The Relationship between Depletion of Intracellular Ca^{2+} Stores and Activation of Ca^{2+} Current by Muscarinic Receptors in Neuroblastoma Cells

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ABSTRACT The relationship between the depletion of IP_3-releasable intracellular Ca^{2+} stores and the activation of Ca^{2+}-selective membrane current was determined during the stimulation of M1 muscarinic receptors in N1E-115 neuroblastoma cells. External Ca^{2+} is required for refilling Ca^{2+} stores and the voltage-independent, receptor-regulated Ca^{2+} current represents a significant Ca^{2+} source for refilling. The time course of Ca^{2+} store depletion was measured with fura-2 fluorescence imaging, and it was compared with the time course of Ca^{2+} current activation measured with nystatin patch voltage clamp. At the time of maximum current density (0.18 + .03 pA/pF; n = 48), the Ca^{2+} content of the IP3-releasable Ca^{2+} pool is reduced to 39 + 3 % (n = 10) of its resting value. Calcium stores deplete rapidly, reaching a minimum Ca^{2+} content in 15–30 s. The activation of Ca^{2+} current is delayed by 10–15 s after the beginning of Ca^{2+} release and continues to gradually increase for nearly 60 s, long after Ca^{2+} release has peaked and subsided. The delay in the appearance of the current is consistent with the idea that the production and accumulation of a second messenger is the rate-limiting step in current activation. The time course of Ca^{2+} store depletion was also measured after adding thapsigargin to block intracellular Ca^{2+} ATPase. After 15 min in thapsigargin, IP3-releasable Ca^{2+} stores are depleted by >90% and the Ca^{2+} current is maximal (0.19 + 0.05 pA/pF; n = 6). Intracellular loading with the Ca^{2+} buffer EGTA/AM (10 μM; 30 min) depletes IP3-releasable Ca^{2+} stores by between 25 and 50%, and it activates a voltage-independent inward current with properties similar to the current activated by agonist or thapsigargin. The current density after EGTA/AM loading (0.61 + 0.32 pA/pF; n = 4) is three times greater than the current density in response to agonist or thapsigargin. This could result from partial removal of Ca^{2+}-dependent inactivation.

INTRODUCTION

Neurotransmitters that activate IP_3 production elicit calcium release from intracellular storage compartments associated with the endoplasmic reticulum. A fraction

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of the calcium that is liberated is pumped back into storage compartments, but most is extruded from the cell by the action of Ca^{2+} ATPases and Na^+/Ca^{2+} exchangers (Carafoli, 1987; Dipolo and Beauge, 1988; Reeves, 1992). This loss of Ca^{2+} to the extracellular volume leaves intracellular stores partially depleted, and the deficit must be recovered by the activation of a membrane Ca^{2+} current. It is suggested that the decrease in luminal calcium concentration is detected in some way and that this leads to the production of a messenger that triggers calcium influx. The process has been termed "capacitative calcium entry" (Putney and Bird, 1993), and according to this model, refilling occurs when Ca^{2+} entering the cytoplasm is pumped back into calcium stores (Muallem, Khademazad, and Sachs, 1990; Montero, Alonso-Torre, Alvarez, Sanchez, and García-Sancho, 1993). One of the questions posed by the capacitative model concerns the quantitative relationship between the depletion of Ca^{2+} stores and the activation of Ca^{2+} current. This relationship has not been measured, but it is important because it defines the characteristics and sensitivity of the postulated luminal Ca^{2+} detector.

We compared the depletion of IP_3-releasable intracellular calcium stores with the activation of calcium influx in N1E-115 mouse neuroblastoma cells. Fura-2 imaging was used to measure changes in $[Ca]_i$ and nystatin patch clamp was used to measure Ca^{2+} current under three experimental conditions: during the activation of M1 muscarinic receptors by carbachol, after applying thapsigargin to inhibit microsomal Ca^{2+}-ATPase, and while loading cells with the Ca^{2+} buffer EGTA/AM. These procedures activate a voltage-independent inward current that is selective for Ca^{2+} and reduced by external Mn^{2+} and Ba^{2+}, and in these respects is similar to depletion-activated Ca^{2+} current or ICRAC (Hoth and Penner, 1992, 1993; Zweifach and Lewis, 1993; Mathes and Thompson, 1994; Vaca and Kunze, 1994). It is demonstrated that receptor-regulated Ca^{2+} current plays a significant role in refilling Ca^{2+} stores after the activation of muscarinic receptors. Muscarinic agonist rapidly releases stored Ca^{2+}, but the activation of Ca^{2+} current begins after a substantial delay and increases more slowly than the rate of release. The results are consistent with the view that a messenger signaling the state of Ca^{2+} stores is produced during Ca^{2+} release and activates Ca^{2+} current with a delay. A preliminary report of these findings has been published (Mathes and Thompson, 1993).

MATERIALS AND METHODS

N1E-115 neuroblastoma cells derived from mouse sympathetic ganglion neurons (Amano, Richelson, and Nirenberg, 1972) were obtained from the UCSF Cell Culture Facility and used in passages 3–8. Cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT) at 37°C with 10% CO_2, plated on glass coverslips, and grown to ~80% confluency before differentiation with dimethylsulfoxide (DMSO) for 5–21 d (Kimhi, Palfrey, Spector, Barak, and Littauer, 1976). The cultures were fed every 2–3 d and were used 1–2 d after feeding.

Ca^{2+} Imaging

Cells were loaded with the Ca^{2+} indicator fura-2 (Molecular Probes, Inc., Eugene, OR) by incubation in saline containing 5 μM fura-2/AM and 0.025% pluronic F-127 (Molecular Probes) for 1 h at 22°C. After rinsing, the cells were transferred to a heated chamber (30°C) on the stage of a Dia-

phot microscope (Nikon, Inc., Instrument Group, Melville, NY) equipped with 20× Fluor objective, SIT camera (model C2400; Hamamatsu, Japan), and a VHS video tape recorder (Sony Corp., Japan). Xenon arc illumination was filtered through 10-nm bandpass interference filters with 340- and 380-nm center wavelengths. Calibration of fluorescence to units of Ca^{2+} was done off-line using a pipeline image processor (Megavision, Santa Barbara, CA). Background-subtracted and frame-averaged F_{340}/F_{380} ratios were calibrated using standard solutions of fura-2 between two coverslips according to the equations of Grynkiewicz, Poenie, and Tsien (1986). Values of the calibration parameters R_{min}, R_{max}, and (F_{min}/F_{max}) at 380 nm are given in the figure legends. Intracellular Ca^{2+} concentrations were measured in regions of interest corresponding to the interiors of individual cell bodies. Calcium kinetics were resolved by monitoring cells continuously for up to 90 s with 380-nm excitation after first obtaining F_{340}/F_{380}. Because dye fade during this period was insignificant, only an initial ratiometric determination was needed at the beginning of each record. The initial F_{380} and $[Ca]_i$ in each individual cell allowed the calculation of F_{min} and F_{max}, corresponding to zero and saturating Ca^{2+} concentrations, using the equations:

$$F_{max} = F_{380}(1 + [Ca]_i/K_d) / (s + [Ca]_i/K_d) \quad (1)$$

$$F_{min} = s^* F_{max}, \quad (2)$$

where $s = F_{380,min}/F_{380,max}$ and is a constant of the imaging system. $[Ca]_i$ at later times could then be calculated from F_{380} using the Henderson-Hasselbalch equation as is done for single wavelength dyes (Kao, Harootunian, and Tsien, 1989).

Saline Solutions

The 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 and 1 mM TTX (pH 7.4; temperature = 30°C). Zero calcium external saline was the same, except $MgCl_2$ was substituted for $CaCl_2$. Carbachol (Sigma Chemical Co., St. Louis, MO) was dissolved in saline and applied at a final concentration of 1 mM (M1 receptor Kd = 100 μM; Wang and Thompson, 1994). The half time for exchange of the chamber volume was ~2 s. Thapsigargin and EGTA/AM (Calbiochem Corp., La Jolla, CA) were dissolved in DMSO and diluted in saline to the appropriate concentration on the day of the experiments. All other chemicals were obtained from Sigma Chemical Co.

Nystatin Patch Clamp

The nystatin perforated patch technique was used for whole-cell voltage clamp (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981; Horn and Marty, 1988; Mathes and Thompson, 1994). The pipette solution contained (in millimolar): 16 CsCl, 70 Cs_2SO_4, 5 $MgSO_4$, 10 HEPES, and 100 sucrose to ~320 mosM at pH 7.2. After filtering, 0.05% pluronic F-127 was added to increase the solubility of nystatin. 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 mg/ml, and this solution was used within the hour. Patch electrodes were made from thick-walled Borofilament glass (outside diameter = 1.5 mm, ID 0.86 mm, model BF150-86-15; Sutter Instrument Co., Novato, CA). The external saline was the same as that used for fura-2 imaging experiments. The calculated reversal potential for Cl^- = −60 mV and for K^+ = −85 mV. A holding voltage of −60 mV was used which is near the normal resting potential (Kato, Anwyl, Quandt, and Narahashi, 1983).

RESULTS

Depletion of Ca^{2+} Stores by Carbachol

Intracellular Ca^{2+} concentration was measured during repeated activation of muscarinic receptors with carbachol in the presence and in the absence of external

Ca^{2+}. The results indicate that refilling of intracellular Ca^{2+} stores after muscarinic receptor activation requires calcium entry. Fig. 1 *A* shows an example from an experiment on an individual cell bathed in normal saline containing 1.8 mM Ca^{2+}. Carbachol (1 mM) was applied for a period of 30 s and then washed away by perfusing the bath, and this was repeated at 4-min intervals. The $[Ca]_i$ signal changes very little in amplitude or time course under these conditions. The response to repeated agonist applications was measured in the cell population by counting the number of cells that responded with a fluoresence change >25% of maximum, equivalent to a $[Ca]_i$ increase of 100–200 nM. The stimulus series was the same as that in Fig. 1 *A*. In the population average, there is a 12% reduction in the number of responding cells after the first stimulus, but little further decline during subsequent trials (Fig. 1 *B*). The initial decrement is probably caused by receptor desensitization, and no decrement is observed when the interval is increased to 15 min (Wang and Thompson, 1994). These results demonstrate that refilling occurs be-

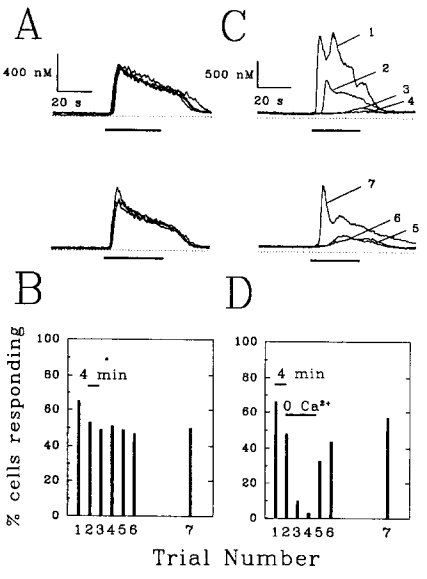

FIGURE 1. Refilling of intracellular calcium stores requires external calcium. Cells were loaded with fura-2/AM (5 μM; 1 h), and video fluorescence imaging was used to measure changes in $[Ca]_i$ during repetitive applications of 1 mM carbachol. The control series is shown in *A* and *B*. The cells were bathed in normal saline (1.8 mM Ca^{2+}) throughout. Carbachol was applied for 30 s and was washed away by rinsing the chamber with normal saline. This was repeated at 4-min intervals for six trials. A 20-min interval separated trials 6 and 7. *C* and *D* illustrate the effect of removing external Ca^{2+}. During the first trial, carbachol was applied in normal saline (1.8 mM Ca^{2+}). After 30 s, the agonist was removed by replacing the bath volume with zero Ca^{2+} saline (Mg^{2+} replaces Ca^{2+}; ~10 μM Ca^{2+}). The next three carbachol trials were done in zero Ca^{2+} saline. At the end of the fourth trial, the chamber was rinsed with zero Ca^{2+} saline for 1 min and then with normal saline (1.8 mM Ca^{2+}) for 2.5 min before trial 5. The interval between trial 6 and trial 7 was 20 min. (*A*) Changes in $[Ca]_i$ under control conditions in a representative cell. The responses to all seven trials are aligned on the time axis (trials 1–4 in the upper panel, 5–7 in the lower). The period in carbachol is indicated by solid bars. (*B*) $[Ca]_i$ responses in the control cell population during repeated carbachol applications. The percentage of responding cells in a field of 189 is indicated for each trial. The criterion for identifying responding cells was a change in fluorescence >25% of $F_{380,min}$ within 10 s of carbachol application. This corresponds to a $[Ca]_i$ elevation of 100–200 nM. The horizontal axis is also a time axis and the scale is shown by the bar. (*C*) Ca^{2+} signals in a representative cell from the group that received the zero Ca^{2+} rinse. The responses to all seven trials are aligned on the same time axis and numbered. (*D*) The percentage of cells in a field of 163 that responded to carbachol with a $[Ca]_i$ increase above criterion level in each of seven trials. The time in zero Ca^{2+} saline is indicated. Values of s, R_{min}, and R_{max} for these experiments were 25.5, 0.03, and 3.51, respectively.

tween trials when external Ca^{2+} is present, and that the refilling process can normally keep up with a repeated schedule of Ca^{2+}-release events.

The experiment was repeated, but this time, the agonist was washed away by perfusing the bath with zero Ca^{2+} saline (Mg^{2+} replaces Ca^{2+}) after the first 30 s of carbachol application, and the cells were bathed continuously in zero Ca^{2+} saline during trials 2–4. Fig. 1 *C* shows the Ca^{2+} response in a single cell. During the stimulus series, there is a progressive decrease in response amplitude and an increase in the time to peak. After the fourth trial, the chamber was washed with zero Ca^{2+} saline for 1 min before reintroducing normal saline (1.8 mM Ca^{2+}). Recovery occurred during subsequent trials. Fig. 1 *D* shows the population response during the same stimulus series. 66% of the cells responded during the first carbachol application, but this decreased to 3% by the fourth trial. Nearly 35% of cells responded on the next trial 2.5 min after reintroducing normal saline, and recovery was almost complete by trial 7, which shows that the decrement is reversible.

The Ca^{2+} current that is activated by carbachol makes a significant contribution to refilling Ca^{2+} stores. This is illustrated in Fig. 2 *A*. In the left panel, the cell was bathed initially in zero Ca^{2+} saline. Carbachol (1 mM), dissolved in the same saline, was applied for 30 s and caused a large increase in $[Ca]_i$ due to intracellular Ca^{2+} release. When the agonist was rapidly removed by rinsing the chamber with normal saline, there was a second peak in $[Ca]_i$ caused by calcium influx, showing that the influx pathway is active at this time. The second peak is absent when the rinse is

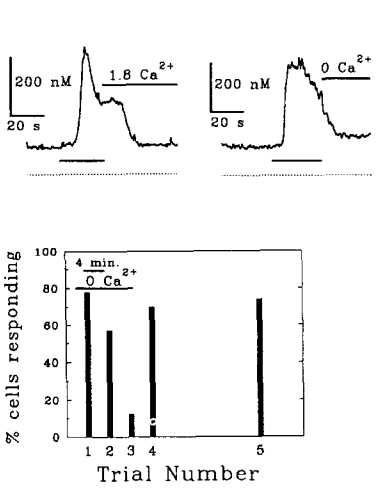

FIGURE 2. Receptor-regulated Ca^{2+} current makes a significant contribution to refilling Ca^{2+} stores. (*A*) Fura-2 imaging was used to measure $[Ca]_i$ in an individual cell bathed in zero Ca^{2+} saline. Carbachol (1 mM in zero Ca^{2+} saline) was applied for the period indicated by the bar under the trace. This caused a large transient increase in $[Ca]_i$ due to intracellular Ca^{2+} release. The agonist was washed away by replacing the solution in the chamber with normal saline containing 1.8 mM Ca^{2+}, which resulted in a second peak in $[Ca]_i$ caused by Ca^{2+} influx. (*B*) The experiment was repeated in a different cell, but this time, the agonist was removed by replacing the solution in the chamber with zero Ca^{2+} saline. Under these conditions, the second peak in $[Ca]_i$ does not occur. The results in *A* and *B* are representative of 10 experiments. (*C*) $[Ca]_i$ responses in a population of 168 cells. The criterion for identifying responding cells was the same as that described in Fig. 1, *B* and *D*. Carbachol (1 mM) was applied for 30-s periods, repeated at 4-min intervals. During the first 3 s, the cells were bathed in zero Ca^{2+} saline, and Ca^{2+} stores became progressively depleted. The time when cells were exposed to zero Ca^{2+} saline is indicated. At the end of the third trial, the agonist was removed by replacing the solution in the chamber with normal saline (1.8 mM Ca^{2+}), and it is seen that refilling is nearly complete after 3.5 min. The horizontal axis is also a time axis, and the scale is indicated by the bar. The interval between trials 4 and 5 was 20 min. s, R_{min}, and R_{max} values were 25.5, 0.03, and 3.51, respectively.

done with zero Ca^{2+} saline (*right panel*). The calcium concentration decays back to the resting level with a half time of 16 ± 4 s (mean ± SD; $n = 10$) when agonist is removed by rinsing with normal saline. The decay is more rapid, 5.2 + 1.3 s ($n = 5$), when the rinse is done with zero Ca^{2+} saline. Our interpretation of this result is that Ca^{2+} influx persists for a period lasting at least 30 s after agonist receptor occupancy is terminated, and that a significant fraction of the Ca^{2+} needed to refill intracellular stores enters during this time.

The experiment illustrated in Fig. 2 *B* supports the conclusion that refilling occurs rapidly. Carbachol (1 mM) was applied for 30-s periods, and this was repeated at 4-min intervals while monitoring the fraction of cells that responded with an increase in $[Ca]_i$. The first three trials were done while the cell was bathed in zero Ca^{2+} saline. At end of the third trial, the agonist was rinsed away with normal saline containing 1.8 mM Ca^{2+}. It is seen that the Ca^{2+} response recovered rapidly and reached nearly the control amplitude by the fourth trial. This shows that the IP_3-releasable Ca^{2+} pool is refilled from 15 to 95% of its resting capacity within 3.5 min when external Ca^{2+} is present. From experiments such as the one illustrated in Fig. 1 *B* in which Ca^{2+} was reintroduced 1 min after the agonist was removed, we estimate that 25–40% of refilling occurs in the first min. The voltage-independent Ca^{2+} current that is activated in response to carbachol recovers slowly after the agonist is removed, decreasing with a half time of 19 ± 23 s (mean ± SD; $n = 22$; Mathes and Thompson, 1994). Because of its slow recovery, receptor-regulated Ca^{2+} current represents a significant source of Ca^{2+} for refilling.

Measuring the Content of IP_3-releasable Ca^{2+} Stores during the Muscarinic Response

Experiments were done to measure the time course of depletion of the IP_3-releasable Ca^{2+} pool during the activation of muscarinic receptors. We took advantage of the fact that N1E-115 cells express both muscarinic and bradykinin receptors, and that both activate IP_3 production and intracellular Ca^{2+} release (Iredale, Martin, Hill, and Kendall, 1992; Mathes, Wang, Vargas, and Thompson, 1992). Because the two receptors do not cross-desensitize, we could use a chase protocol to measure the depletion time course (Fatatis, Caporaso, Iannotti, Bassi, Renzo, and Annunziato, 1994). Carbachol was applied to stimulate IP_3 production, and after a variable interval, bradykinin was applied to generate renewed IP_3 production and a second episode of Ca^{2+} release. Bradykinin was chosen as the second agonist because it causes a large Ca^{2+} signal that begins with a short delay in >98% of cells, indicating that bradykinin receptors are expressed in large numbers. In fact, the peak $[Ca]_i$ increase in response to bradykinin was always greater than the peak response to carbachol (Coggan, Kovacs, and Thompson, 1994). A saturating concentration of bradykinin (100 nM) was used to generate the largest possible increment in IP_3. This appears to cause a maximal response since the Ca^{2+} release resulting from 100 nM bradykinin plus 1 mM carbachol applied together is no greater than the release caused by bradykinin alone (Coggan, J. C., and S. H. Thompson, unpublished observation).

The experimental procedure was as follows. Carbachol (1 mM) was applied and the change in $[Ca]_i$ was measured with fura-2 imaging. After a variable time in carbachol, bradykinin (100 nM) was applied to elicit a second calcium release event

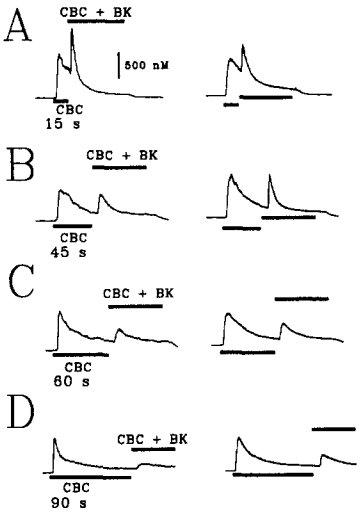

FIGURE 3. Time course of depletion of the IP_3-releasable Ca^{2+} pool by carbachol measured in normal saline with 1.8 mM Ca^{2+}. Cells were loaded with fura-2/AM as before. Carbachol (*CBC*; 1 mM) was applied for a variable period before rapidly replacing the solution in the chamber with normal saline containing carbachol (1 mM) plus bradykinin (100 nM). 1 min later, agonist action was terminated by rinsing the chamber with normal saline. The left column shows representative Ca^{2+} responses in individual cells and the right column shows averaged responses from 10 cells. Carbachol was applied for various times before introducing bradykinin; 15 s in *A*, 45 s in *B*, 60 s in *C*, and 90 s in *D*. The data describing the time course of Ca^{2+} store depletion by carbachol are gathered in Table I. The values of s, R_{min}, and R_{max} were 18.8, 0.072, and 4.02, respectively.

and the maximum change in $[Ca]_i$ due to bradykinin was recorded. The ratio of this value to the maximum change in $[Ca]_i$ due to bradykinin alone measured in the same cell population was used as an indicator of the remaining IP_3-releasable calcium pool on the assumption that a saturating concentration of bradykinin releases all of the pool in a single trial. This assumption is supported by the fact that the maximum change in $[Ca]_i$ in response to bradykinin is equal to the maximum seen with 1 μM ionomycin. The results are illustrated in Fig. 3 for cells bathed in normal saline. Example records from individual cells are shown in the left column, and the average response in 10 cells is shown on the right. Bradykinin was added

TABLE I

Time Course of Depletion of Intracellular Calcium Stores by Carbachol, Values of $[Ca]_i$ in Nanomolar

	Measured in normal saline			
Time (s)	CBC (1 mM) (peak)	BK (0.1 μM) + CBC (rel. peak)*	BK‡	Percent of depletion
15	943 ± 141	546 ± 174	1,475 ± 85	63 ± 5
	(n = 11)	(n = 11)	(n = 4); $P < 0.01$	
30	633 ± 88	591 ± 190	1,595 ± 102	63 ± 3
	(n = 10)	(n = 10)	(n = 10); $P < 0.001$	
45	926 ± 130	638 ± 174	1,650 ± 124	61 ± 3
	(n = 10)	(n = 10)	(n = 8); $P < 0.001$	
60	794 ± 126	343 ± 49	1,472 ± 76	77 ± 1
	(n = 10)	(n = 10)	(n = 9); $P < 0.001$	
90	855 ± 92	265 ± 79	1,081 ± 108	75 ± 3
	(n = 10)	(n = 10)	(n = 9); $P < 0.001$	

* Relative amplitudes measured as the change in $[Ca]_i$.

‡ Response to bradykinin (BK) alone.

± SEM; statistical test for difference between test response and BK response (Student's t test). CBC, carbachol.

15, 30, 45, 60, and 90 s after carbachol. It is seen that the content of the IP_3-releasable Ca^{2+} pool decreases rapidly reaching a steady level equal to 23% of the initial content by 1 min (Table I).

The experiment was repeated on cells bathed in zero Ca^{2+} saline to prevent Ca^{2+} influx, and the results are similar to what was seen when Ca^{2+} was present (Fig. 4). After 30 s in carbachol, the response to bradykinin was 34% of the control response to bradykinin alone, indicating that 34% of the IP_3-releasable pool remains. More pronounced store depletion is evident after 45 s. After 60 s in carbachol, the $[Ca]_i$ increase caused by Ca^{2+} release had decayed 80% of the way back to the resting level, and the response to bradykinin showed partial recovery. This suggests that at this time IP_3-releasable Ca^{2+} stores are beginning to refill, and this is most apparent in the averaged response (Fig. 4 *C*). Because the cells in this experiment were bathed in zero Ca^{2+} saline, there are only two possible explanations for partial refilling of stores, either Ca^{2+} ions released into the cytoplasm are taken back into the IP_3-releasable pool since agonist action slows down due to receptor desensitization, or Ca^{2+} moves from another intracellular Ca^{2+} store into the IP_3-releasable pool.

A second method was used to measure the depletion of intracellular Ca^{2+} stores by carbachol. The experimental design was the same, except that ionomycin was used in place of bradykinin. In this experiment, the cells were again bathed in zero Ca^{2+} saline to prevent Ca^{2+} entry due to ionomycin acting at the plasma membrane. Ionomycin (1 μM) was added at various times after carbachol (1 mM) and the results are illustrated in Fig. 5. After 30 s in carbachol, the $[Ca]_i$ increase in response to ionomycin was 21% of the control response, indicating that 21% of the initial Ca^{2+} store remains. When normal saline was reintroduced to rinse away the agonist, there was an additional increase in $[Ca]_i$ resulting either from ionophore in the membrane or from the depletion-activated Ca^{2+} pathway. 2 out of 10 cells showed no additional increase in $[Ca]_i$ in response to ionomycin. Because ionomycin releases stored Ca^{2+} from every cell under control conditions, the result from

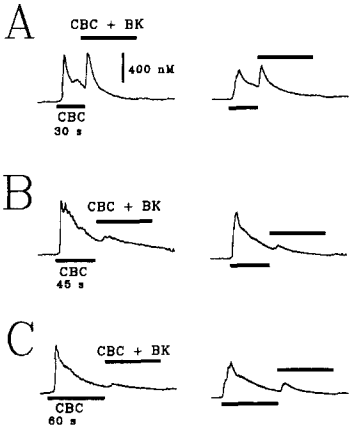

FIGURE 4. Time course of depletion of the IP_3-releasable Ca^{2+} pool by carbachol measured in zero Ca^{2+} saline. Carbachol (*CBC*; 1 mM) was applied for a variable period before rapidly replacing the solution in the chamber with zero Ca^{2+} saline containing carbachol (1 mM) plus bradykinin (100 nM). 1 min later, agonist action was terminated by rinsing the chamber with zero Ca^{2+} saline. The left column shows Ca^{2+} responses in individual cells, and the right column shows the average responses from 10 cells. (*A*) Carbachol was applied for 30 s before introducing bradykinin. (*B*) The period in carbachol was lengthened to 45 s. 5 of 10 cells showed no additional increase in Ca^{2+} when carbachol plus bradykinin was applied. (*C*) After lengthening the interval between carbachol application and carbachol + bradykinin to 60 s, there is indication that stores are beginning to refill. 2 of 10 cells showed no additional response to carbachol + bradykinin. The values of s, R_{min}, and R_{max} were 18.8, 0.072, and 4.02, respectively.

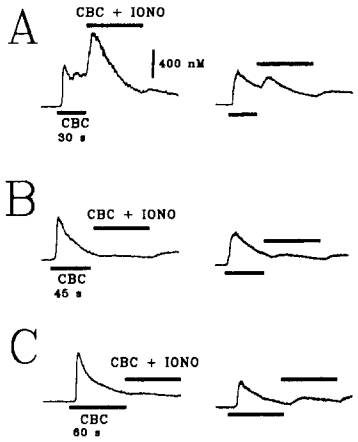

FIGURE 5. Time course of depletion of the IP_3-releasable Ca^{2+} pool measured with ionomycin. The protocol was the same as that described in Fig. 4, except that ionomycin (1 μM) was used in place of bradykinin. The two columns show $[Ca]_i$ responses in individual cells (*left*) and averaged responses (*right*; n = 10 in *A*, 5 in *B*, 3 in *C*). The experiment was conducted in zero Ca^{2+} saline. Carbachol (*CBC*; 1 mM) was applied for a 30-s interval before replacing the bath solution with saline containing carbachol + ionomycin in *A*. The interval was lengthened to 45 s in *B*, and 60 s in *C*. In each case the chamber was rinsed with normal saline containing 1.8 mM Ca^{2+} 1 min after adding carbachol + ionomycin. The values of s, R_{min}, and R_{max} were 18.8, 0.072, and 4.02, respectively.

these two cells indicates that carbachol can empty Ca^{2+} stores in less than 30 s. After 45 s in carbachol, ionomycin elicits a smaller increase in $[Ca]_i$, but when ionomycin is added after 60 s, there is evidence that Ca^{2+} stores are beginning to refill. The time course and the magnitude of Ca^{2+} store depletion by carbachol was approximately the same whether measured with bradykinin or with ionomycin. This suggests that IP_3 and ionomycin release Ca^{2+} from the same pool even though they engage very different mechanisms. Because ionomycin is not expected to select one endoplasmic reticulum Ca^{2+} store over another, we conclude that in N1E-115 cells most of the stored Ca^{2+} is in an IP_3-releasable pool.

Relationship between Depletion of Ca^{2+} Stores and Activation of Ca^{2+} Current

The increase in $[Ca]_i$ in response to carbachol, the Ca^{2+} current, and the content of the IP_3-releasable Ca^{2+} pool expressed as a percentage are shown together on the same time base in Fig. 6. The data sets were generated using the same agonist concentration, bathing saline, and temperature. Fig. 6 A shows that the change in $[Ca]_i$ rises rapidly to a peak within 10 s and then begins to slowly decline (average response in nine cells, three experiments). The IP_3-releasable Ca^{2+} pool empties equally rapidly (Fig. 6 *B*), reaching 82% of the maximum change in Ca^{2+} content by 15 s, approximately the time that the cytoplasmic Ca^{2+} concentration reaches its peak. There is, therefore, good correspondence between the time course of Ca^{2+} store depletion and the time course of the $[Ca]_i$ increase measured with fura-2 imaging. Calcium current, on the other hand, activates much more slowly. Fig. 6 *C* shows the average Ca^{2+} current density measured in 15 cells with nystatin patch clamp. The onset of the current lags behind Ca^{2+} release, the current increases more gradually than release, and the Ca^{2+} current continues to increase steadily, even after Ca^{2+} release and $[Ca]_i$ have peaked (Table I).

Depletion of Ca^{2+} Stores by Thapsigargin

Thapsigargin (1 μM), an inhibitor of microsomal Ca^{2+} ATPase, causes $[Ca]_i$ to increase. This begins within 30 s and reaches a peak (220 nM in this example) by

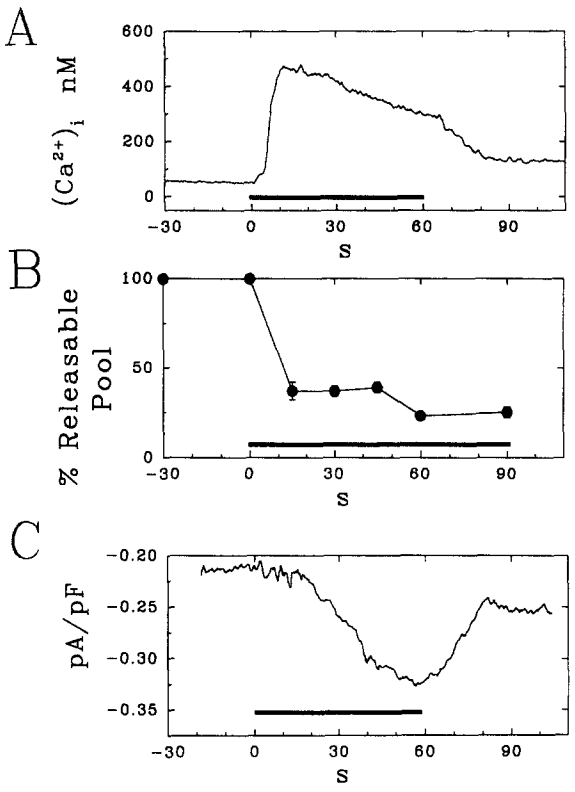

FIGURE 6. The increase in $[Ca]_i$, the content of IP_3-releasable stores, and Ca^{2+} current are shown on the same time base during carbachol stimulation. All measurements were done in normal saline at 30°C. (*A*) Change in $[Ca]_i$ in response to 1 mM carbachol applied for 1 min. Average of nine cells (these data appeared in Fig. 5 *B* of Mathes and Thompson, 1994). (*B*) Time course of depletion of IP_3-releasable Ca^{2+} stores in response to carbachol. Store content was measured using the bradykinin chase protocol (see Fig. 3), and it is expressed as a percentage of the peak change in $[Ca]_i$ during responses to bradykinin alone (Table I). Error bars were determined using a bootstrapping method (10 repetitions) to determine the SEM (Effron, 1982). (*C*) Time course of Ca^{2+} current activation measured with nystatin patch voltage clamp. Average response in 15 cells expressed in units of current density.

2 min (Fig. 7 *A*). After the peak, [Ca]i falls to a sustained value 45 nM above the resting level (average steady-state elevation; 82 + 35 nM, n = 14; Mathes and Thompson, 1994). When thapsigargin is applied to cells bathed in zero Ca^{2+} saline, [Ca]i again increases and the increase occurs at about the same rate and reaches an equally high peak value (210 nM in the example). In zero Ca^{2+} saline, however, the response is transient and $[Ca]_i$ decays back to the resting level with little evidence of sustained elevation (Fig. 7 *B*). Because the rate of rise of $[Ca]_i$ and the maximum increase are the same in the presence and absence of external Ca^{2+}, we conclude that the rising phase of the response to thapsigargin is caused by Ca^{2+} moving into the cytosol from Ca^{2+} storage compartments. The sustained elevation of $[Ca]_i$ in normal saline, on the other hand, must result from continued Ca^{2+} influx (Takemura, Ohshika, Yokosawa, Oguma, and Thastrup, 1991; Zweifach and Lewis, 1993; Mathes and Thompson, 1994).

The time course of depletion of the IP_3-releasable Ca^{2+} pool by thapsigargin was measured in a chase experiment. Carbachol (1 mM) was applied at various times after introducing thapsigargin, and the peak change in $[Ca]_i$ in response to carbachol was measured. This was divided by the peak $[Ca]_i$ increase during stimulation with carbachol alone, measured in the same cells before thapsigargin was applied, to calculate the fraction of the IP_3-releasable Ca^{2+} pool remaining at each time

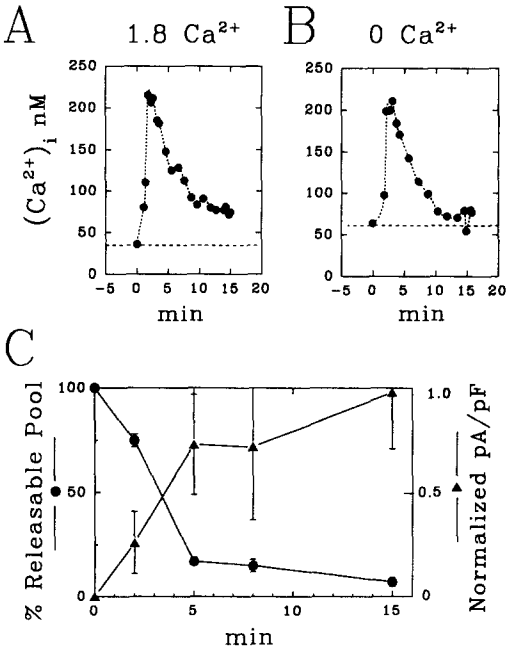

FIGURE 7. Depletion of Ca^{2+} stores and activation of Ca^{2+} current by thapsigargin. (*A*) Thapsigargin (1 μM) was applied to cells loaded with fura-2/AM and bathed in normal saline (1.8 mM Ca^{2+}). Ratiometric determinations of $[Ca]_i$ were made at intervals and plotted as a function of time in thapsigargin. (*B*) The experiment was repeated on a different cell bathed in zero Ca^{2+} saline (Mg^{2+} replaces Ca^{2+}). Comparison of *A* and *B* show that the sustained phase of $[Ca]_i$ elevation requires external Ca^{2+}. (*C*) Time course of depletion of IP_3-releasable Ca^{2+} stores by thapsigargin (*filled circles*) plotted together with the time course of Ca^{2+} current activation (*filled triangles*). Store depletion was measured using the carbachol chase protocol described in the text and plotted as a percentage of the change in $[Ca]_i$ in response to 1 mM carbachol alone (Table II). Error bars were determined using a bootstrapping method (10 repetitions; Effron, 1982). The values of s, R_{min}, and R_{max} were 21.0, 0.072, and 2.44, respectively. Ca^{2+} current was measured using nystatin patch voltage clamp and is shown in units of current density normalized to the maximum current observed in each cell after 15 min in thapsigargin. The average maximum current density was 0.19 ± 0.05 pA/pF (mean ± SEM; $n = 6$).

point (Table II). Fig. 7 *C* shows that the content of the Ca^{2+} pool declines steadily to < Δ10% by 15 min. In 69% of cells (18 out of 26), carbachol added 8 or 15 min after thapsigargin failed to activate additional Ca^{2+} release, and for the others, the value of DF/F was < Δ25%. The time course of Ca^{2+} current activation during in-

TABLE II

Time Course of Depletion of Ca Stores by Thapsigargin, Values of [Ca]$_i$ in Nanomolar Measured in Normal Saline

	Measured in normal saline		
Time (min)	CBC (peak)	CBC during THAPS (peak)	Percent of depletion
2	865 ± 91	568 ± 58	25 ± 3
	($n = 10$)	($n = 10$) $P < 0.05$	
5	603 ± 56	102 ± 12	83 ± 2
	($n = 10$)	($n = 10$) $P < 0.001$	
8	700 ± 92	105 ± 59	85 ± 3
	($n = 12$)	($n = 12$) $P < 0.001$	
15	729 ± 70	51 ± 20	93 ± 2
	($n = 15$)	($n = 13$) $P < 0.001$	

± SEM; statistical analysis by Student's *t* test. Carbachol (CBC), 1 mM; thapsigargin (THAPS), 1 μM.

cubation with thapsigargin (1 μM) was measured in nystatin patch experiments. Average current density is plotted as a function of time in Fig. 7 *C*, and the values were normalized to the current density measured 15 min after adding thapsigargin. The figure shows that when Ca^{2+} stores empty gradually after blocking the endoplasmic reticulum Ca^{2+} ATPase with thapsigargin, the time course of Ca^{2+} current activation closely approximates the time course of Ca^{2+} store depletion.

EGTA Loading Activates Ca^{2+} Current

Cells were loaded with EGTA by adding the acetoxymethyl ester form of the buffer to normal saline from a 1 mM DMSO stock solution. Loading with AM-APTRA (half-BAPTA/AM; Molecular Probes, Inc.) prepared in the same way served as a control for nonspecific effects of ester loading, and it was found that this did not affect membrane current. Fig. 8 *A* shows that EGTA/AM (10 μM for 30 min) activates an inward current that is blocked by adding 2 mM Mn^{2+} to the external solution (Fig. 8 *A*). Other experiments showed that the inward current is increased by elevating the external Ca^{2+} concentration and that it is reduced by adding external Ba^{2+}. Voltage pulses were applied before and during the inward current to construct the difference I(V) shown in Fig. 8 *B*. The dotted line in the figure is the predicted I(V) curve for a Ca^{2+} current from the Goldman-Hodgkin-Katz current equation. The positive slope of the I(V) curve indicates that EGTA/AM loading causes a conductance increase, but it was not possible to extend the curve to voltages more positive than −30 mV or to measure a reversal potential because of the activation of voltage dependent Ca^{2+} currents. The sensitivity to external Ca^{2+}, Mn^{2+}, and Ba^{2+} is similar to what is seen with the currents activated by carbachol and thapsigargin, and this is consistent with the idea that these three procedures activate the same inward current pathway (Mathes and Thompson, 1994). EGTA/AM loading, however, appears to activate the current to a greater extent. The average steady state current density after incubation with EGTA/AM was 0.61 ± 0.32 pA/pF (n = 4; mean ± SEM) compared to 0.18 ± 0.03 pA/pF for carbachol (n = 48) and 0.19 ± 0.05 pA/pF for thapsigargin (n = 6).

Fura-2 imaging experiments were done to measure the depletion of Ca^{2+} stores by EGTA/AM. Fig. 8 *B* shows the change in $[Ca]_i$ in response to carbachol measured before and 30 min after EGTA/AM loading (10 μM) in a representative cell. Incubation with EGTA/AM had little effect on the time course of the response, but it decreased the maximum amplitude by 25–35%, a decrease that might be expected from the increase in cytoplasmic Ca^{2+} buffer strength because of added EGTA. The fraction of the IP_3-releasable Ca^{2+} pool that remains during EGTA loading was measured by applying carbachol at different times and comparing the peak change in $[Ca]_i$ at each time point with the control response measured in the same cell before buffer loading (Table III). Because no correction was made for the increase in cytosolic Ca^{2+} buffer strength resulting from the introduction of EGTA, this measure overestimates the magnitude of Ca^{2+} store depletion. With this caveat in mind, the results plotted in Fig. 7 *C* suggest that the IP_3-releasable Ca^{2+} pool is reduced by only 30% after a 30-min incubation with EGTA/AM. The inward current activated by EGTA loading is plotted on the same axis after normalizing to the amplitude measured at 30 min. It appears that the current activates over ap-

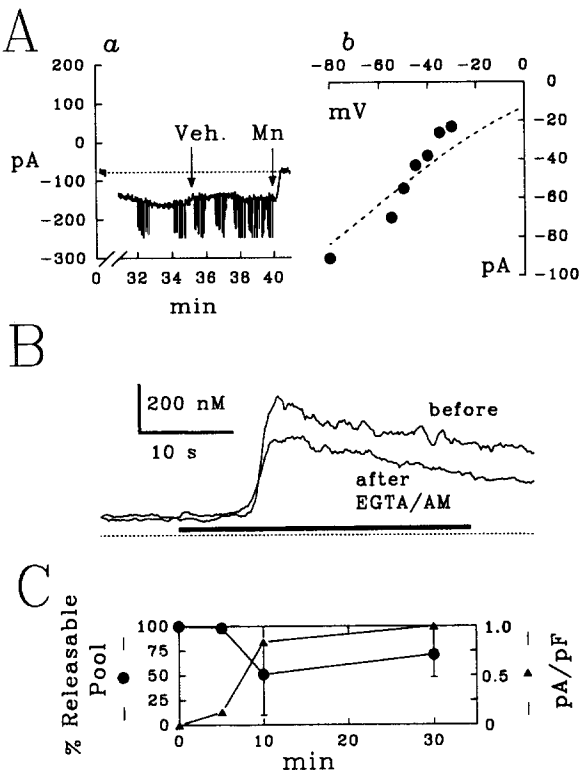

FIGURE 8. Activation of Ca^{2+} current and depletion of Ca^{2+} stores by EGTA/AM. (*A*) Inward current after 30 min incubation with 10 μM EGTA/AM. The chamber was rinsed with normal saline after 35 min (*veh.*) and with saline containing 2 mM Mn^{2+} (*Mn*) after 40 min. Downward deflections in the record are responses to test voltage pulses. Representative of four experiments. (*A*) Difference I(V) curve for the current activated by EGTA/AM. Currents in response to a series of voltage pulses measured before EGTA/AM loading were subtracted from currents measured after the loading treatment to construct the curve. The dotted line is the solution of the Goldman-Hodgkin-Katz current equation for $[Ca^{2+}]_{out}$ = 1.8 mM, $[Ca^{2+}]_{in}$ = 70 nM. (*B*) Change in $[Ca]_i$ in response to carbachol (1 mM) before and 30 min after adding EGTA/AM (10 μM) in a representative cell loaded with fura-2. Time in carbachol shown by the bar. Dotted line denotes zero $[Ca]_i$. (*C*) Time course of depletion of the IP_3-releasable Ca^{2+} pool by EGTA/AM. Store depletion was measured using the chase protocol described in the text, and it is expressed as a percentage of the peak change in $[Ca]_i$ in response to carbachol before EGTA/AM loading (Table II). Statistical error was estimated using a bootstrapping method to determine the SEM. The error bars are smaller than the size of the symbols. The values of s, R_{min}, and R_{max} were 21.0, 0.072, and 2.44, respectively. Ca^{2+} current density is plotted on the same axis normalized to the maximum current after 30 min (n = 10 for each time point). The cellular variability in measurements of current density was large.

proximately the same time course as Ca^{2+} store depletion, but the large variability in the measurement of current density makes it difficult to determine the exact form of that relationship.

DISCUSSION

Depletion of intracellular Ca^{2+} stores activates voltage-independent Ca^{2+} currents in a variety of electrically inexcitable cells (Hoth and Penner, 1992; Vaca and Kunze, 1994; Zweifach and Lewis, 1993), and it is becoming apparent that a similar process occurs in excitable cells as well (Putney and Bird, 1993; Mathes and Thompson, 1994; Felder, Singer-Lahat, and Mathes, 1994). The capacitative model proposes that Ca^{2+} store depletion leads to the production of a messenger that activates Ca^{2+} current. The activating messenger(s) has not been identified, although

TABLE III

Time Course of Depletion of Ca Stores by EGTA/AM, Values of $[Ca]_i$ in Nanomolar Measured in Normal Saline

Time (min)	CBC (peak)	CBC during EGTA/AM (peak)	Percent of depletion
5	—	870 ± 60 (n = 10); NS	2 ± 3
10	—	435 ± 49 (n = 10); $P < 0.01$	49 ± 3
30	890 ± 92	636 ± 45 (n = 10); NS	29 ± 2
60	—	913 ± 44 (n = 19); NS	8 ± 2

± SEM; statistical analysis by Student's t test (NS, not significant).
Carbachol (CBC), 1 mM; EGTA/AM, 10 mM.

several candidates have been proposed including a diffusible anionic molecule of <500 mol wt (Randriamampita and Tsien, 1993; Parekh, Teriau, and Stuhmer, 1993), cGMP (Pandol and Schoeffield-Payne, 1990; Bahnson, Pandol and Dionne, 1993; Xu, Star, Tortorici, and Muallem, 1994), and a monomeric G protein (Fasolato, Hoth, and Penner 1993; Bird and Putney, 1993). Other evidence suggests that phosphorylation may be involved (Vostal, Jackson, and Schulman, 1991; Gusovsky, Lueders, Kohn, and Felder, 1993).

In N1E-115 cells, external Ca^{2+} is required for refilling the IP_3-releasable Ca^{2+} pool and refilling occurs rapidly, replenishing the Ca^{2+} content to 95% of the resting value in less than 4 min. This is similar to a refilling time of 3 min reported for PC12 cells (Clementi, Scheer, Zacchetti, Fasolato, Pozzan, and Meldolesi, 1992), although a wide range of refilling times is seen in other cell types from 8 s in Ehrlich ascites tumor cells (Montero, Alvarez, and García-Sancho, 1990) to 40 min in adrenal glomerulosa cells (Kojima, Shibata, and Ogata, 1987; see Tsunoda, 1993).

When transmitter action is stopped by rinsing the chamber with zero Ca^{2+} saline, the intracellular Ca^{2+} concentration decays back to the resting level with a half time of 5.2 ± 1.3 s (n = 5; mean ± SD) . This is similar to the 9 ± 2 s half-life of IP_3 in N1E-115 cells (Wang, Alousi, and Thompson, 1994), which suggests that in the absence of Ca^{2+} influx, the decay of the intracellular Ca^{2+} signal is governed by metabolic processing of IP_3 and transport of Ca^{2+} out of the cell or back into stores. The decay is prolonged when external Ca^{2+} is present because of the prolonged recovery time course of the Ca^{2+} current.

During carbachol stimulation, the IP_3-releasable Ca^{2+} pool is depleted by 61–77% at the time that the current reaches its maximum amplitude. With thapsigargin, the pool is 90–95% emptied when the current is maximal. These results are consistent with the hypothesis that the Ca^{2+} current is activated subsequent to Ca^{2+} store depletion and lends strong support to the capacitative model for Ca^{2+} current activation. It is apparent that Ca^{2+} stores need not be empty or in equilibrium with the cytosol for the Ca^{2+} current to be activated and, therefore, if there is a Ca^{2+} de-

tector associated with internal stores, it must be able to operate over a wide range of luminal calcium concentrations.

A comparison of the time course of depletion of the IP_3-releasable Ca^{2+} pool with the time course of Ca^{2+} current activation in response to agonist showed that store depletion is much more rapid than current activation. This is in agreement with studies on hepatocytes (Kass, Llopic, Chow, Duddy, and Orrenius, 1990), parotid acinar cells (Hiramatsu, Baum, and Ambudkar, 1992), and avian exocrine cells (Shuttleworth, 1994). The increase in current follows the onset of Ca^{2+} release with a 10–15-s latency and the current increases more gradually than Ca^{2+} release. Ca^{2+} release is over 10–15 s after applying the agonist, while the current continues to increase for as long as 60 s. From this, it is apparent that the rate-limiting step in current activation is not store depletion. The results are consistent with a model in which the current is activated subsequent to the production of a second messenger, with messenger production and accumulation being rate limiting. In contrast, Ca^{2+} current activation and Ca^{2+} store depletion follow the same time course when thapsigargin is used to block intracellular Ca^{2+} ATPases. Ca^{2+} release in this case is very slow, requiring several minutes to reach completion, compared to 10–15 s for agonist. Our interpretation is that when Ca^{2+} release is slow, the production and acccumulation of the second messenger responsible for modulating the current is no longer rate limiting.

A number of studies have shown that a voltage-independent Ca^{2+} current is activated in whole-cell voltage clamp experiments when cells are internally perfused with pipette solutions containing EGTA or BAPTA (Zweifach and Lewis, 1993; Hoth and Penner, 1992, 1993; Lückhoff and Clapham, 1994). The explanation has been that this lowers the Ca^{2+} content of stores because of Ca^{2+} leakage into the strongly buffered environment supplied by the pipette solution. A somewhat different situation arises when cells are loaded with EGTA/AM. In this case, the free buffer concentration is limited by partitioning of the compound across the membrane and the activity of intracellular esterases. In N1E-115 cells, loading with EGTA/AM increases a voltage-independent inward current that resembles the Ca^{2+} current activated by carbachol and thapsigargin in its sensitivity to external Ca^{2+} and to block by external Mn^{2+} and Ba^{2+}. Using fura-2, we estimated that the content of the IP_3-releasable Ca^{2+} pool is 50–70% of the resting value when the current is maximally activated. This measurement, however, underestimates the Ca^{2+} content of stores because of competition between EGTA and fura-2 for intracellular Ca^{2+}, and the result is consistent with the observation that BAPTA/AM loading of parotid acinar cells does not deplete Ca^{2+} stores (Foskett, Gunter-Smith, Melvin, and Turner, 1989). With carbachol or thapsigargin, the IP_3-releasable Ca^{2+} pool is reduced to 5–25% of the resting value when the current reaches its peak. It would appear, therefore, that Ca^{2+} stores are less strongly affected by EGTA/AM loading than by agonist or thapsigargin. In contrast, the current density after EGTA loading is almost three times larger than it is with agonist or thapsigargin. This suggests that inward current activation is more sensitive to store depletion when EGTA is used. Experiments in N1E-115 cells and in other cells suggest that receptor-regulated Ca^{2+} currents may be subject to Ca^{2+}-dependent inactivation (Lewis and Cahalan, 1989; Hoth and Penner, 1993; Mathes and Thompson, 1994; Zweifach and Lewis,

1995). This provides a possible explanation for the larger current density after buffer loading since the added Ca^{2+} buffer could protect against Ca^{2+}-dependent inactivation. The combination of partial store depletion and increased Ca^{2+} buffering might allow a larger current amplitude, even in the unperfused condition of nystatin patch voltage clamp. The present experiments were not designed to resolve small changes in $[Ca]_i$, but this idea merits further testing, for example, by measuring the Ca^{2+} dependence of inactivation.

Like many neurons, N1E-115 cells express voltage-activated Ca^{2+} channels (Yoshii, Tsunoo, and Narahashi, 1988). We have shown that receptor-regulated Ca^{2+} influx is necessary for refilling stores, but these tissue culture cells are rarely spontaneously active. In an active neuron, the Ca^{2+} influx during action potentials should be sufficient to refill intracellular Ca^{2+} stores and, therefore, one should consider what additional physiological roles receptor-regulated Ca^{2+} currents might have. We suggest that the Ca^{2+} current plays its primary role in signal transduction contributing, for example, to nitric oxide and cGMP production (Thompson, Mathes, and Alousi, 1995) and to the local regulation of ion channels. Signal transduction can be spatially localized, for example, by the activation of receptors at discrete locations on a dendritic tree (Koch and Zador, 1993). Local Ca^{2+} release (Yuste, Gutnick, Saar, Delaney, and Tank, 1994) creates a need for a locally regulated Ca^{2+} refilling mechanism that does not require a distributed signal such as a dendritic Ca^{2+} spike.

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