Calcium Channels Activated by Depletion of Internal Calcium Stores in A431 Cells

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ABSTRACT Depletion of intracellular calcium stores induces transmembrane Ca^{2+} influx. We studied Ca^{2+}- and Ba^{2+}-permeable ion channels in A431 cells after store depletion by dialysis of the cytosol with 10 mM BAPTA solution. Cell-attached patches of cells held at low (0.5 μM) external Ca^{2+} exhibited transient channel activity, lasting for 1–2 min. The channel had a slope conductance of 2 pS with 200 mM $CaCl_2$ and 16 pS with 160 mM $BaCl_2$ in the pipette. Channel activity quickly ran down in excised inside-out patches and was not restored by $InsP_3$ and/or $InsP_4$. Thapsigargin induced activation in cells kept in 1 mM external Ca^{2+} after BAPTA dialysis. These channels represent one Ca^{2+} entry pathway activated by depletion of internal calcium stores and are clearly distinct from previously identified calcium repletion currents.

INTRODUCTION

Ca^{2+} may enter nonexcitable cells through several different mechanisms. However, studies utilizing fluorescent Ca^{2+} indicators have revealed a general principle in many cell types: depletion of internal calcium stores induces Ca^{2+} influx (Putney, 1986, 1990; Meldolesi et al., 1991). No second messenger that mediates this influx has been unequivocally identified (Clapham, 1993), although new candidates have been proposed (Randriamampita and Tsien, 1993). Particularly, inositol 1,4,5 trisphosphate ($InsP_3$) seems to affect Ca^{2+} influx in most cases only indirectly, by emptying calcium stores but not by direct activation of membrane Ca^{2+} channels.

The A431 cell, a human epidermal cancer cell line, is widely used as a model for signaling by epidermal growth factor (EGF). These cells express EGF receptors that are coupled, via tyrosine kinases, to phospholipase C and the production of $InsP_3$ (Carpenter, 1987; Schlessinger, 1988; Wahl and Carpenter, 1988). However, the role of $InsP_3$ in EGF-induced increases in the intracellular free calcium concentration ($[Ca^{2+}]_i$) is not clear. EGF activates Ca^{2+}-permeable ion channels in intact A431 cells (Peppelenbosch et al., 1991, 1992), and leukotriene C4 activates these channels in outside-out patches. Thus, EGF may affect $[Ca^{2+}]_i$ independently of $InsP_3$. On the other hand, Ca^{2+}-permeable channels sensitive to $InsP_3$ and GTPγS have been described (Mozhayeva et al., 1989). Finally, a recent study has shown that the intracellular $InsP_3$ antagonist heparin inhibits $[Ca^{2+}]_i$ responses to EGF in A431 cells and that all of the effects of EGF on $[Ca^{2+}]_i$ can be accounted for by EGF-induced production of $InsP_3$ (Hughes et al., 1991). It was argued that the effects of $InsP_3$ on Ca^{2+} influx might be due to depletion of calcium stores because thapsigargin, an inhibitor of Ca^{2+} uptake into these stores, elicited an influx similar to EGF.

Ca^{2+} influx induced by store depletion is in the range of a few pA in mast cells (Penner et al., 1988), lymphocytes (Lewis and Cahalan, 1989; Zweifach and Lewis, 1993), and pancreatic acinar cells (Bahnson et al., 1993). Studies in frog oocytes also found depletion-induced Ca^{2+} currents that were small in relation to the cell size (Parekh et al., 1993), as expected for a calcium store repletion pathway (Neher, 1991). Depletion of stores in mast cells was achieved by dialyzing the cells with the Ca^{2+} chelator EGTA (Hoth and Penner, 1992, 1993; Fasolato et al., 1993), BAPTA (Hoth and Penner, 1993), by addition of $InsP_3$ to the dialysate (Hoth and Penner, 1993), or in lymphocytes by thapsigargin (Zweifach and Lewis, 1993). Thapsigargin (Thastrup et al., 1990) and EGTA presumably inhibit Ca^{2+} uptake, such that the stores are depleted by continuous discharge of Ca^{2+}, similar to the condition of continuous stimulation with an $InsP_3$-dependent agonist (Parekh et al., 1993). The transmembrane Ca^{2+} current in mast cells was named I_{CRAC} (Hoth and Penner, 1992) and proved to be selective for Ca^{2+} over Ba^{2+} and even more so over Mn^{2+} and other cations (Hoth and Penner, 1992; Fasolato et al., 1993). Similar permeability ratios for divalent cations were found in oocytes, but in these cells, the currents were also carried by K^+ (Parekh et al., 1993). Noise analysis of the current fluctuations suggested that the responsible channels had a conductance of much less than 1 pS (Hoth and Penner, 1993) or 24 fS (Lewis and Cahalan, 1993), making single-channel analysis impossible.

In this study we obtained electrophysiological evidence for a Ca^{2+} entry pathway regulated by the filling state of internal calcium stores in A431 cells. We report Ca^{2+}- and Ba^{2+}-permeable channels that are specifically activated by store depletion. These channels are distinct from channels directly activated by EGF because they have a lower conductance and are uniquely regulated. The entry pathway reported here has some characteristics similar to I_{CRAC} in mast cells (Hoth and Penner, 1992), I_{DAC} (depletion activated current) in lymphocytes (Lewis and Cahalan, 1989; Zweifach and Lewis, 1993), and the depletion current in oocytes

Received for publication 23 February 1994 and in final form 1 April 1994.
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0006-3495/94/07/177/06 $2.00

(Parekh et al., 1993), but differs in some important aspects such as conductance, permeability ratios, and inactivation.

MATERIALS AND METHODS

Cell culture

A431 cells were kept in culture as described elsewhere (Wahl and Carpenter, 1988) or were seeded on coverslips for patch-clamp experiments. Single-channel analysis was usually performed on confluent cell layers, whereas separate cells from subconfluent monolayers were used for whole-cell experiments to avoid electrical coupling between cells.

Patch clamp experiments

Standard patch clamp techniques were used (Hamill et al., 1981). Cells were covered with bathing solution (1-mm depth) and a layer of silicone fluid (Dow Corning 200^R Fluid, 20 cSt; Rae and Levis, 1992). Measurements were performed using an EPC-9 patch clamp amplifier (HEKA Electronics, Lambrecht, Germany) connected to an Atari ST Computer. Data were digitized at 10 kHz, stored on a videotape, and filtered at 0.4 kHz for display and analysis.

Pipettes were pulled from KG12 glass (Garner glass, Claremont, CA) to a tip resistance (in isotonic NaCl) of 1.5–3 MOhm and coated with Sylgard (Dow Corning, Midland, MI). In inside-out experiments, the pipette with the patch was pulled back just under the oil-water interface to reduce noise (Rae and Levis, 1992). All experiments were done at 22 ± 2°C.

Channel activity is expressed as NP_o, i.e., the product of the number of channels present in the patch (N) and the open probability (P_o). NP_o, mean channel amplitudes, and open time distributions were calculated with the M-TAC program (Instrutech, Elmont, NY) on an Atari ST computer.

Solutions

For single-channel recordings, low Ca^{2+} bath solution contained (in mM) NaCl 140, KCl 4, $CaCl_2$ 1, $MgCl_2$ 2, EDTA 2, HEPES 10, glucose 5, pH 7.4 (calculated $[Ca^{2+}]$ 10 μM). EDTA was omitted for physiological Ca^{2+} concentrations. The pipette solution for BAPTA dialysis contained KCl 120, $CaCl_2$ 1, $MgCl_2$ 2, BAPTA 10, ATP 1.5, GTP 0.1, pH (KOH) 7.2 (calculated $[Ca^{2+}] < 10$ nM). In many experiments, ATP and/or GTP were omitted, with no obvious effects on the results. The pipette solution for channel recordings contained either $CaCl_2$ 200, $CaCl_2$ 80, $BaCl_2$ 160, or $BaCl_2$ 80, plus $MgCl_2$ 1, HEPES 5, pH (KOH) 7.4. For whole-cell experiments, the bath solution contained *N*-methyl-D-glucamine (NMDG) 140, $CaCl_2$ 1, $MgCl_2$ 2, EGTA 2 or 1.2, HEPES 10, glucose 5, pH (HCl) 7.4 (calculated $[Ca^{2+}] < 100$ nM or 0.4 μM, respectively). The pipette solution contained *N*-methyl-D-glucamine 140, $CaCl_2$ 1, $MgCl_2$ 2, EGTA 20, ATP 1.3, HEPES 10, pH (HCl) 7.4 (calculated $[Ca^{2+}] < 10$ nM).

Ca^{2+} concentrations in the solutions were calculated with a computer program using the algorithm described by Fabiato and Fabiato (1979).

Drugs

Thapsigargin (Sigma Chemical Co., St. Louis, MO) was dissolved in DMSO and diluted 1:100 into the bath to final concentrations of 3–10 μM. 2′,5′-di (tert-butyl)-1,4-benzohydroquinone (buBHQ, Serva, Heidelberg, Germany) was dissolved in ethanol and diluted 1:300 into the bath to final concentrations of 10–30 μM. The solvents alone had no effect on channel activity or whole-cell currents in control experiments. The chloride channel blocker 4,4′-diisothiocyanatostilbene-2,2′disulfonic acid (DIDS, Sigma) was dissolved in DMSO.

RESULTS

BAPTA dialysis induces activity of Ca^{2+}- and Ba^{2+}-permeable channels

As in the study by Hoth and Penner (1993), calcium stores were depleted by dialysis with Ca^{2+} chelator: a single A431 cell kept in low external Ca^{2+} was patch-clamped in the whole-cell configuration (series resistance 1.5–10 MOhm) with a pipette containing 10 mM BAPTA ($[Ca^{2+}] < 10$ nM). After 1–2 min, the dialysis pipette was removed and a second pipette, containing 200 mM $CaCl_2$, was sealed in cell-attached mode to the same cell. Under these conditions, we observed Ca^{2+} channel activity in 29 out of 104 technically adequate attempts (Fig. 1, *a* and *b*). Technically adequate attempts were characterized by a noise level of <180 fA at a bandwidth of 3 kHz (in the absence of channel activity). Channel activity typically lasted 1–3 min.

When the cell-attached pipette solution contained $BaCl_2$ (80–160 mM) instead of $CaCl_2$, similar channel activity was observed, also lasting 1–3 min (n = 17/50). However, the channel amplitude was ~10-fold higher. The slope conductance (Fig. 2) was approximately 2 pS with 200 mM $CaCl_2$ in the pipette, less than 1 pS with 80 mM $CaCl_2$, 16 pS with 160 mM $BaCl_2$ and 10 pS with 80 mM $BaCl_2$. There were no obvious voltage-dependent changes in the channel activity over the voltage range shown in Fig. 2 *a*. No such channels were observed with 140 mM NaCl (n = 12) or 120 mM $MnCl_2$ (n = 12) in the pipette. Furthermore, channel activity

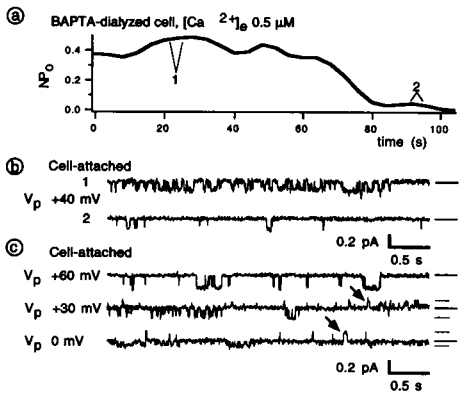

FIGURE 1 Ca^{2+} channel activity in A431 cells after BAPTA dialysis. A431 cells were bathed in 10 μM $[Ca^{2+}]$ and internally dialyzed with 10 mM BAPTA in whole-cell patch clamp for ~60 s. The dialysis pipette was removed, and another pipette containing 200 mM $CaCl_2$ was sealed to the same cell for cell-attached single-channel recording. (*a*) Activity of Ca^{2+} permeable channels. Channel activity is given as NP_o. (*b*) Channel recordings obtained during two different time periods (as indicated in panel *a*). The pipette potential (V_p) was set to +40 mV. Cell membrane potential was measured as −20 mV at the end of the dialysis (transmembrane potential ca. −60 mV). Current amplitude, 0.16 pA. The closed level is indicated by lines. (*c*) Channel recordings from another BAPTA-dialyzed cell at various holding potentials. Two different channels were present, one passing inward currents (*downward deflections*) carried by Ca^{2+} and another passing outward currents (*arrows*). The latter channel is similar to a previously described (Peppelenbosch et al., 1991) Ca^{2+}-activated K^+ channel (18 pS). The membrane potential of the cell during dialysis was −15 mV, so that the transmembrane potentials in the patch are estimated as −75, −45, and −15 mV.

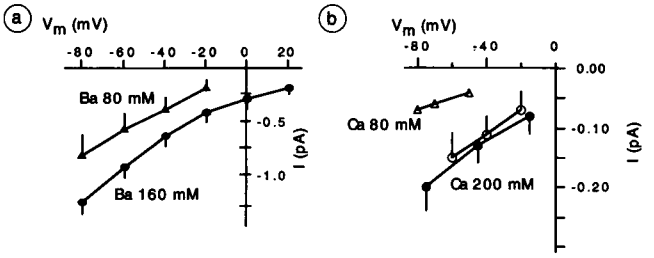

FIGURE 2 Current-voltage relationship of calcium entry channel events after dialysis with BAPTA. (*a*) Currents were carried by Ba^{2+} (80 and 160 mM); (*b*) currents were carried by Ca^{2+} (200 and 80 mM). Data are obtained by analysis of experiments performed with the protocol of Fig. 1. Closed symbols denote cell-attached patches (membrane potential determined at the end of BAPTA dialysis). Open symbols denote inside-out patches (n = 2 for each concentration, bath [Ca^{2+}] 50 nM). Only long openings (threshold arbitrarily set ≥8 ms, f_o = 400 Hz) were analyzed. Error bars denote SD of individual openings. The slopes between −40 and 80 mV yield conductances of 10 and 16 pS for $BaCl_2$ 80 and 160 mM, and 2 and <1 pS for $CaCl_2$ 200 and 80 mM, respectively.

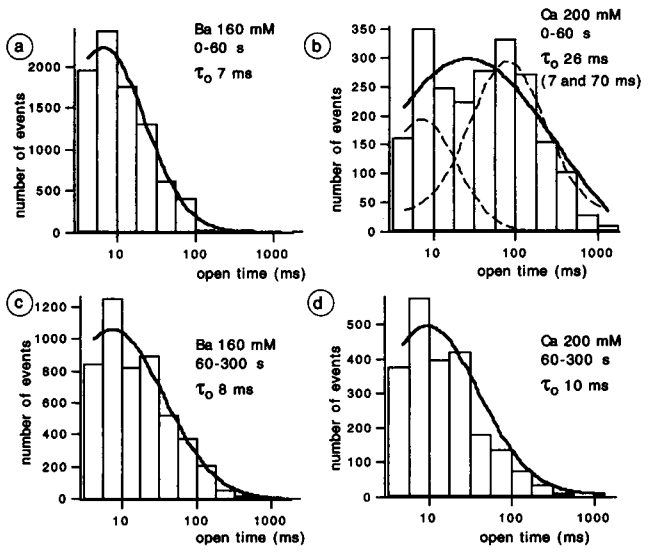

FIGURE 3 Open time distribution of channel events after dialysis with BAPTA. Open times are plotted in logarithmic bins and fitted to a Gaussian distribution (or, in *b*, to the sum of two Gaussian distributions) of the logarithmic mean of each bin, yielding the estimates of the mean open time (τ_o) (Sigworth and Sine, 1987). Each panel represents data from analysis of three experiments performed with a similar protocol as in Figs. 1 or 2. *a* and *b* represent data obtained 0–60 s after establishing the seal; *c* and *d* 60–300 s after establishing the seal. (*a*, *c*) Pipette solution 160 mM $BaCl_2$; (*b*, *d*) pipette solution 200 mM $CaCl_2$.

was not seen without BAPTA dialysis (n = 12) or with low BAPTA concentration (0.1 mM, with no added $CaCl_2$; n = 12) in the dialysis pipette.

The current-voltage relation (Fig. 2) excludes the possibility that the inward currents were carried by Cl^-. Even if one assumes that the internal Cl^- concentration was equal to that of the BAPTA dialysate (126 mM), the reversal potential would be −25 mV with 160 mM $BaCl_2$ in the pipette and expected to be shifted to more positive values if the Cl^- concentration in the pipette were reduced. Likewise, the conductance of chloride channels should be the same with $CaCl_2$ and $BaCl_2$ in the pipette, unlike the 10-fold observed difference. Furthermore, channel activity was not affected by the addition of the chloride channel blocker DIDS (0.3 mM) to the pipette solution.

Because fluorimetric studies in A431 cells have shown that depletion-induced calcium influx is sustained for at least 10 min (Hughes et al., 1991), we tested the possibility that the rapid inactivation of the channels in our experiments is related to the high divalent cation concentrations in the pipette and the accordingly higher current amplitude and divalent entry than that expected under physiological conditions. To reduce the currents temporarily, the holding potential of five cell-attached patches (pipette solution 80 mM $BaCl_2$) of BAPTA-dialyzed cells was set to −100 mV (transmembrane potential across the patch ~+80 mV) as soon as channel activity was observed after sealing. At this small electrochemical gradient, no channel activity was visible. However, when the holding potential was reset to +20 mV (transmembrane potential ca. −40 mV) for 5 s every 30 s, channel activity remained high in all patches for several minutes. The mean NP_o (±SD) after 4 min was 0.41 ± 0.24.

An analysis of the single-channel open times is shown in Fig. 3. The longest open times were found with Ca^{2+} as charge carrier during the first minute after sealing. The open time distribution under these conditions was best fit as the sum of two log-Gaussian distributions (Sigworth and Sine, 1987), whereas after one minute, or with Ba^{2+} as charge carrier, a reasonable fit was possible assuming a single log-Gaussian distribution.

Excision of patches to the inside-out configuration abolished channel activity within ~5 s in all but five experiments (n > 50). Changing Ca^{2+} concentrations in the bath (facing the cytosolic side of the patch) to 10, 0.5, or 0.05 μM (n = 12), or adding both $InsP_3$ and 1,3,4,5 $InsP_4$ (30 μM, n = 6) did not restore activity. Rarely (n = 5), channel activity was preserved in inside-out patches for sufficient time for further analysis. In two such patches, when activity lasted for ~1 min, $InsP_3$ + $InsP_4$ (10 μM) did not enhance activity at bath Ca^{2+} concentrations of 0.5 or 0.05 μM. The channels reported here are clearly distinct from our previously reported Ca^{2+} and $InsP_4$-dependent channels in endothelial cells (Lückhoff and Clapham, 1992).

To demonstrate more directly that the channel activity was caused by the BAPTA dialysis, cells were patched with two pipettes at the same time, one filled with BAPTA and the other with Ba^{2+}. Initiating dialysis by obtaining the whole-cell configuration with the BAPTA pipette rapidly induced channel activity, measured in the Ba^{2+}-filled cell-attached pipette, in 7 of 19 attempts (Fig. 4). To enhance depletion, these cells had been kept in the low Ca^{2+} medium for at least 5 min.

Occasionally, we measured another channel type conducting outward currents during recordings of the Ca^{2+}-permeable channel. This channel was not observed when the pipette contained $BaCl_2$ and was active in BAPTA-dialyzed cells only as long as Ca^{2+} entry channels were also active.

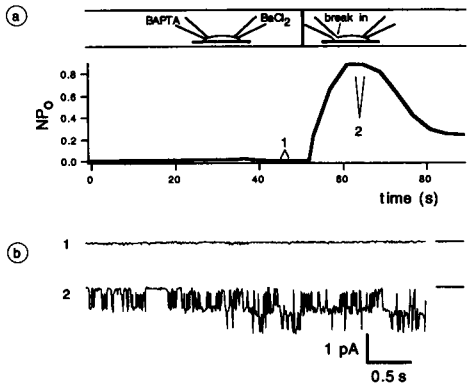

FIGURE 4 Channel activity induced by BAPTA dialysis (cell-attached recording from one A431 cell before and during dialysis with BAPTA (10 mM) solution through another patch clamp pipette). (*a*) Single-channel activity (expressed as NP_o) in the cell-attached pipette (containing 160 mM $BaCl_2$), before and after obtaining the whole-cell configuration in the dialysis pipette. The transmembrane potential was set to −40 mV with the dialysis pipette. (*b*) Single-channel recordings during periods 1 and 2 in *a*.

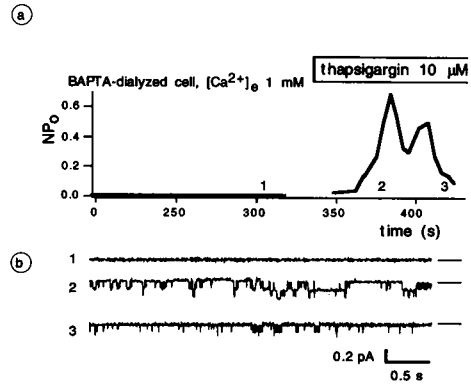

FIGURE 5 Channel activity induced by thapsigargin. An A431 cell was dialyzed with BAPTA before cell-attached recording (pipette solution 200 mM $CaCl_2$) began in 1 mM external Ca^{2+} (bath solution without EDTA). Thapsigargin was added (final concentration 10 μM) 340 s after obtaining the cell-attached patch. (*a*) Channel activity (expressed as NP_o) as a function of time. (*b*) Sample single-channel recordings during periods 1–3 in *a*. Note the change in channel open time as activation declines.

In inside-out patches, this channel was furthermore characterized as a Ca^{2+}-dependent K^+ channel, because it was activated by 10 μM Ca^{2+} in the bath but was inactive in 10 nM Ca^{2+} bathing solution. The channel had a conductance of 22 pS in 120 mM KCl and is probably the same Ca^{2+}-dependent K^+ channel (18 pS) that was described by Peppelenbosch et al. (1991). We speculate that its activity in Ca^{2+} buffered cells is due to local elevations of $[Ca^{2+}]_i$ induced by local Ca^{2+} channel activation.

Thapsigargin and buBHQ induce channel activity

Up to this point it was not entirely clear whether channel activation was due to store depletion, cytoplasmic Ca^{2+} depletion, or to some other effects of dialysis. In previous studies, the inhibitors of microsomal Ca^{2+} ATPase pumps, thapsigargin (Thastrup et al., 1990), or 2′,5′-di (tert-butyl)-1,4-benzohydroquinone (buBHQ, Moore et al., 1987) were frequently used to deplete stores. However, we failed to detect any significant channel activity after addition of thapsigargin (10 μM, $n = 9$) or buBHQ (30 μM, $n = 8$) to cells not dialyzed with BAPTA. Similarly, addition of thapsigargin or buBHQ to cells in low Ca^{2+} before, during, or after dialysis had no effect on channel activity ($n = 12$). Because BAPTA dialysis alone should be sufficient to deplete stores at low external Ca^{2+} (Hoth and Penner, 1993), we performed a series of experiments with BAPTA-dialyzed cells in physiological (1 mM) bath Ca^{2+}. Basal channel activity was observed in only 2 out of 33 experiments and, when present, was fairly low ($NP_o < 0.1$). Thapsigargin (3–10 μM, $n = 3/14$) and buBHQ (30 μM, $n = 3/14$) induced, or enhanced, channel activity under these conditions (Fig. 5). In seven experiments, NP_o increased from 0.02 to 0.45 ± 0.13 30 s after application of the compounds and to 0.14 after 90 s. In a parallel control series with low (0.5 μM) bath Ca^{2+} without thapsigargin, channel activity was observed in 4/11 experiments. Thus, both methods of store depletion induced channel activity with comparable efficiency. The effects of thapsigargin occurred after a lag time of 10–20 s and were transient, lasting for about 2 min. Interestingly, chelation of external Ca^{2+} with EGTA transiently activated the channels (in BAPTA-dialyzed cells), in the absence of thapsigargin ($n = 3/6$) as well as after the thapsigargin activation had subsided ($n = 3$).

Whole-cell Ba^{2+} currents

Calcium entry whole-cell current was studied in voltage-clamped cells dialyzed with strongly buffered internal Ca^{2+} solution (20 mM EGTA) for at least 4 min. Addition of $BaCl_2$ (25 mM) induced an inward current with peak values of 21 ± 7 pA (0.8 ± 0.2 pA/pF, $n = 12$) at a holding potential of −30 mV (Fig. 6, *a* and *c*). The currents were transient, lasting 20–180 s. The current-voltage relation (Fig. 6, *b* and *d*) indicates that the currents were predominantly carried by Ba^{2+}. No such currents were observed with $CaCl_2$ (25 or 50 mM), perhaps because they were smaller than the whole-cell detection limit (~5 pA) in our system. However, $CaCl_2$ (5 mM) rapidly abolished Ba^{2+} currents (Fig. 6 *c*) when added to cells exhibiting sustained currents in response to $BaCl_2$ ($n = 6$) and prevented Ba^{2+} currents when added before $BaCl_2$ ($n = 6$).

The magnitude of the currents was dependent on the time allowed in the whole-cell configuration before $BaCl_2$ was added and also on the time over which the cells were kept in medium with low Ca^{2+} concentration. Cells kept at low Ca^{2+} for 10 min produced currents of 17 ± 2 pA ($n = 3$) when $BaCl_2$ was added 15 s after obtaining the whole-cell configuration. When the time of low Ca^{2+} exposure was reduced to 1 min, $BaCl_2$ induced no, or only small, inward currents (4 ± 3 pA, $n = 8$). In all of these eight cells, thapsigargin (3 μM) elicited inward currents amounting to 26 ± 8 pA (0.8 ± 0.2 pA/pF). However, these were the only conditions under which we found thapsigargin to have an effect.

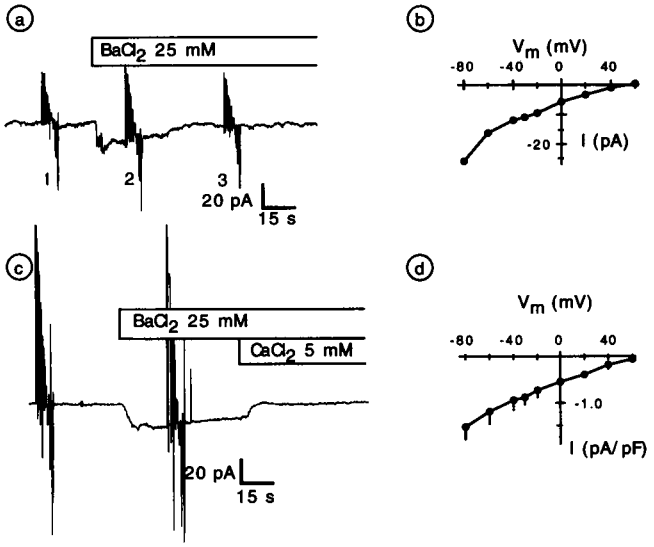

FIGURE 6 Whole-cell Ba^{2+} currents in A431 cells. (*a*) $BaCl_2$ was added (final concentration 25 mM) to an A431 cell held in whole-cell voltage clamp at −30 mV for 4 min. $BaCl_2$ induced a transient inward current that declined to less than 10% of its peak within 32 s. During periods 1, 2, and 3, pulses to various holding potentials (from +60 to −80 mV, step 20 mV, 500 ms each) were applied. (*b*) From the difference between 1 and 2, the voltage dependence of the currents was calculated. (*c*) In a different experiment, $CaCl_2$ (5 mM) was added after $BaCl_2$. Holding potential −30 mV. (*d*) The relation of $BaCl_2$-induced inward currents (corrected for the cell capacity, ±SD) to the holding potential, as calculated from voltage pulses applied before and during application of $BaCl_2$ (n = 5).

In cells dialyzed for 4 min, thapsigargin neither prevented the inhibition of Ba^{2+} currents by Ca^{2+} (n = 2) nor enhanced Ba^{2+} currents (n = 6).

DISCUSSION

We have demonstrated a Ca^{2+}- and Ba^{2+}-permeable channel in A431 cells dialyzed with the Ca^{2+} chelator BAPTA. Channel activity required either a low external Ca^{2+} concentration or the addition of inhibitors of Ca^{2+} uptake into internal calcium stores. These results strongly suggest that depletion of stores activates the channel.

Calcium entry channel activity was always transient. One reason for this observation may be the high concentration of pipette Ca^{2+} that was required to resolve the unitary currents through channels with a fairly low conductance. The opening of Ca^{2+}-dependent K^+ channels in some of the patches suggests submembrane elevations of $[Ca^{2+}]_i$ even in the presence of high internal BAPTA concentrations. Our data, therefore, are consistent with the view that cytosolic Ca^{2+} exerts a negative control over the Ca^{2+} channel. In agreement with this is the fact that not only were no Ca^{2+} currents observed in whole-cell experiments, but external Ca^{2+} also rapidly inhibited Ba^{2+} currents. Therefore, it is not surprising that thapsigargin did not induce consistent and long-lasting effects in cells not loaded with BAPTA. In normal cells with only intrinsic Ca^{2+} buffers in the cytosol, in which 98–99% of all Ca^{2+} influx may be buffered (Neher and Augustine, 1992), the slight elevation of $[Ca^{2+}]_i$ by thapsigargin would require no more than ~1 pA of Ca^{2+} influx, even if Ca^{2+} release did not occur. Ca^{2+} currents through single channels in the patch alone amounted to about 0.2 pA in many experiments. We interpret the transience of calcium entry channel activation as due to a negative regulation mechanism, reducing channel activity to undetectable levels after an initial elevation of activity.

Ba^{2+} whole-cell currents, as well as the activity of single Ba^{2+} channels, were also transient. The inactivation of channels could be delayed by decreasing the electrical driving force for divalent influx. Therefore, Ba^{2+} may also inhibit divalent cation entry, although to a lesser extent than Ca^{2+}. It is not unexpected that divalent cations can substitute for Ca^{2+} in processes normally specific for Ca^{2+}. For example, in cells incubated in isotonic $MnCl_2$, Mn^{2+} accumulated in calcium stores and was mobilized by $InsP_3$ (Fasolato et al., 1993).

It seems unlikely that the concentration of divalent cations in the cytosol is the main regulatory determinant of these channels because they were also stimulated by thapsigargin and buBHQ. In the presence of high internal BAPTA concentrations, thapsigargin and buBHQ presumably do not grossly affect $[Ca^{2+}]_i$ but, if anything, should increase $[Ca^{2+}]_i$. Taken together, the evidence supports the hypothesis that channel activation is governed by the filling state of the internal stores and not solely by the levels of cytosolic divalent cations.

At present we do not know the mechanism by which the store filling state communicates to the membrane channels. The fact that channel activity immediately stopped in nearly all experiments when the patch was pulled back into the inside-out configuration suggests a soluble factor as the stimulating agent, such as the recently proposed Ca^{2+} influx factor CIF (Randriamampita and Tsien, 1993) or a phosphorylation-sensitive second messenger (Parekh et al., 1993). However, the concentration of any soluble factors should have been dramatically decreased by dialysis, particularly in the experiments of Fig. 4, when dialysis with BAPTA initiated channel activity.

An alternative idea proposed by Irvine (1990) suggests that there is direct interaction between the Ca^{2+} entry channel in the cell membrane, the $InsP_4$ receptor, and an endoplasmic Ca^{2+} channel, the $InsP_3$ receptor. In unstimulated cells, the two proteins would be coupled, preventing channel activity. This inhibition would be reversed in the presence of $InsP_3$, $InsP_4$, and a low Ca^{2+} concentration in the lumen of the ER, resulting in Ca^{2+} release simultaneously with transmembrane Ca^{2+} influx. This protein-protein model is analogous to the interaction of the dihydropyridine receptor and the ryanodine receptor in heart and skeletal muscle (Block et al., 1987; Takeshima et al., 1989). However, in our experiments, Ca^{2+} influx occurred without stimulation of $InsP_3$ and $InsP_4$ levels. Furthermore, these second messengers failed to activate the channels in inside-out patches, in contrast to endothelial Ca^{2+} channels activated by $InsP_4$ in the presence of elevated cytosolic Ca^{2+} (Lückhoff and Clapham, 1992).

Our study was not designed to compare store depletion-operated channels in stimulated cells to other Ca^{2+}-permeable channels reported previously (Peppelenbosch

et al., 1991, 1992). We have confirmed that addition of EGF to the bath elicited activity of 9 pS channels in cell-attached patches (with 80 mM $CaCl_2$ or $BaCl_2$ in the pipette solution), the same type of channel observed by Peppelenbosch et al. (1992). This channel would obscure the presence of the store depletion-dependent channel in EGF-stimulated cells.

The present study provides the first single-channel analysis of a Ca^{2+} entry pathway that is controlled by internal calcium stores. The entry pathway in A431 cells bears some regulatory characteristics similar to that described in mast cells (Hoth and Penner, 1992), in which internal Ca^{2+} buffering was required to measure Ca^{2+} currents. Single-channel conductance was small, but not as small as was estimated for I_{CRAC} in mast cells (Hoth and Penner, 1993) and I_{DAC} in lymphocytes (Zweifach and Lewis, 1993). There are also major differences. The most important one appears to be that the currents in mast cells and lymphocytes were sustained, not transient as in A431 cells. The difference might reflect different regulation by cytosolic Ca^{2+} and might have some consequences for the temporal and spatial pattern of cytosolic Ca^{2+} levels in stimulated cells. Furthermore, we found that the permeability to Ba^{2+} was higher than that to Ca^{2+}, unlike the very Ca^{2+}-selective permeability found in the other cell types (Fasolato et al., 1993; Zweifach and Lewis, 1993; Parekh et al., 1993).

In summary, we have described Ca^{2+}-permeable ion channels in A431 cells that constitute a Ca^{2+} entry pathway regulated by the filling state of internal calcium stores. This pathway co-exists with another pathway that is under direct, calcium-independent control by EGF and is distinct in many aspects from I_{CRAC} in mast cells and I_{DAC} in lymphocytes. Thus, there are multiple mechanisms by which Ca^{2+} might enter the cytosol in stimulated cells, perhaps providing for subtle local control of the intracellular calcium release mechanisms.

We thank Dr. G. Carpenter for the gift of A431 cells, L. Stehno-Bittel for critical review of the manuscript, and S. Manahan for assistance with the cell culture.

This work was supported by National Institutes of Health. D. E. Clapham is an Established Investigator of the American Heart Association. A. Lückhoff is a Heisenberg fellow of the Deutsche Forschungsgemeinschaft.

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