The Store-operated Calcium Entry Pathways in Human Carcinoma A431 Cells: Functional Properties and Activation Mechanisms

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ABSTRACT Activation of phospholipase C (PLC)-mediated signaling pathways in nonexcitable cells causes the release of Ca$^{2+}$ from intracellular Ca$^{2+}$ stores and activation of Ca$^{2+}$ influx across the plasma membrane. Two types of Ca$^{2+}$ channels, highly Ca$^{2+}$-selective I$_{CRAC}$ and moderately Ca$^{2+}$-selective I$_{SOC}$, support store-operated Ca$^{2+}$ entry process. In previous patch-clamp experiments with a human carcinoma A431 cell line we described store-operated I$_{min}$/I$_{CRAC}$ plasma membrane Ca$^{2+}$ influx channels. In the present paper we use whole-cell and single-channel recordings to further characterize store-operated Ca$^{2+}$ influx pathways in A431 cells. We discovered that (a) I$_{CRAC}$ and I$_{SOC}$ are present in A431 cells; (b) I$_{CRAC}$ currents are highly selective for divalent cations and fully activate within 150 s after initiation of Ca$^{2+}$ store depletion; (c) I$_{SOC}$ currents are moderately selective for divalent cations (P$_{Ba}$/P$_{Ca}$ = 14.5) and require at least 300 s for full activation; (d) I$_{CRAC}$ and I$_{SOC}$ currents are activated by PLC-coupled receptor agonists; (e) I$_{SOC}$ currents are supported by I$_{min}$/I$_{CRAC}$ channels that display 8.5–10 pS conductance for sodium; (f) I$_{CRAC}$ single channel conductance for sodium is estimated at 0.9 pS by the noise analysis; (g) I$_{min}$/I$_{CRAC}$ channels are activated in excised patches by an amino-terminal fragment of InsP$_3$R1 (InsP$_3$R1N); and (h) InsP$_3$ binding to InsP$_3$R1N is necessary for activation of I$_{min}$/I$_{CRAC}$ channels. Our findings provide novel information about store-operated Ca$^{2+}$ influx pathways in A431 cells.

KEY WORDS: calcium signaling • patch-clamp • inositol 1,4,5-trisphosphate • calcium channels • whole-cell

INTRODUCTION

Activation of PLC-mediated signaling pathways in nonexcitable cells causes the release of Ca$^{2+}$ from intracellular Ca$^{2+}$ stores and promotes Ca$^{2+}$ influx across the plasma membrane via capacitative Ca$^{2+}$ entry (CCE)* or store-operated Ca$^{2+}$ entry (SOC) processes (Berridge, 1995; Parekh and Penner, 1997; Putney et al., 2001; Venkatachalam et al., 2002). Two types of Ca$^{2+}$ channels have been implicated in store-operated Ca$^{2+}$ entry in nonexcitable cells. Highly Ca$^{2+}$-selective channels (P$_{D/M}$ > 1,000) named “Ca$^{2+}$ release activated channels” (I$_{CRAC}$) have been initially discovered in studies of Jurkat and RBL cells (Hoth and Penner, 1992; Zweifach and Lewis, 1993; Premack et al., 1994). Moderately Ca$^{2+}$-selective channels have been later identified in a number of cells and grouped under the name I$_{SOC}$ (Berridge, 1995; Parekh and Penner, 1997; Nilius and Droogmans, 2001; Putney et al., 2001; Venkatachalam et al., 2002). When compared with I$_{CRAC}$, I$_{SOC}$ channels display lower selectivity for divalent cations (P$_{D/M}$ ~10), at least 10-fold higher single channel conductance for divalent and monovalent cations, and different kinetic and pharmacological properties (Berridge, 1995; Parekh and Penner, 1997; Nilius and Droogmans, 2001; Putney et al., 2001; Venkatachalam et al., 2002). The molecular identity of I$_{CRAC}$ remains unclear (Clapham, 1996; Parekh and Penner, 1997; Putney et al., 2001; Venkatachalam et al., 2002). Mammalian nP channels of TRPC family are the most likely candidates for the role of I$_{SOC}$ channels (Birnbaumer et al., 1996; Clapham et al., 2001; Nilius and Droogmans, 2001; Montell et al., 2002; Venkatachalam et al., 2002; Zitt et al., 2002).

The mechanisms of I$_{SOC}$ and I$_{CRAC}$ channel activation have been under intense investigation (Birnbaumer et al., 1996; Clapham et al., 2001; Nilius and Droogmans, 2001; Montell et al., 2002; Venkatachalam et al., 2002; Zitt et al., 2002). Activation of I$_{SOC}$/I$_{CRAC}$ channels by a diffusible messenger CIF (Ca$^{2+}$-influx factor) released by depleted Ca$^{2+}$ stores (Putney and Bird, 1993; Randriamampita and Tsien, 1993; Kim et al., 1995; Csutora et al., 1999; Trepakova et al., 2000), via “conformational coupling” with the intracellular InsP$_3$R (Irvine, 1990; Kiselyov et al., 1998; Zubov et al., 1999), by cleavage of PIP$_2$ (Kaznacheyeva et al., 2000; Estacion et al., 2001), and by regulated insertion of channels into plasma membrane (Patterson et al., 1999; Yao et al., 1999) have been considered. Direct association of

*Abbreviations used in this paper: CCE, Ca$^{2+}$ entry; DVF, divalent-free; SOC, store-operated Ca$^{2+}$ entry.
TrpC3 and TrpC4 with the InsP$_3$R amino terminus (Boulay et al., 1999; Tang et al., 2001) provided a biochemical support to conformational-coupling model of $I_{\text{SOC}}$ activation. The mechanisms of $I_{\text{GRAC}}$ activation remain poorly understood.

In experiments with a human carcinoma A431 cell line we previously described $I_{\text{min}}$ plasma membrane Ca$^{2+}$ channels that are activated by application of uridine triphosphate and bradykinin to cell-attached patches or by application of InsP$_3$ to excised inside-out patches (Kiselyov et al., 1997, 1999b; Mozhayeva et al., 1990). We found that major functional properties of $I_{\text{min}}$ channels, such as small conductance (1–1.5 pS for divalent cations) and sensitivity to block by SKF95365 are similar to $I_{\text{GRAC}}$ channels (Kiselyov et al., 1999b; Zubov et al., 1999). We further demonstrated that activation of $I_{\text{min}}$ channels by InsP$_3$ in inside-out patches is facilitated by anti-PIP$_2$ antibodies and suggested a InsP$_3$R–PIP$_2$-dependent signaling complex in these cells (Kaznacheyeva et al., 2000). More recently we demonstrated activation of $I_{\text{min}}$ channels in A431 cells by depletion of intracellular Ca$^{2+}$ stores and renamed these channels $I_{\text{GRAC}}$ (Kaznacheyeva et al., 2001).

Our previous characterization of $I_{\text{min}}$/CRAC channels in A431 cells was performed at the single-channel level by patch-clamp technique (Mozhayeva et al., 1990; Kiselyov et al., 1997, 1999b; Kaznacheyeva et al., 2000, 2001; Zubov et al., 1999). In these experiments we determined that the monovalent single-channel conductance of $I_{\text{min}}$/CRAC channels is in the range 8.5–10 pS (Kaznacheyeva et al., 2001). The CRAC channels were described in Jurkat and RBL cells in whole-cell recordings (Hoth and Penner, 1992; Zweifach and Lewis, 1993; Premack et al., 1994) and the channels with 40 pS conductance for monovalent cations were identified in Jurkat cells (Kerschbaum and Cahalan, 1999). Recently, Mg$^{2+}$ and ATP-sensitive I$_{\text{MagNaM}}$ (Mg$^{2+}$, and nucleotide-regulated metal) channels encoded by TRPM7 were described in RBL and Jurkat cells (Nadler et al., 2001; Runnels et al., 2001; Hermosura et al., 2002). Channels with identical properties were also uncovered in RBL and Jurkat cells by other groups and called I$_{\text{MISC}}$ (Mg$^{2+}$-inhibited channels) (Kozak et al., 2002; Prakriya and Lewis, 2002). It appears that TRPM7 protein encodes both I$_{\text{MagNaM}}$ and I$_{\text{MISC}}$ channels, and in our paper we will use the originally proposed I$_{\text{MagNaM}}$ nomenclature (Nadler et al., 2001). I$_{\text{MagNaM}}$ channels display 40 pS conductance for monovalent cations (Prakriya and Lewis, 2002), and it appears that the previously measured 40 pS channels present in Jurkat cells (Kerschbaum and Cahalan, 1999) correspond to I$_{\text{MagNaM}}$, not to I$_{\text{GRAC}}$ (Kozak et al., 2002). In fact, recent noise measurements suggested that the monovalent conductance of I$_{\text{GRAC}}$ channels in Jurkat cells is no more than 0.2 pS (Prakriya and Lewis, 2002).

These results pose a number of questions. What is a relationship between $I_{\text{GRAC}}$, $I_{\text{SOC}}$, I$_{\text{MagNaM}}$, and $I_{\text{min}}$/CRAC channels in A431 cells? What is a mechanism of $I_{\text{min}}$/CRAC activation? To answer these questions, we analyzed store-operated Ca$^{2+}$ influx pathways in A431 cells by whole-cell and single-channel recordings. We discovered that both $I_{\text{min}}$/CRAC and I$_{\text{GRAC}}$ channels are activated by intracellular Ca$^{2+}$ and by cytosolic InsP$_3$ and by PLC-linked agonists. However, I$_{\text{GRAC}}$ channels are activated by Ca$^{2+}$ store-depletion faster than $I_{\text{min}}$/CRAC channels and are more selective for divalent cations than $I_{\text{min}}$/CRAC channels. In some experiments we also observed channels with the properties typical of I$_{\text{MagNaM}}$ (Clapham, 2002; Hermosura et al., 2002; Kozak et al., 2002; Prakriya and Lewis, 2002). These channels were clearly distinct from $I_{\text{min}}$/CRAC channels. Thus, we concluded that $I_{\text{min}}$/CRAC channels correspond to $I_{\text{SOC}}$ and not to I$_{\text{GRAC}}$ or I$_{\text{MagNaM}}$ channels in A431 cells.

In additional experiments we demonstrated activation of $I_{\text{min}}$/CRAC channels by the amino-terminal recombinant fragment of InsP$_3$R1, in support of a conformational-coupling activation model of $I_{\text{SOC}}$ in A431 cells.

**MATERIALS AND METHODS**

**Cells**

Human carcinoma A431 cells (Cell Culture Collection) were kept in culture as described elsewhere (Kiselyov et al., 1999b). For patch clamp experiments cells were seeded onto coverslips and maintained in culture for 1 to 3 d before use.

**Electrophysiology**

All electrophysiological experiments were performed using a PC-501A patch clamp amplifier (Warner Instruments) with a conventional 10 GΩ feed-back resistance in the head stage. Resistance of sylgard-coated, fire-polished glass microelectrodes varied from 3 to 5 MΩ. Series resistance was not compensated. During recording the currents were sampled at 2.5 kHz and filtered at 500 Hz. In all whole-cell experiments the holding potential was 0 mV. Periodically (once every 4–30 s) the membrane potential was stepped to −100 mV (for 30 ms) and a 170 ms voltage ramp to 70 mV was applied. Traces recorded before I$_{\text{GRAC}}$ and I$_{\text{SOC}}$ current activation were used as a template for leak subtraction. The recorded currents were normalized to the cell capacitance. Mean value of cell capacitance was 25 ± 4 pF.

During recording the currents were sampled at 2.5 kHz and filtered at 100 Hz for analysis and presentation. Unitary current amplitude was determined from current records and all-point amplitude histograms. The experiments were performed at room temperature (22–24°C). Data are...
given as mean ± SE. Error bars denoting SE are shown where they exceed the symbol size.

**Current Fluctuation Analysis**

The whole-cell recordings in DFV media were performed as described above before and after application of UTP in the bath. The cells were maintained at 0 mV holding potential and currents were recorded by applying 700-ms voltage steps to −50- and −100-mV test potentials every 5 s. The current records at each test potential were sampled at 5 kHz and filtered at 1 kHz. The mean current and variance at each test potential were calculated from 200-ms segments of the digitized current using a software written on the basis of Microsoft Excel. The background noise and leak current at every potential were subtracted from mean current and variance.

The stationary noise analysis was performed as described in (Jackson and Strange, 1995; Prakriya and Lewis, 2002) with the following assumptions: (a) the I_{CRAC} channels in the membrane are identical and independent; (b) the channels have two conductance states, open and closed; and (c) the single-channel conductance of channels in the membrane stays constant during current activation. With these assumptions, the current variance $\sigma^2$ is related to the single-channel properties as

$$\sigma^2 = I \cdot I - I^2/N + \sigma^2_o,$$  
(2)

where $\sigma^2$ is the current variance, $\sigma^2_o$ is the variance of background noise, $i$ is the single-channel current, $N$ is a number of active channels in the membrane, and $I$ is a whole cell current:

$$I = N \cdot i \cdot P_o,$$  
(3)

where $P_o$ is the single channel open probability.

Combination of Eqs. 2 and 3 leads to:

$$\sigma^2 - \sigma^2_o = i \cdot I \cdot (1 - P_o).$$  
(4)

In general case the Eq. 4 (and the equivalent Eq. 2) is parabolic. However, the Eq. 4 adapts linear form if open channel probability is low:

$$\sigma^2 - \sigma^2_o = i \cdot I, \text{ if } P_o \approx 1.$$  
(5)

**Solutions**

For whole-cell experiments, pipette solution contained (in mM): 145 NMDG aspartate, 10 Cs-EGTA (or 12 mM Cs-BAPTA), 10 Cs-HEPES, pH 7.3, 1.5 MgCl$_2$, and either 4.5 CaCl$_2$ (pCa 7.0) or no CaCl$_2$ added (pCa > 9). 10 µM InsP$_3$ was included in the pCa 7.0 pipette solution as indicated. Extracellular solution contained (in mM) 140 NMDG aspartate, 10 BaCl$_2$, 10 Cs-HEPES, pH 7.3. The divalent-free (DFV) solution contained (in mM): 145 Na methanesulfonate, 10 NaCl, 10 HEDTA, 0.5 EGTA, and 10 HEPES pH 7.3. For single-channel experiments, the pipette solution contained (in mM): 105 BaCl$_2$ or 140 NaCl as indicated and 10 Tris-HCl (pH 7.3). In inside-out experiments the intracellular solution contained (in mM): 140 K glutamate, 5 NaCl, 1 MgCl$_2$, 10 K-HEPES pH 7.4, 2 EDTA and 1.13 CaCl$_2$ (pCa 7.0), with or without InsP$_3$ as indicated. In cell-attached experiments, the bath solution contained (in mM): 140 KCl, 5 NaCl, 10 K-HEPES, 1 MgCl$_2$, and 2 CaCl$_2$. In outside-out experiments the same solutions were used as in whole-cell experiments. The drugs and recombinant proteins were applied to the patches by bath perfusion. The time required for a complete change of solution around the patch was less than 1 s.

**Expression and Purification of Recombinant CBD-InsP$_3$R1N Proteins**

CBD-InsP$_3$R1N and CBD-InsP$_3$R1N-K508R proteins were expressed in 0.5 liter of LB media for 14 h at 30°C in protease-deficient BL21 E. coli strain by 1 mM IPTG induction according to manufacturer’s (Amersham Pharmacia Biotech) protocols. Cells were cooled on ice, collected by 20 min centrifugation at 4,500 rpm (Beckman JLA-10.5), and resuspended in 30 ml of wash buffer (20 mM TrisCl pH 8.0, 40 mM EDTA) with addition of 0.2 mg/ml lysozyme and protease inhibitors (1 mg/ml leupeptin, 200 µg/ml aprotinin, 1 mM PMSF). Cells were disrupted by repetitive 60 s sonication bursts on

**Figure 1.** Activation of I_{CRAC} and I_{SOC} currents in A431 cells by intracellular store-depletion. Whole-cell recordings were performed at 0-mV holding potential using ramp protocol (test potentials from −100 to 70 mV; duration of the ramp, 200 ms; inter-ramp interval is 10 s). Pipette solution contained (in mM) 10 Cs-HEPES pH 7.3, 145 NMDG aspartate, 10 Cs-EGTA (pCa > 9.0), 1.5 MgCl$_2$. Extracellular solution contained (in mM) 10 Cs-HEPES pH 7.3, 140 NMDG aspartate, 10 BaCl$_2$. (A) The amplitudes of peak currents recorded at each ramp at −80 mV (filled circles), 30 mV (open circles) and 50 mV (filled triangles) test potentials are plotted as a function of time after break-in. (B) Current-voltage relationships recorded at 159 s (curve a) and 275 s (curve b). Data from the same experiment are shown in A and B. Ramps corresponding to curves a and b in B are indicated by arrows in A. The data are representative of 19 experiments.
ice (Branson Ultrasonics). After centrifugation (4,500 g for 30 min; Beckman J-20 rotor) the pellet was twice washed with TED buffer (10 mM TrisCl pH 8.0, 1 mM EDTA, 1 mM DTT) and solubilized in 5 ml denaturing buffer (10 mM TrisCl pH 8.0, 8 M urea) for 3 h at RT. 5 ml of denatured protein was added to 250 ml of refolding buffer (50 mM TrisCl pH 8.0, 2 mM EDTA, 1.25 M NaCl, 0.5 M L-arginine) and incubated with stirring overnight in cold room. The resulting soluble fraction was concentrated to 5 ml on Amicon YM50 filters under nitrogen pressure and dialyzed against PBS.

**[3H]InsP3 Binding Assay**

Specific [3H]InsP3 binding was performed with minor modifications of a procedure described previously (Glouchankova et al., 2000). Briefly, 10–20 μg of purified CBD-InsP3 R1N protein was incubated on ice with 10 nM [3H]InsP3 (Amersham Pharmacia Biotech) in the binding buffer (50 mM Tris-HCl, pH 8.3, 1 mM EDTA, 1 mM DTT, 100 mM NaCl) and precipitated with 12.5% PEG and 1.2 mg/ml γ-globulin at 14,000 g. Precipitates were quickly washed with the binding buffer, dissolved in Soluene (Packard Instrument Co.) and their [3H] content was determined by liquid scintillation counting. Nonspecific counts, determined in the presence of 25 μM nonlabeled InsP3, were subtracted from the total to yield specific binding.

**Chemicals**

HEPES, NMDG, and Na-methanesulfonate were from Sigma-Aldrich; EGTA and HEDTA were from Fluka Chemie AG; and UTP and InsP3 were from Calbiochem.

**RESULTS**

ICRAC and ISOC Divalent Whole-cell Currents in A431 Cells

To investigate store depletion–activated divalent cation influx pathways in A431 cells, we performed a series of whole-cell current recordings using 10 mM Ba2+ as a current carrier. To stimulate store-depletion–activated

![Graph A](image1.png)

**Figure 2.** “ICRAC-only” and “ISOC-only” currents are activated by intracellular store-depletion. (A) Whole-cell recordings were performed as on Fig. 1 with the inter-ramp interval equal to 30 s. Pipette solution is the same as in Fig. 1 with addition of 4.5 CaCl2 (pCa 7.0) and 10 μM InsP3. Extracellular solution is the same as in Fig. 1. The amplitudes of the peak currents recorded at each ramp at −80 mV (filled circles), 30 mV (open circles), and 50 mV (filled triangles) test potentials are plotted as a function of time after break-in. (B) Current-voltage relationship recorded at 156 s (curve a) and 486 s (curve b). Ramps corresponding to curves a and b in A are indicated by arrows in A. The data are representative of 5 experiments. (C) Whole-cell recordings were performed as in Fig. 1 with the interramp interval equal to 10 s. The pipette and extracellular solutions are the same as on Fig. 2 A. The amplitudes of peak currents recorded at each ramp at −80 mV (filled circles), 30 mV (open circles), and 50 mV (filled triangles) test potentials are plotted as a function of time after break-in. (D) Current-voltage relationship recorded at 100 s (curve a) and 600 s (curve b) of the experiment. Ramps corresponding to curves a and b in D are indicated by arrows in C. The data are representative of seven experiments.

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currents, the pipette solution contained 10 mM EGTA or 12 mM BAPTA and 1.5 mM Mg$^{2+}$ (pCa > 9.0). The A431 cells in these experiments were maintained at 0-mV holding potential and currents were recorded using 170-ms ramps from −100 to 70 mV. The inter-ramp interval was in the range from 4 to 30 s. The amplitudes of divalent currents recorded in a representative experiment at −80-, 30-, and 50-mV test potentials are plotted as a function of time on Fig. 1 A (time = 0 at the moment of break-in). The complete current-voltage curves measured at 159 s after break-in (curve a) and 275 s after break-in (curve b) in the same experiment are shown on Fig. 1 B. It is apparent that the current-voltage curves change not only in amplitude but also in shape. The curve a (measured at 159 s) displays strong inward rectification, with no detectable outward current with test potentials as positive as 70 mV. In contrast, the curve b (measured at 275 s) is practically linear and shows significant outward current at test potentials more positive than 30 mV. Curve a (Fig. 1 B) has the shape expected for a highly Ca$^{2+}$-selective I$_{\text{CRAC}}$ current described in Jurkat and RBL cells (Hoth and Penner, 1992; Zweifach and Lewis, 1993; Premack et al., 1994). The shape of curve b (Fig. 1 B) corresponds to a less Ca$^{2+}$-selective current.

Two alternatives could account for the observed results. The first is that A431 cells express two types of Ca$^{2+}$ influx channels: highly Ca$^{2+}$-selective I$_{\text{CRAC}}$ and less Ca$^{2+}$-selective store-operated channels (I$_{\text{SOC}}$). The second possibility is that I$_{\text{CRAC}}$ channels activated by store depletion became less Ca$^{2+}$ selective in the course of an experiment. Our data indicate that the coexistence of two different store-operated Ca$^{2+}$ channel types in A431 cells is a more likely possibility. Indeed, in some cells (5/36 experiments, 14%) only highly Ca$^{2+}$-selective I$_{\text{CRAC}}$ channels (Fig. 2, A and B) were observed. To facilitate Ca$^{2+}$ store depletion, 10 $\mu$M InsP$_3$ was included in the pipette solution in addition to 10 mM EGTA and 4.5 mM CaCl$_2$ (pCa = 7.0) in the experiment shown on Fig. 2, A and B. In other cells (7/36 experiments, 19%) only moderately selective I$_{\text{SOC}}$ currents were observed using the same pipette solution (Fig. 2, C and D). The combination of I$_{\text{CRAC}}$ and I$_{\text{SOC}}$ currents (Fig. 1) was observed in 19/36 experiments (53%). We could not identify the reasons for variability in I$_{\text{CRAC}}$ and I$_{\text{SOC}}$ current expression in A431 cells. Nevertheless, we reasoned that the existence of “I$_{\text{CRAC}}$ only” (Fig. 2, A and B) and “I$_{\text{SOC}}$ only” (Fig. 2, C and D) A431 cells argues against the possibility of I$_{\text{CRAC}}$ to I$_{\text{SOC}}$ interconversion during our recordings.

Both types of channels were activated by store depletion in our experiments, but with the different time course. Fig. 3 A illustrates an average time-course of I$_{\text{CRAC}}$ (filled circles, n = 4) and I$_{\text{SOC}}$ (filled triangles, n = 6) currents activation. It is apparent that I$_{\text{CRAC}}$ current reaches maximum value within 200 s after break-in (Fig. 3 A, curve a), whereas I$_{\text{SOC}}$ requires at least 300 s to reach its maximum value (Fig. 3 A, curve b). To further compare I$_{\text{CRAC}}$ and I$_{\text{SOC}}$ divalent currents in A431 cells, we calculated average current-voltage relation-
Figure 4. Activation of I_{CRAC} and I_{SOC} currents by extracellular UTP. Whole-cell recordings were performed with the pipette and extracellular solutions as in Fig. 2 without addition of InsP$_3$. (A) The amplitudes of peak currents recorded at each ramp at $-80$ mV (filled circles) and $+50$ mV (open triangles) test potentials are plotted as a function of time after break-in. 100 $\mu$M UTP was applied to the cells at 208 s (arrow). (B) Current-voltage relationships recorded at 231 s (curve a) and 300 s (curve b) of the experiment. Ramps corresponding to curves a and b in B are indicated by the arrows in A. (C) The amplitudes of peak currents recorded at each ramp at $-80$ mV (filled circles) and $+50$ mV (open triangles) test potentials are plotted as a function of time after break-in. 100 $\mu$M UTP was applied to the cells at 630 s (arrow). (D) The current-voltage relationships recorded at 650 s (curve a), 850 s (curve b), and 1140 s (curve c) of the experiment. Ramps corresponding to curves a–c in D are indicated by the arrows in C.

ships measured in cells containing only I_{CRAC} (Fig. 3 B, curve a, $n = 4$) or I_{SOC} (Fig. 3 B, curve b, $n = 6$) currents. To generate curve a, the current voltage-relationship was determined when I_{CRAC} channels were fully activated (Fig. 3 A, curve a). To generate curve b, I_{SOC} currents were measured at least 340 s after break-in (Fig. 3 A, curve b). On average, the amplitude of I_{CRAC} currents at $-80$ mV was equal to $-0.96 \pm 0.02$ pA/pF ($n = 4$), and the amplitude of I_{SOC} currents at $-80$ mV was equal to $-2.1 \pm 0.16$ pA/pF ($n = 6$). Thus, an average I_{SOC} current is twice the size of an average I_{CRAC} current. The reversal potential of I_{CRAC} currents could not be measured as we could not detect any significant outward current via I_{CRAC} channels at test potentials as positive as 60 mV in our recording conditions (Fig. 3 B, curve a). For I_{SOC} currents an average reversal potential ($E_{rev}$) was equal to $30 \pm 3$ mV ($n = 6$) (Fig. 3 B, curve b). After correction for liquid junction potential we calculated P$_{Ba}/P_{Ca}$ selectivity ratio of I_{SOC} currents equal to 14.5 using GHK equation (Hille, 2001) as described in MATERIALS AND METHODS. Thus, divalent selectivity of I_{SOC} channels in A431 cells is similar to the divalent selectivity of I_{SOC} channels described in other nonexcitable cells (Berridge, 1995; Parekh and Penner, 1997; Nilius and Droogmans, 2001; Putney et al., 2001; Venkatachalam et al., 2002).

I_{CRAC} and I_{SOC} currents were observed in most (31/36) of the whole-cell recording experiments (86%). In a small fraction of cells (5/36, 14%), currents with very different properties were observed (unpublished data). In these cells, inward currents at $-80$-mV test potentials reached 10–20 pA/pF, 2–5-fold larger than typical I_{CRAC} or I_{SOC} currents (Fig. 3 B). At positive test potentials these cells displayed outward rectification leading to even larger currents at positive potentials (up to 30 pA/pF), in striking contrast with I_{CRAC} or I_{SOC} (Fig. 3 B). The reversal potential for these currents was close to 30 mV, similar to I_{SOC} currents (Fig. 3 B). Based on the large size of inward currents, moderate selectivity for divalent cations and characteristic outward rectification, we reasoned that the currents observed in these experiments correspond to I_{MagNuM} currents in A431 cells (Clapham, 2002; Hermosura et al., 2002; Kozak et al., 2002; Prakriya and Lewis, 2002). Detailed character-
ization of \(I_{\text{MagNuM}}\) currents in A431 cells was precluded by their rare occurrence in our experiments, and in the remainder of this paper we focus on the analysis of more prevalent \(I_{\text{CRAC}}\) and \(I_{\text{SOC}}\) currents.

**Activation of \(I_{\text{CRAC}}\) and \(I_{\text{SOC}}\) Currents by Extracellular UTP**

In experiments shown on Figs. 1–2 \(I_{\text{CRAC}}\) and \(I_{\text{SOC}}\) currents were activated by depletion of intracellular Ca\(^{2+}\) stores. This was achieved by buffering cytosolic Ca\(^{2+}\) to pCa 9.0 with 12 mM BAPTA or 10 mM EGTA (Fig. 1) or by addition of 10 \(\mu\)M InsP\(_3\) to cytosolic solution buffered to pCa 7.0 (Fig. 2). In physiological situations, Ca\(^{2+}\) influx in nonexcitable cells is activated in response to activation of PLC-coupled receptors. Therefore, in the next series of experiments we used whole-cell recordings in A431 cells to measure Ba\(^{2+}\) currents activated by application of extracellular UTP. The intracellular Ca\(^{2+}\) concentration in these experiments was clamped to pCa 7.0 as in the experiments shown on Fig. 2, but no InsP\(_3\) was added. In these conditions passive store depletion is slow and \(I_{\text{CRAC}}\) and \(I_{\text{SOC}}\) currents were not activated within the first 200 s after break-in (Fig. 4 A) and within 600 s (Fig. 4 C). However, we found that 100 \(\mu\)M extracellular UTP efficiently activated both \(I_{\text{CRAC}}\) and \(I_{\text{SOC}}\) currents in A431 cells (Fig. 4).

Similar to experiments with store-depletion (Figs. 1 and 2), “\(I_{\text{CRAC}}\)-only” (Fig. 4, A and B) and “\(I_{\text{SOC}}\)-only” (Fig. 4, C and D) currents were induced in some A431 cells in response to extracellular UTP. The current-voltage relationship of UTP-activated currents (Fig. 4, B and D) was identical to currents activated by Ca\(^{2+}\) store depletion (Fig. 3 B). Thus, we concluded that both \(I_{\text{CRAC}}\) and \(I_{\text{SOC}}\) currents could be activated in A431 cells by Ca\(^{2+}\) store depletion or in response to activation of PLC-coupled receptors.

**\(I_{\text{CRAC}}\) and \(I_{\text{SOC}}\) Monovalent Whole-cell Currents in A431 Cells**

To further characterize \(I_{\text{CRAC}}\) and \(I_{\text{SOC}}\) currents in A431 cells, we performed a series of whole-cell recording experiments in DVF media containing 140 mM Na\(^+\). In the absence of divalent cations, Na\(^+\) and other monovalent cations carry a substantial current through voltage-gated Ca\(^{2+}\) channels (Almers et al., 1984; Hess and Tsien, 1984) and \(I_{\text{CRAC}}\) (Hoth and Penner, 1993; Hermosura et al., 2002; Prakriya and Lewis, 2002). In most of the experiments performed in DVF media, large monovalent inward and outward currents were observed (Fig. 5 A). Similar to experiments with Ba\(^{2+}\) as a current carrier (Fig. 1), the shape of the current-voltage relationship recorded in DVF media was changing in a course of the experiments. Within the first 10 s after addition of DVF solution the current-voltage relationship displayed inward rectification, and outward currents were absent with test potentials as positive as 80 mV (Fig. 5 B, curve a). At later times, the current-voltage relationship became more linear, with a reversal potential at 0 mV and large outward currents (Fig. 5 B, curve b). The amplitude of Na\(^+\)-currents continued to rise during first 10 min of recordings (Fig. 5 A). Thus, similar to recordings with Ba\(^{2+}\), recordings in DVF media revealed two types of channels in A431 cells activated in our recording conditions. The inwardly rectifying monovalent current observed in our experiments in DVF media (Fig. 5 B, curve a) is similar to Na\(^{+}\)-current recorded in Jurkat and RBL cells (Hoth and Penner, 1993; Hermosura et al., 2002; Prakriya and Lewis, 2002). The large nonselective current observed in our experiments in DVF media (Fig. 5 B, curve b) is likely to correspond to Na\(^{+}\)-\(I_{\text{SOC}}\) currents.

**Figure 5.** \(I_{\text{CRAC}}\) and \(I_{\text{SOC}}\) currents in DVF medium. Whole-cell recordings were performed as described in Fig. 1 with the interramp interval of 11 s. Pipette solution contained (in mM) 120 Cs-Aspartate, 10 BAPTA (pCa > 9.0), 1.5 MgCl\(_2\), pH 7.3. Extracellular DVF solution contained (in mM): 135 Na-methanosulfonate, 5 NaCl, 10 HEDTA, 0.5 EGTA, pH 7.3. (A) Amplitudes of peak currents recorded at each ramp at –80 mV (filled circles), 0 mV (open circles), and 80 mV (open triangles) test potentials are plotted as a function of time after break-in. Extracellular solution was replaced by DVF medium at 100 s after break-in (arrow). (B) Current-voltage relationship recorded at 120 s (curve a) and 130 s (curve b) of the experiment. Ramps corresponding to curves a and b in B are indicated by arrows in A. The data are representative of four experiments in DVF medium.
To gain insight into properties of channels supporting I_{CRAC} and I_{SOC} currents in A431 cells, we performed a series of single-channel recordings. In these experiments, we took advantage of increased conductance of these channels in DVF media (Fig. 5). Because of different time course of I_{CRAC} and I_{SOC} activation (Fig. 3A), we reasoned that the channels recorded within first 100 s after break-in are more likely to correspond to I_{CRAC} and the channels recorded between 300–600 s after break-in are more likely to correspond to I_{SOC}. To measure single-channel currents in whole-cell experiments, we applied 10-s test pulses from −100 to 50 mV from the holding potential of 0 mV. In these experiments, we failed to observe any channel activity within the first 100 s after break-in. However, with 12 mM BAPTA and 1.5 mM Mg^{2+} in the pipette (pCa > 9.0), we observed activity of multiple channels 200–600 s after break-in (Fig. 6A). On average, the single-channel current through the channels was equal to 0.7 ± 0.05 pA at −70 mV test potential (Fig. 7, triangles, n = 4).

Channels with similar properties were recorded in excised patches after application of 2.5 μM InsP₃ (inside-out, Fig. 6B, n = 8), and after application of 1 μM thapsigargin and 1 mM BAPTA-AM (cell-attached, Fig. 6C, n = 4; see also Kaznacheyeva et al., 2001) and in outside-out configuration in response to application of extracellular UTP (n = 4, current records not shown). The single-channel slope conductances for channels

**Figure 6.** Patch-clamp recordings of single-channel activity. Single-channel activity recorded in whole-cell (A), inside-out (B), and cell-attached (C) patch-clamp mode. 140 mM Na⁺ is a current carrier in all experiments (see materials and methods for details). The single-channel activity was induced by: (A) store-depletion (12 mM BAPTA and 1.5 mM Mg^{2+} in the pipette, pCa > 9.0), representative of four whole-cell experiments; (B) 2.5 μM intracellular InsP₃, representative of 8 inside-out experiments; (C) BAPTA-AM and 100 μM Tg in cell-attached mode, representative of four cell-attached experiments. The unitary current amplitudes were measured manually or determined from all-point amplitude histograms (shown on the right).
measured in all these experimental conditions were in the range 8.5–10 pS (Fig. 7). The properties of these channels are identical to the properties of $I_{\text{min/ICRAC}}$ channels that we described previously (Kaznacheyeva et al., 2001). Importantly, $I_{\text{min/ICRAC}}$ single-channels were observed in our whole-cell experiments from 100 s until 20 min after break-in, that is during the time period corresponding to $I_{\text{SOC}}$ activation (Fig. 3 A).

We did not observe $I_{\text{min/ICRAC}}$ channel activity in whole-cell experiments within the first 100 s after break-in, that is in the time window of $I_{\text{GRAC}}$ current development (Fig. 3 A). From these experiments we concluded that the previously described $I_{\text{min/ICRAC}}$ channels (Mozhayeva et al., 1990; Kiselyov et al., 1997, 1999b; Zubov et al., 1999; Kaznacheyeva et al., 2000, 2001) support $I_{\text{SOC}}$ currents in A431 cells.

Current Fluctuation Analysis of Na-ICRAC

As described above, we were unable to measure single-channel activity of $I_{\text{GRAC}}$ channels using DVF media, most likely due to small conductance of these channels. Current fluctuation analysis has been used previously in studies of $I_{\text{GRAC}}$ channels in Jurkat cells (Zweifach and Lewis, 1993; Prakriya and Lewis, 2002). Here we applied the stationary current fluctuation analysis method (see MATERIALS AND METHODS) to estimate the size of single-channel sodium currents via $I_{\text{GRAC}}$ channels activated by application of 100 μM UTP to A431 cells (Fig. 8 A). To perform fluctuation analysis, we selected experiments in DVF media with “$I_{\text{GRAC-only}}$” cells as determined by the inward rectification shape of current-voltage relationship (Fig. 8, A and B). The intracellular Ca$^{2+}$ concentration in these experiments was clamped to pCa 7.0 by 10 mM EGTA as in the experiments shown on Fig. 2. The mean macroscopic current (I) and corresponding variance ($\sigma^2$) were measured at holding potential of 0 mV and at −50 and −100 mV test potentials as described in MATERIALS AND METHODS. Application of UTP activated $I_{\text{GRAC}}$ (Fig. 8 A) and caused a significant increase in the noise of the current recorded at −100 mV test potential (Fig. 8 C).

To determine the single-channel conductance of Na-ICRAC, we measured variance of the UTP-activated DVF current at 0, −50, and −100 mV membrane potentials. Plots of the current variance against mean current amplitude at 0, −50, and −100 mV could well be fitted by the straight lines (Fig. 8 D). Because of the observed linear relationship between $\sigma^2$ and I we used linear approximation for relationship between $\sigma^2$ and I (MATERIALS AND METHODS, Eq. 5) in our calculations. The underlying assumption for linear approximation is that Na-ICRAC open channel probability Po is low (Jackson and Strange, 1995; Prakriya and Lewis, 2002). According to Eq. 5, the slope of $\sigma^2$ versus I linear fit is equal to i, where i is the size of the Na-ICRAC single channel current. The fit to the data yielded the slope equal to −0.09 pA at 0 mV (Fig. 8 D, bottom), −0.14 pA at −50 mV (Fig. 8 D, middle), and −0.18 pA at −100 mV (Fig. 8 D, top). Obtained measurements are consistent with the slope single-channel conductance of Na-ICRAC channels in A431 cells equal to 0.9 pS (Fig. 8 E), which is 5 times greater than the chord conductance estimated for Na-ICRAC currents in Jurkat cells (Prakriya and Lewis, 2002), and 10 times smaller than the single-channel conductance of Na-$I_{\text{unc}}$ in A431 cells (Fig. 7). The reasons for differences between our estimates and the results of Prakriya and Lewis (2002) may reflect the differences in $I_{\text{GRAC}}$ properties in A431 and Jurkat cells, or may be related to assumptions about channel open probability and gating properties inherent to the current fluctuation analysis approach.

Activation of $I_{\text{min/ICRAC}}$ Channels by InsP$_3$RIN

What mechanism is responsible for activation of $I_{\text{min/ICRAC}}$ channels in A431 cells? Our previous results suggested that $I_{\text{min/ICRAC}}$ channels may be activated via di-
rect conformational coupling with the InsP₃R (Zubov et al., 1999) or with the InsP₃R–PIP2 complex (Kaznacheyeva et al., 2000). Which domain of the InsP₃R is required for Iᵢₘᵟᵡ/ᵢₐᵡᵣᵡᵡ activation? Based on its functional properties Iᵢₘᵟᵡ/ᵢₐᵡᵣᵡᵡ is likely to be encoded by a member of the TRPC family (see Discussion). Activation of TRPC3 channels by the amino-terminal region of InsP₃R1 (InsP₃R1N) has been reported (Kiselyov et al., 1999a). Can InsP₃R1N gate Iᵢₘᵟᵡ/ᵢₐᵡᵣᵡᵡ? To answer this question, we expressed the InsP₃R1N fragment (aa 2–604) in bacteria as CBD-fusion protein (see materials and methods). In addition, we also generated and expressed a CBD-InsP₃R1N-K508R mutant, which has been reported to lack specific InsP₃ binding activity (Yoshikawa et al., 1996).

When expressed in E. coli BL21 cells, both CBD-InsP₃R1N and CBD-InsP₃R1N-K508R proteins were obtained in this purification scheme, but only CBD-InsP₃R1N protein displayed specific [³H]-InsP₃ binding activity (unpublished data). Thus, in agreement with the report published previously (Yoshikawa et al., 1996) and with the recently reported structure of the InsP₃R1 ligand-binding core (Bosanac et al., 2002), the K508 residue plays a critical role in InsP₃ binding.

To test functional effects of InsP₃R1N, we applied the CBD-InsP₃R1N protein to inside-out patches excised from A431 cells using 105 mM Ba²⁺/H₁₁₀₀₁ in the pipette as a current carrier. As described previously (Mozhayeva et al., 1990; Kiselyov et al., 1997, 1999b; Zubov et al., 1999; Kaznacheyeva et al., 2000, 2001), a low activity of Iᵢₘᵟᵡ/ᵢₐᵡᵣᵡᵡ channels was observed in excised patches in the presence of 2.5 µM of InsP₃ (Figs. 9 A and 10 B). Addition of CBD-InsP₃R1N protein to the cytosolic surface of the patch resulted in rapid facilitation of Iᵢₘᵟᵡ/ᵢₐᵡᵣᵡᵡ channel activity (Figs. 9 A and 10 B). These channels displayed 1.5 pS conductance in 105 mM Ba²⁺ (Fig. 9 C), as described previously for Iᵢₘᵟᵡ/ᵢₐᵡᵣᵡᵡ (Mozhayeva et al., 1990; Kiselyov et al., 1997, 1999b; Zubov et al., 1999; Kaznacheyeva et al., 2000, 2001).
Can InsP$_3$R$_1$N activate $I_{\text{min}}/I_{\text{CRACL}}$ channels independently from InsP$_3$? When CBD-InsP$_3$R$_1$N was added to excised patches in the absence of InsP$_3$, no channel activity was observed (Fig. 10 A). However, addition of InsP$_3$ to the same patch resulted in an immediate activation of $I_{\text{min}}/I_{\text{CRACL}}$ channels (Fig. 10 A). Thus, InsP$_3$ is required for InsP$_3$R$_1$N activation of $I_{\text{min}}/I_{\text{CRACL}}$ channels. Is InsP$_3$ binding to InsP$_3$R$_1$N important for the observed functional effects? When CBD-InsP$_3$R$_1$N-K508R protein was added to inside-out patches in the presence of 2.5 $\mu$M InsP$_3$, no channel activity was observed (Fig. 10 C). In contrast, addition of CBD-InsP$_3$R$_1$N to the same patch induced $I_{\text{min}}/I_{\text{CRACL}}$ activity (Fig. 10 B). These data suggest that the InsP$_3$-liganded form of InsP$_3$R$_1$N is a true activator of $I_{\text{min}}/I_{\text{CRACL}}$ channels.

DISCUSSION

Store-operated Ca$^{2+}$ influx pathways in A431 cells were analyzed in this paper by whole-cell and single-channel recordings. We discovered that $I_{\text{CRAC}}$ and $I_{\text{SOC}}$ currents are present in A431 cells. The main functional properties of $I_{\text{CRAC}}$ and $I_{\text{SOC}}$ ($I_{\text{min}}/I_{\text{CRACL}}$) channels in A431 and other nonexcitable cells are briefly summarized in Table I. Implications of our findings are discussed below.

$I_{\text{min}}/I_{\text{CRACL}}$ and $I_{\text{CRAC}}$

In the previous patch-clamp studies we described store-operated and InsP$_3$-activated $I_{\text{min}}/I_{\text{CRACL}}$ channels in A431 cells (Mozhayeva et al., 1990; Kiselyov et al., 1997, 1999b; Zubov et al., 1999; Kaznacheyeva et al., 2000, 2001). Some of the properties of $I_{\text{min}}/I_{\text{CRACL}}$ channels, such as sensitivity to store-depletion, small conductance for divalent and monovalent cations, and sensitivity to block by SKF95365, indicated that $I_{\text{min}}/I_{\text{CRACL}}$ channels may correspond to $I_{\text{CRAC}}$ channels in A431 cells. However, a number of differences between $I_{\text{min}}/I_{\text{CRACL}}$ channels and $I_{\text{CRAC}}$ channels were uncovered in the present study. The main differences between $I_{\text{min}}/I_{\text{CRACL}}$ channels and $I_{\text{CRAC}}$ channels are (Table I): (a) $I_{\text{min}}/I_{\text{CRACL}}$ conductance for divalent cations is 1–1.5 pS, for $I_{\text{CRAC}}$ it is estimated at 10–25 fS; (b) $I_{\text{min}}/I_{\text{CRACL}}$ conductance for sodium is 8.5–10 pS, for $I_{\text{CRAC}}$ it is estimated at 0.2–0.9 pS; (c) $I_{\text{min}}/I_{\text{CRACL}}$ channels are moderately selective for divalent cations ($P_{\text{Ba}}/P_{\text{Na}} = 14.5$), $I_{\text{CRAC}}$ channels are highly selective for divalent cations ($P_{\text{Ca}}/P_{\text{M}} > 1,000$); (d) $I_{\text{min}}/I_{\text{CRACL}}$ channels display linear current-voltage relationship, $I_{\text{CRAC}}$ currents display inward rectification; and (e) $I_{\text{min}}/I_{\text{CRACL}}$ currents require at least 300 s after initiation of Ca$^{2+}$ store depletion for complete activation, $I_{\text{CRAC}}$ currents activate within 100–200 s. Thus, we concluded that $I_{\text{min}}/I_{\text{CRACL}}$ channels and $I_{\text{CRAC}}$ channels are different entities. In support of this conclusion, $I_{\text{CRAC}}$-only cells were found in 5 of 36 whole-cell experiments (14%) and $I_{\text{min}}/I_{\text{CRACL}}$-only currents were found in 7 of 36 experiments (19%). In 19 of 36 experiments (53%), both types of currents were present in the same cell. The different rates of $I_{\text{CRAC}}$ and $I_{\text{min}}/I_{\text{CRACL}}$ activation in response to store-depletion (Fig. 3 A) suggest that these two Ca$^{2+}$ entry pathways could be gated by different mechanisms. Another possibility is that both $I_{\text{CRAC}}$ and $I_{\text{min}}/I_{\text{CRACL}}$ are activated by a similar "conformational coupling" mechanism (see below), but coupled to different Ca$^{2+}$ pools, so that $I_{\text{CRAC}}$ is coupled to the pool that is depleted rapidly, and $I_{\text{min}}/I_{\text{CRACL}}$ is...
coupled to the pool that is depleted more slowly. The differences in the rates of pool depletion can be related to the differences in distribution of InsP$_3$R and Ca$^{2+}$ pumps, as well as to localized expression of different InsP$_3$R isoforms. Future experiments will be needed to discriminate between these possibilities.

**Figure 10.** InsP$_3$ binding to InsP$_3$R1N is required for $I_{\text{min}}$/$I_{\text{CRAC}}$ activation. Inside-out patch clamp recordings of $I_{\text{min}}$/$I_{\text{CRAC}}$ channel activity were performed as described in Fig. 9 at $-70$ mV. (A) InsP$_3$ is required for $I_{\text{min}}$/$I_{\text{CRAC}}$ channel activation by CBD-InsP$_3$R1N. $I_{\text{min}}$/$I_{\text{CRAC}}$ currents are shown on compressed (top) and expanded (bottom) time scales. Data are representative of eight experiments. (B) InsP$_3$R1N, but not InsP$_3$R1N-K508R, evokes $I_{\text{CRAC}}$ activity. $I_{\text{min}}$/$I_{\text{CRAC}}$ current activity in response to application of InsP$_3$, CBD-InsP$_3$R1N-K508R, and CBD-InsP$_3$R1N to the patch is shown as indicated. $I_{\text{min}}$/$I_{\text{CRAC}}$ currents are shown on compressed (top) and expanded (bottom) time scales.

**Table I**

| Property                          | $I_{\text{CRAC}}$ Functional Properties | ISOC ($I_{\text{min}}$/$I_{\text{CRAC}}$) Functional Properties |
|-----------------------------------|-----------------------------------------|---------------------------------------------------------------|
| Conduction                        | ~25 fS (Zweifach and Lewis, 1993)       | 1.5 pS (Kaznacheyeva et al., 2001)                           |
| DVF                               | 0.2–0.9 pS (Fig. 8 E; Prakriya and Lewis, 2002) | 8.5–10 pS (Fig. 7; Kaznacheyeva et al., 2001)               |
| Divalent current size in A431 cells | 1 pA/pF (Fig. 3 B)                   | 2 pA/pF (Fig. 3 B)                                         |
| Permeability ratio                |                                        |                                                               |
| $P_{\text{D/F}}$                   | >1,000 (Hoth and Penner, 1993; Zweifach and Lewis, 1993) | 14.5 (Fig. 3 B)                                             |
| $P_{\text{Na/Cs}}$                | ~10 (Hermosura et al., 2002; Prakriya and Lewis, 2002) | ~1 (Fig. 5 B)                                               |
| IVC                               | Inward rectification                   | Linear (Fig. 3 B)                                           |
|                                 | (Fig. 3 B; Hoth and Penner, 1993; Zweifach and Lewis, 1993) |                                                               |
|                                 | Inward rectification                   | Linear (Fig. 5 B)                                           |
|                                 | (Fig. 5 B; Hermosura et al., 2002; Prakriya and Lewis, 2002) |                                                               |
| Block by Mg$^{2+}$                | No (Fig. 2 A)                          | No (Fig. 2 C)                                               |
| Activation by store depletion     | Yes (Fig. 1; Hoth and Penner, 1993; Zweifach and Lewis, 1993) | Yes (Figs. 1, 6 A, 6 C; Kaznacheyeva et al., 2001)       |
| By InsP$_3$                       | Yes (Fig. 2 A; Hoth and Penner, 1993; Zweifach and Lewis, 1993) | Yes (Figs. 2 C and 6 B; Kaznacheyeva et al., 2001)       |
| By PLC-linked agonists            | Yes (Fig. 4 A; Hoth and Penner, 1993; Zweifach and Lewis, 1993) | Yes (Figs 4 C; Kaznacheyeva et al., 2001)                   |
| PIP$_2$                           | ND                                      | Inhibits (Kaznacheyeva et al., 2000)                        |
| Onset of current                  | <100 s (Fig. 3 A; Hermosura et al., 2002; Prakriya and Lewis, 2002) | >200 s (Fig. 3 A)                                           |
| Block by SKF96365                 | Complete block by 20 µM (Franzius et al., 1994; Prakriya and Lewis, 2002) | Complete block by 25 µM (Zubov et al., 1999)                |
Our results lead us to conclude that $I_{\text{min}}/I_{\text{GRACL}}$ channels support $I_{\text{SO}}$ current in A431 cells. What is the molecular identity of $I_{\text{min}}/I_{\text{GRACL}}$ channels? Recent results support the hypothesis that members of the TRPC family are essential components of $I_{\text{SO}}$ channels in nonexcitable cells. Indeed, genetic knockout of the TRPC4 subunit in mouse resulted in dramatic reduction of $I_{\text{SO}}$ currents in endothelial cells (Freichel et al., 2001).

Similar results were obtained when TRPC1 was genetically deleted in chicken B cell lymphocytes (Mori et al., 2002). Thus, it is likely that $I_{\text{SO}}$ currents in other nonexcitable cells are also supported by the members of TRPC family. The main functional properties of $I_{\text{min}}/I_{\text{GRACL}}$ channels are consistent with the properties of channels formed by TRPC family members when expressed in holotoxigenous expression system (for review see Birnbaumer et al., 1996; Clapham et al., 2001; Nilius and Droogmans, 2001; Montell et al., 2002; Venkataram et al., 2002; Zitt et al., 2002). Future molecular studies will be required to test this hypothesis and to identify the TRPC protein that encodes $I_{\text{min}}/I_{\text{GRACL}}$ channels in A431 cells.

$I_{\text{min}}/I_{\text{GRACL}}$ and InsP$_3$R

What is a mechanism of $I_{\text{min}}/I_{\text{GRACL}}$ activation? Our previous results (Zubov et al., 1999; Kaznatcheyeva et al., 2000) supported the conformational coupling model of $I_{\text{min}}/I_{\text{GRACL}}$ activation. Here we provide additional evidence in favor of the conformational coupling model. We established that the amino-terminal fragment of InsP$_3$R1 (InsP$_3$R1N) was able to activate $I_{\text{min}}/I_{\text{GRACL}}$ in excised inside-out patches (Fig. 9) and that InsP$_3$R1N association with InsP$_3$ is critical for $I_{\text{min}}/I_{\text{GRACL}}$ channel activation (Fig. 10). Importantly, the InsP$_3$R1N fragment used in our experiments (amino acids 2–604) does not contain previously identified TRPC3- and TRPC4-binding sites, which are located within 669–821 region (Boulay et al., 1999; Tang et al., 2001). Thus, it is unlikely that the direct association of InsP$_3$R1N with a member of TRPC family can account for an ability of InsP$_3$R1N to activate $I_{\text{min}}/I_{\text{GRACL}}$. Most likely, an additional adaptor protein is involved in the conformational coupling between InsP$_3$R and $I_{\text{min}}/I_{\text{GRACL}}$ (TRPC). One potential candidate for this role is a Homer protein (Xiao et al., 2000) that binds to the residues 48–55 of the InsP$_3$R1 amino-terminal region (Tu et al., 1998), which are present within InsP$_3$R1 sequence. Identification of additional InsP$_3$R1 amino-terminal binding partners may provide interesting insights into InsP$_3$R1-$I_{\text{min}}/I_{\text{GRACL}}$ conformational coupling mechanism.

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