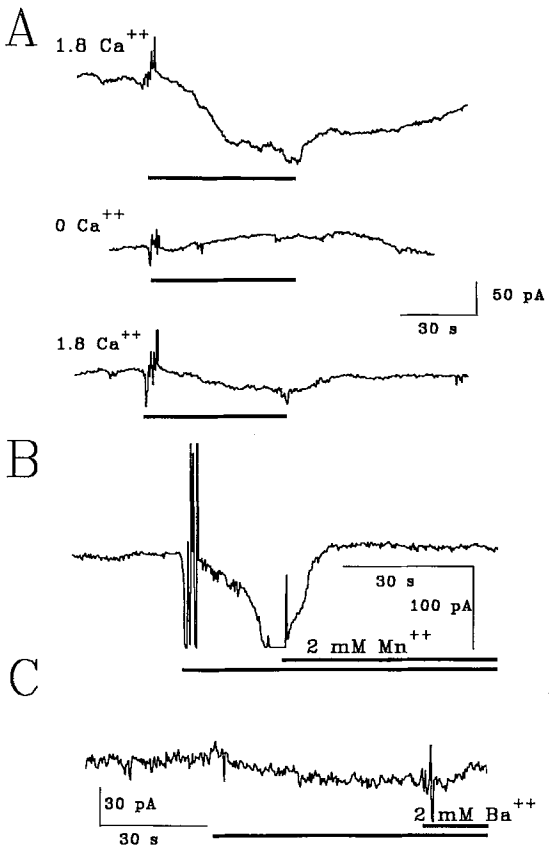


FIGURE 2. The inward current is carried by calcium in normal physiological saline. (*A*) Receptor operated inward current is absent in $Ca^{2+}$-free external saline ($Mg^{2+}$ substituted for $Ca^{2+}$). Three carbachol responses in the same cell are illustrated. Carbachol was applied first in normal saline. The chamber was rinsed with $Ca^{2+}$-free saline and 4 min later, carbachol was applied a second time (a small outward current was observed in two of four experiments). The third record shows the response to carbachol 4 min after returning to normal saline. The capacitance of this cell was 567 pF. (*B*) Manganese (2 mM), added to the bath as the receptor operated inward current developed, immediately blocked the current. This effect was reversible ($n = 4$). (*C*) Barium (2 mM) added to the bath in the presence of external $Ca^{2+}$ reduced the inward current ($n = 3$). In these experiments $SO_4^-$ was excluded to prevent precipitation and $Cs^+$ replaced $K^+$ in the pipette solution.

strongly reduced (Fig. 3 *B*). This effect was reversible (Fig. 3 *C*). Barium substitution reduced the amplitude of the receptor operated inward current by 70% ($n = 3$). It completely blocked $Ca^{++}$-dependent $K^+$ current.

*Calcium Current in Zero $Na^+$ Saline and after BAPTA/AM Loading*

The current-voltage relationship characterizing the receptor operated current was measured in an external saline containing high calcium, zero sodium, and TEA. $Cs^+$

replaced $K^+$ in the patch pipette (Fig. 4 *A*). The cells were loaded with 10 μM BAPTA/AM (1 h) to block calcium dependent conductances. Under these conditions the current reaches its maximum in 31 ± 11 s ($n$ = 4) after the agonist is applied. A series of voltage pulses was presented from a holding potential of −60 mV before applying agonist (Fig. 4 *B, left*) and at the peak of the inward current activated by carbachol (Fig. 4 *B, right*). The pulses were limited to negative potentials (−95 to −50 mV) to avoid activating voltage-gated $Ca^{++}$ channels (Yoshii et al., 1988). There is no indication of time-dependent gating during the pulses. Control currents were subtracted from the currents measured during the agonist response to generate the difference $I(V)$ curve in Fig. 4 *C*. The $I(V)$ curve is approximately linear and extrapolation of a least-squares fit to the data suggests an apparent reversal potential of +95 mV. The positive slope of the $I(V)$ curve is taken as evidence that muscarinic

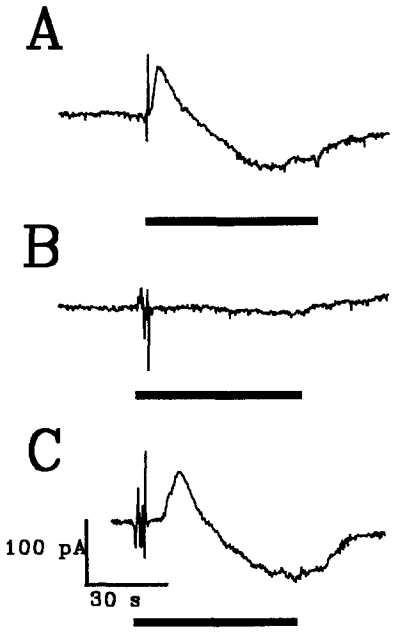


FIGURE 3. Effects of barium replacement on receptor-operated currents. (*A*) Biphasic response during a 1-min exposure to 1 mM carbachol in normal saline. The pipette solution contained 156 mM $K^+$. (*B*) The chamber was rinsed with external saline containing $Ba^{2+}$ in place of $Ca^{2+}$ and after 4 min carbachol was applied again. In $Ba^{2+}$ saline the outward component was blocked and the inward component was reduced by 70%. (*C*) Response to 1 mM carbachol 4 min after returning to normal saline. Cell capacitance = 395 pF. These results are representative of three experiments.

activation causes an increase in conductance and under the conditions of this experiment it is most likely that calcium ions carry the current.

*Time Course of $(Ca^{++})_i$ Elevation*

The $Ca^{++}$ indicator fura-2 was used in video imaging experiments to measure the $Ca^{++}$ influx activated by carbachol in single cells. This required a difference measurement because carbachol also elicits intracellular $Ca^{++}$ release (Mathes et al., 1992; Fig. 5). Cells loaded with fura-2/AM (5 μM; 1 h) were exposed to $Ca^{++}$-free external saline for 1–2 min before applying carbachol. An example from a single cell is shown in Fig. 5 *A* and an average of nine cells from three different experiments is shown in Fig. 5 *B*. The agonist stimulated a rapid elevation of $(Ca^{++})_i$ that peaked and then declined. After a rest period, carbachol was applied again but this time the

external saline contained 1.8 mM $Ca^{++}$. The peak amplitudes of the two responses are approximately the same but after the peak the $(Ca^{++})_i$ measured in 1.8 mM $Ca^{++}$ remains at a higher level for a greater period of time. The difference between the two responses represents the change in $(Ca^{++})_i$ that results from $Ca^{++}$ influx. The time to peak calcium influx measured in this way is 43 ± 12 s (mean ± SD; $n$ = 15) and the average steady state elevation is 89 ± 41 nM (range, 47–186 nM; $\Delta F/$

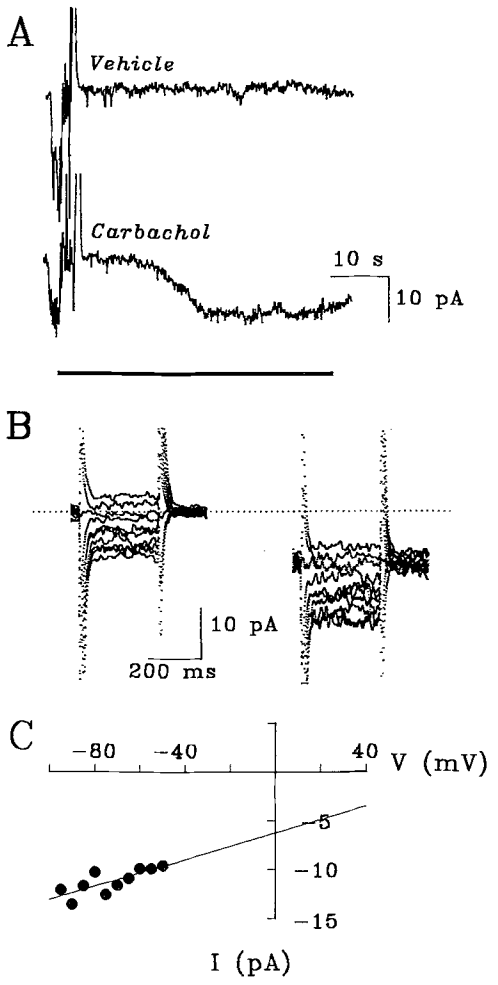


FIGURE 4. Current-voltage relationship for receptor operated inward current in an external saline that isolates $Ca^{2+}$ as the current carrier. (*A*) Inward current activated by carbachol in saline containing high calcium (25 mM), zero sodium (90 mM Tris-HCl), and TEA (10 mM). $Cs^+$ replaced $K^+$ in the pipette solution. This cell was loaded with 10 μM BAPTA/AM (Calbiochem Corp.) for 1 h before the experiment. (*B*) In a different cell, 200-ms voltage pulses from −95 to −50 mV were applied from the holding potential of −60 mV. The pulse series was repeated before applying agonist (*left*) and during the inward current activated by 1 mM carbachol (*right*). The dotted line shows the holding current at −60 mV (−15 pA). (*C*) Difference $I(V)$ curve obtained from the data in *B* by subtracting control currents from currents measured during the agonist response. The line drawn through the data points is a linear regression ($r$ = 0.78).

$F$ = 0.13 ± 0.14, SD). It is apparent that the time course of calcium influx is very similar to the time course of the carbachol activated calcium current.

*Depletion Activated Calcium Influx*

Thapsigargin treatment (1 μM; 15 min) also activates calcium entry. Fig. 6*A* illustrates the effect of thapsigargin on the intracellular $Ca^{++}$ signal during stimula-

tion with agonist. A carbachol response was first measured in normal saline. Thapsigargin was introduced and after 15 min the agonist was applied again. This treatment eliminated the $Ca^{++}$ response to carbachol which is consistent with depletion of $Ca^{++}$ stores. Thapsigargin also elevated resting $(Ca^{++})_i$ by 82 ± 35 nM (mean ± SD, $n$ = 7) and the sustained nature of this increase suggests that thapsigargin treatment activates $Ca^{++}$ influx. This is demonstrated in Fig. 6 *B* where it is seen that perfusion with $Ca^{++}$ free saline after thapsigargin causes a rapid decrease in $(Ca^{++})_i$ back to the control level. The figure also shows that when external $Ca^{++}$ is

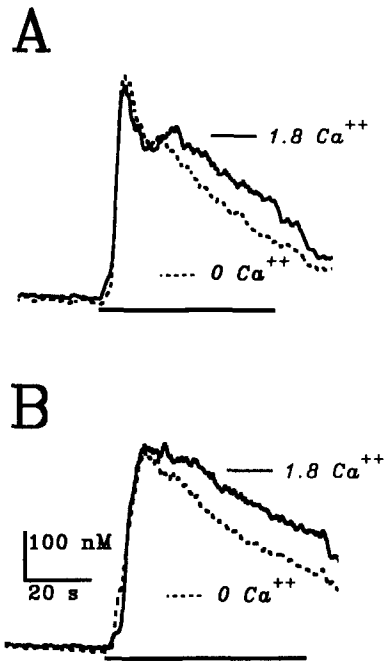


FIGURE 5. Receptor operated $Ca^{2+}$ influx resolved with fura-2 imaging in single cells. (*A*) Two responses in the same cell are superimposed. In the first response (*dashed line*), the cell was exposed to $Ca^{2+}$-free saline for 2 min before the application of 1 mM carbachol (*bar*). The chamber was then rinsed with normal saline and after a 20-min rest carbachol was applied again (response shown by the *solid line*). The intracellular calcium concentration reached approximately the same peak value in normal and $Ca^{2+}$-free saline, but after the peak the $(Ca^{2+})_i$ was greater when external $Ca^{2+}$ was present. (*B*) Average response of nine cells from three different experiments. Experiments were done the same way as in *A*. The resting $(Ca^{2+})_i$ in $Ca^{2+}$-free saline was 74 ± 53 nM (mean ± SD, $n$ = 11) compared to 84 ± 45 nM in normal saline ($n$ = 22). In $Ca^{2+}$-free saline, 55 ± 2% of the cells responded to carbachol versus 63 ± 2% in normal saline (error determined by the binomial test, see Mathes et al., 1992). The fraction of cells that responded to agonist with an elevation in intracellular calcium (60 ± 1%) is similar to the fraction that expressed receptor-operated $Ca^{2+}$ current (51 ± 5%).

reintroduced, $(Ca^{++})_i$ exhibits a pronounced transient rebound. Rebound was observed in each of 15 cells in three separate experiments. It appears that lowering $(Ca^{++})_i$ in a thapsigargin treated cell causes a further activation of the influx pathway.

The calcium current that is activated by thapsigargin was measured in nystatin patch clamp experiments (Fig. 7 *A*). It reaches a maximum amplitude of 65 ± 61 pA (mean ± SD, $n$ = 6) and a current density of 0.22 ± 0.20 pA/pF, similar to the values obtained with muscarinic receptor agonist. Like the response to carbachol, the current is reduced reversibly by adding $Ba^{++}$ or $Mn^{++}$ to the external saline (Fig.

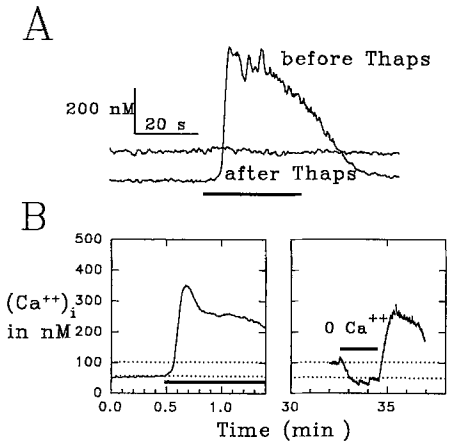


FIGURE 6. Thapsigargin depletes intracellular $Ca^{2+}$ stores and activates calcium influx. In 441 cells tested in nine experiments, thapsigargin emptied $Ca^{2+}$ stores and eliminated the $Ca^{2+}$ response to carbachol. (*A*) Representative fura-2 experiment on a single cell. In the control, before thapsigargin, 1 mM carbachol activated a large increase in $(Ca^{2+})_i$. After a 15-min treatment with 1 μM thapsigargin the resting $(Ca^{2+})_i$ increased by ~100 nM and the response to carbachol was eliminated. (*B*) The elevation of resting $(Ca^{2+})_i$ by thapsigargin is due to calcium influx. Representative results obtained from an individual cell. The response to 1 mM carbachol in normal saline was measured with fura-2 and is illustrated in the left panel. The bar marks the agonist application. The cell was then exposed to 1 μM thapsigargin for 15 min. This resulted in an increase in resting $(Ca^{2+})_i$ (*right*, compare *dashed lines*). Resting $(Ca^{2+})_i$ returned rapidly to the control level when the cell was rinsed with $Ca^{2+}$-free saline. When normal saline was reintroduced, there was a large rebound in $(Ca^{2+})_i$ that was almost as large as the carbachol response.

7 *A*). These results suggest that muscarinic receptors and thapsigargin activate the same process. We reasoned that if thapsigargin and carbachol activate separate calcium pathways, then the calcium influx in response to the two stimuli should sum. This is not the case. In each of 20 thapsigargin treated cells, carbachol failed to

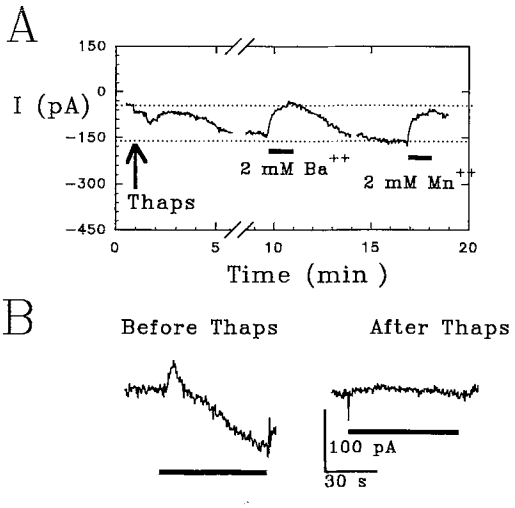


FIGURE 7. Thapsigargin activates an inward current with similar properties to muscarinic receptor activated calcium current. (*A*) Inward current measured with nystatin patch voltage clamp at −60 mV during the addition of 1 μM thapsigargin to the external medium (representative of six cells). The capacitance of this cell was 308 pF. Barium and manganese (2 mM) added directly to the bath in the presence of calcium both reduced the current amplitude (representative of three experiments). (*B*) The currents activated by thapsigargin and carbachol do not sum. On the left is a biphasic response to 1 mM carbachol. 15 min after adding thapsigargin, carbachol no longer activated a calcium response.

activate additional calcium current in voltage clamp experiments (Fig. 7 *B*; $n = 4$) or additional calcium influx in fura-2 experiments (Fig. 6 *A*; $n = 16$).

Whole cell break-in with $IP_3$ (10 μM) and EGTA (10 mM) in the pipette is expected to deplete intracellular calcium stores. We found that this procedure also activates calcium current. Fig. 8 *A* shows an example of the inward current that develops during dialysis with a pipette solution containing $IP_3$ and EGTA. The maximum amplitude was 46 ± 10 pA (mean ± SEM; $n = 9$) and this value is not statistically different from the values measured after muscarinic receptor activation or thapsigargin treatment (*t* test; $P = 0.7$ [muscarinic]; $P = 0.5$ [thapsigargin]). The current developed slowly, reaching a peak in 66 ± 46 s (mean ± SD) and the current density

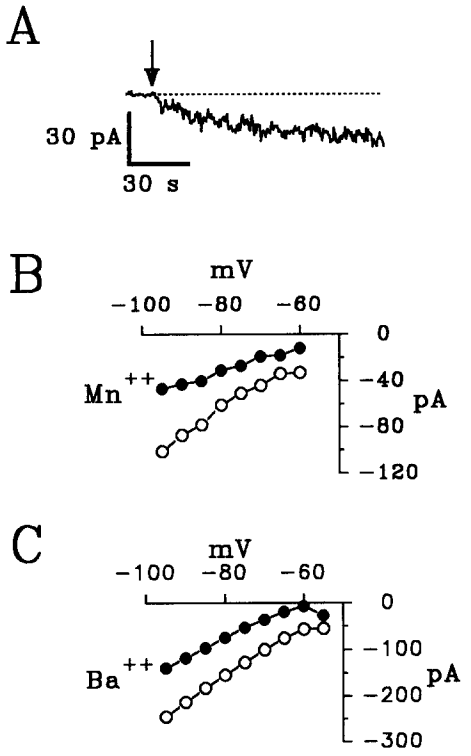


FIGURE 8. Whole cell break-in with $IP_3$ (10 μM) and EGTA (10 mM) in the pipette activates an inward current with similar properties to the calcium current activated by muscarinic receptor agonist and thapsigargin. (*A*) Inward current measured after whole cell break-in (*arrow*). The current reached a maximum amplitude of 20 pA and a current density of 0.53 pA/pF. (*B*) *I*(*V*) relationship for the inward current in a different cell before (*closed circles*) and during (*open circles*) the addition of 2 mM $Mn^{2+}$ to the bath. Manganese reduced the amplitude of the inward current at each voltage and this effect was reversible ($n = 5$). (*C*) *I*(*V*) relationship for the inward current before (*closed circles*) and after (*open circles*) exchanging the bath volume with an external saline containing $Ba^{2+}$ (2 mM) instead of $Ca^{2+}$. Barium reduced the amplitude of inward current at each voltage and this effect was reversible ($n = 3$).

was 0.32 ± 0.31 pA/pF. The inward current is reduced by $Mn^{++}$ ($n = 5$) and $Ba^{++}$ ($n = 3$) (Fig. 8, *B* and *C*). Application of carbachol after the current reached its maximum amplitude failed to activate additional inward current ($n = 6$). These results suggest that muscarinic receptors and depletion of intracellular calcium stores turn on the same inward current process.

## DISCUSSION

Muscarinic agonist and thapsigargin activate a voltage-independent calcium current in N1E-115 cells, an excitable cell line derived from mouse sympathetic ganglion.

The current can be recorded in normal physiological saline using nystatin-patch voltage clamp to avoid intracellular dialysis and the need for exogenous $Ca^{++}$ buffers. It can also be recorded after break-in with a pipette solution containing $IP_3$ and EGTA. The receptor-operated current is identified as a calcium current by several criteria. It is not observed in $Ca^{++}$-free external saline, it is enhanced transiently by high $Ca^{++}$ saline, the current is strongly reduced by the calcium channel blocker $Mn^{++}$, and the time course of the current matches the time course of $Ca^{++}$ influx measured with fura-2. Furthermore, the current is observed in $Na^+$ free saline with $Cs^+$ in the pipette solution, a condition where $Ca^{++}$ is expected to be the sole current carrier.

Yellen (1982) reported a non-selective cation channel in N1E-115 cells that is activated by micromolar $(Ca^{++})_i$ and has a conductance of 17 pS. It is unlikely that this channel is responsible for the $Ca^{++}$ current we measured for several reasons. This cation current is expected to reverse at −20 mV under the conditions of our experiments. More importantly, the nonselective cation channel is permeable to $Na^+$ but not to $Ca^{++}$. We observe receptor-operated inward current in $Na^+$ free saline, which should greatly reduce the amplitude of the nonselective cation current. We also observe receptor-operated calcium current in cells heavily loaded with BAPTA sufficient to eliminate calcium-dependent potassium current activation, and presumably any other calcium-dependent conductances.

The amplitude of the receptor operated current is reduced by addition of $Ba^{++}$ in the presence of $Ca^{++}$ and when $Ba^{++}$ replaces $Ca^{++}$, suggesting a competition between $Ba^{++}$ and $Ca^{++}$ at some site associated with the pathway. Partial block by $Ba^{++}$ distinguishes this calcium influx pathway from the voltage gated L- and T-type calcium channels that are known to be expressed in N1E-115 cells (Yoshii et al., 1988) because it is generally found that the amplitudes of $Ba^{++}$ currents in L-type and T-type channels are the same or greater than the corresponding $Ca^{++}$ current amplitudes (Almers and McCleskey, 1984; Hess and Tsien, 1984; Tsien, Lipscombe, Madison, Bley, and Fox, 1988).

The observation that $Ba^{++}$ substitution reduces the inward current is similar to the effect of $Ba^{++}$ on calcium-release-activated $Ca^{++}$ influx in Jurkat T-cells (Zweifach and Lewis, 1993) and mast cells (Hoth and Penner, 1992), which suggests that the $Ca^{++}$ influx in N1E-115 cells reflects the same or a similar process. The current density observed during muscarinic activation in N1E-115 cells (0.11 pA/pF) is also similar to reports of approximately 0.6 pA/pF in mast cells and transfected T-lymphocytes (Hoth and Penner, 1993; McDonald et al., 1993).

In the several reports of receptor-operated and/or depletion-operated calcium currents, there are differences with regard to the effects of $Mn^{++}$ and $Ba^{++}$ on current amplitude. In some preparations, $Ba^{++}$ and/or $Mn^{++}$ do not reduce calcium influx (Lückhoff and Clapham, 1992; McDonald et al., 1993; Randriamampita and Tsien, 1993), while in other preparations they do (Hoth and Penner, 1993; Parekh, Foguet, Lübbert, and Stühmer, 1993; Parekh, Teriau, and Stühmer, 1993; Vaca and Kunze, 1993; Zweifach and Lewis, 1993). The differences might be explained by variations between cell types, but they could also be explained if a nonselective cation current is activated concurrently (Yellen, 1982; Fasolato, Hoth, Matthews, and

Penner, 1993). In mast cells, for example, the $Ca^{++}$ influx pathway and a 50 pS cation channel coexist and both are activated by agonist (Hoth and Penner, 1993; Fasolato et al., 1993).

The amplitude of the calcium current in response to carbachol was consistently smaller when cells were bathed in $Na^+$ free external saline and loaded with BAPTA. This might suggest partial $Na^+$ permeability, but there is another likely explanation. It has been reported that exogenous $Ca^{++}$ buffers introduced into mast cells (Hoth and Penner, 1992, 1993), and T-lymphocytes (McDonald et al., 1993; Zweifach and Lewis, 1993) activate the calcium influx pathway, possibly by depleting internal stores of $Ca^{++}$. We observed a similar phenomenon in N1E-115 cells, after loading with EGTA/AM or BAPTA/AM (Mathes and Thompson, 1993). Under these conditions, the pathway may be partially activated and not available for additional activation by agonist. The expectation is that the amplitude of the agonist dependent component will be reduced.

Carbachol and thapsigargin activate currents with very similar properties. In both cases the inward current is reduced by $Mn^{++}$ and $Ba^{++}$ and altered by changing external $Ca^{++}$. The maximum amplitude of the inward current activated by thapsigargin treatment (62 ± 25 pA, [SEM]) was not statistically different than the amplitude of calcium current activated by carbachol (35 ± 7 pA (SEM); *t* test, $P = 0.2$). The fact that carbachol did not activate additional inward current or stimulate an additional rise in $(Ca^{++})_i$ in thapsigargin treated cells, indicates that agonist and thapsigargin engage the same conductance mechanism.

The mechanism of $Ca^{++}$ current activation is presently not known and there may be multiple levels of regulation. The slow time course of the current is consistent with there being one or more second messengers in the activation pathway. Results from BAPTA loaded cells show that the pathway is not activated by $Ca^{++}$ directly. In nonexcitable cells, it has been shown that $Ca^{++}$ influx is activated by depletion of cytoplasmic calcium stores (Takemura, Hughes, Thastrup, and Putney, 1989; Mason, Garcia-Rodriguez, and Grinstein, 1991; Hoth and Penner, 1992, 1993; Montero, Alonso-Torre, Alvarez, Sanchez, and Garcia-Sancho, 1993). This process has been called "capacitative calcium entry" (Putney, 1993; Putney and Bird, 1993*a*, *b*) and it has been suggested that depletion increases production of an intracellular signal that turns on calcium current at the membrane (Penner, Matthews, and Neher, 1988; Lewis and Cahalan, 1989; Hoth and Penner, 1993; Parekh, Teriau, and Stühmer, 1993; Randriamampita and Tsien, 1993). This intracellular signal may involve a small G-protein (Bird and Putney, 1993; Fasolato, Hoth, and Penner, 1993). Cyclic GMP (Bahnson, Pandol, and Dionne, 1993), protein kinase C (Montero, Garcia-Sanchez, and Alvarez, 1993) and $IP_4$ (Bird, Rossier, Hughes, Shears, Armstrong, and Putney, 1991) have also been identified as potential modulators of the $Ca^{++}$ influx pathway. In addition, it was suggested that $IP_3$ itself activates calcium current in T-lymphocytes (Kuno and Gardner, 1988).

The receptor-operated calcium current may have several physiological roles. For example, it appears to provide a source for refilling intracellular $Ca^{++}$ stores after they are depleted. The calcium current may also trigger events in signal transduction cascades, such as cGMP production (Study et al., 1978; El-Fakahany and Richelson, 1983). Receptor-operated calcium current in neurons could also accelerate calcium

waves, as reported in oocytes (Girard and Clapham, 1993). In addition, the activation of calcium dependent ion channels may be influenced by this current (Marty, Tan, and Trautman, 1984; Felder et al., 1992; Leinders and Vijverberg, 1992). In this report we have shown that receptor and depletion activated calcium influx occurs in excitable cells and this process may be a general feature of cells that express the $IP_3$ pathway.

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