Activation of calcium entry in human carcinoma A431 cells by store depletion and phospholipase C-dependent mechanisms converge on I_{CRAC}-like calcium channels

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Activation of phospholipase C in nonexcitable cells causes the release of calcium (Ca^{2+}) from intracellular stores and activation of Ca^{2+} influx by means of Ca^{2+} release-activated channels (I_{CRAC}) in the plasma membrane. The molecular identity and the mechanism of I_{CRAC} channel activation are poorly understood. Using the patch-clamp technique, here we describe the plasma membrane Ca^{2+} channels in human carcinoma A431 cells, which can be activated by extracellular UTP, by depletion of intracellular Ca^{2+} stores after exposure to the Ca^{2+}-pump inhibitor thapsigargin, or by loading the cells with Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetate. The observed channels display the same conductance and gating properties as previously described I_{min} channels, but have significantly lower conductance for monovalent cations than the I_{CRAC} channels. Thus, we concluded that the depletion-activated Ca^{2+} current in A431 cells is supported by I_{CRAC-like} (I_{CRACL}) channels, identical to I_{min}. We further demonstrated synergism in activation of I_{CRACL} Ca^{2+} channels by extracellular UTP and intracellular inositol (1,4,5)-triphosphate (IP_{3}), apparently because of reduction in phosphatidylinositol 4,5-bisphosphate (PIP_{2}) levels in the patch. Prolonged exposure of patches to thapsigargin renders I_{CRACL} Ca^{2+} channels unresponsive to IP_{3} but still available to activation by the combined action of IP_{3} and anti-IP_{3} antibody. Based on these data, we concluded that phospholipase C-mediated and store-operated Ca^{2+} influx pathways in A431 cells converge on the same I_{CRACL} Ca^{2+} channel, which can be modulated by PIP_{2}.

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 by means of capacitative Ca^{2+} entry or store-operated Ca^{2+} entry processes (1–3). These processes are mediated by plasma membrane Ca^{2+} channels termed “Ca^{2+} release activated channels” (I_{CRAC}) (4–7). The molecular identity of I_{CRAC} remains unclear, with mammalian trp channels (mTrp) usually considered the most likely candidate for the role of I_{CRAC} (1–3, 8, 9). When compared with I_{CRAC}, mTrp channels display relatively low selectivity for divalent cations, higher single channel conductance, and different kinetic and pharmacological properties. In experiments with a human carcinoma A431 cell line, we previously described plasma membrane Ca^{2+} channels (I_{min}) that are activated by application of uridine triphosphate and bradykinin to cell-attached patches or by application of inositol (1,4,5)-trisphosphate (IP_{3}) to excised inside-out (i/o) patches (10–12). IP_{3}-gated channels that share some common properties with I_{min} have been also observed in experiments with human T cells (13), rat macrophages (12), and endothelial cells (14, 15). Major functional properties of I_{min} channels, such as small conductance (1 pS for divalent cations), high selectivity for divalent cations (F_Ca/K > 1,000), inward rectification, and sensitivity to block by SKF95365 are similar to I_{CRAC} channels (12, 16). Thus, we previously suggested that I_{min} and I_{CRAC} may in fact be the same channels (17).

The mechanism of I_{CRAC} activation remains similarly controversial (1–3). When studied in a heterologous expression system, activation of mTrp channels by IP_{3} appears to be mediated by direct conformational coupling between the cytosolic carboxy-terminal tail of mTrp and the amino-terminal ligand-binding domain of intracellular IP_{3} receptor (IP_{3}R) (18–21). However, whether mTrp can serve as an appropriate model system for understanding I_{CRAC} activation is unresolved (18, 21, 22). In previous studies, we demonstrated that activity of I_{min} in i/o patches is potentiated by addition of IP_{3}R-enriched microsomes as predicted by an I_{min}-IP_{3}R conformational coupling model (16). More recently, we discovered that anti-IP_{3} antibody (PI_{3}Ab) sensitizes I_{min} to IP_{3} activation and proposed an I_{min}-IP_{3}-RI_{n} (IP_{3}R) functional coupling model based on these findings (17). In parallel with our results, a potential role of IP_{3} in trp-like (trpl) channel activation has been recently demonstrated in Sf9 cells (23). The I_{min}-IP_{3}-RI_{n} coupling model can adequately explain activation of I_{min} channels by direct action of PLC but not the activation of I_{CRAC} channels resulting from Ca^{2+} store depletion (4–6).

A number of critical questions related to a depletion-activated Ca^{2+} influx pathway remain unanswered. Most importantly, do store-depletion and PLC-dependent pathways activate the same or a different channel type? To answer this question, we compare the effects of PLC-linked agonist UTP, Ca^{2+} pump inhibitor thapsigargin (Tg), and Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetate (BAPTA) on plasma membrane Ca^{2+} channel activity in patch-clamp experiments performed with human carcinoma A431 cells. We conclude that PLC activation and depletion of intracellular Ca^{2+} stores activate the same Ca^{2+} channel in A431 cells. We found that the conductance and selectivity properties of the store-operated channel in A431 cells are identical to the properties of I_{min} and somewhat different from the properties of I_{CRAC} channels described in Jurkat T cells (5–7). Thus, we will refer to store-operated channels in A431 cells as I_{CRACL} (“crac-like”). We also concluded that IP_{3} plays a role in modulation of I_{CRACL} activity.

Materials and Methods

Electrophysiological Recordings. Human carcinoma A431 cells (Cell Culture Collection, Institute of Cytology, St. Petersburg, §1734 solely to indicate this fact.

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Abbreviations: PLC, phospholipase C; I_{CRAC}, Ca^{2+} release-activated channel; I_{CRACL}, I_{CRAC-like}; IP_{3}, inositol 1,4,5-triphosphate; IP_{3}R, IP_{3} receptor; PI_{P}, phosphatidylinositol (1,4,5)-biphosphate; c/a, cell-activated; i/o, inside-out; Tg, thapsigargin; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetate.

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Russia) were kept in culture, as described elsewhere (12). For patch-clamp experiments, cells were seeded onto coverslips and maintained in culture for 1–3 days before use. Single-channel currents were recorded by using the cell-attached and i/o configuration of the patch-clamp technique (24). Currents filtered at 500 Hz were recorded with a PC-501A patch-clamp amplifier (Warner Instruments, Hamden, CT) with a conventional resistance feedback in the headstage (10 GΩ). The currents were digitized at 2.5 kHz. For data analysis and presentation, currents were additionally digitally filtered at 100 Hz.

NPo was determined by using the following equation: \[ \text{NPo} = \frac{I}{i}, \]
where \( I \) and \( i \) are the mean channel current and unitary current amplitude, respectively. \( I \) was estimated from the time integral of the current above the baseline, and \( i \) was determined from current records and all-point amplitude histograms. Data were collected from current records after channel activity reached steady state. Because channel activity was transient and displayed significant fluctuations, we used NPo collected during 30 s of maximal activity (NPomax30) as a standard way to compare open channel probability among different experiments. Average NPomax30 values of channel activity from several experiments are presented in the text and on the figures as mean ± SEM. The pipette solution contained (in mM): 105 BaCl2, 105 CaCl2, or 140 NaCl as indicated, and 10 Tris/HCl (pH 7.4). For i/o experiments, patches were excised into the standard intracellular solution containing (in mM): 140 K glutamate, 5 NaCl, 1 MgCl2, 10 Hepes/KOH, 1.13 CaCl2, and 2 EGTA (pCa 7, pH 7.4), with or without IP3 as indicated. The cell-attached and i/o recordings were performed at ~70 mV holding potential. All experiments were carried out at room temperature (22–24°C).

Materials. Monoclonal anti-PiP2 antibody (PiP2Ab) (25) was from PerSeptive Biosystems (Framingham, MA), and monoclonal anti-PiP antibody (PiPAb) was from Assay Designs (Ann Arbor, MI). PiP2Ab and PiPAb were reconstituted in PBS (titer 1:1,500), diluted 1:100 by intracellular solution and used for chamber perfusion. Hepes, UTP, and Tg were from Sigma; EGTA was from Fluka Chemie AG (Buchs, Switzerland). IP3 and BAPTA-AM were from Calbiochem.

Results

Exposure to Extracellular UTP Sensitizes Imin to IP3 Activation. When cell-attached (c/a) recordings of Imin in A431 cells were performed in control recording conditions, the channel activity was very low with NPomax30 equal to 0.08 ± 0.06 (n = 12) (Fig. 1 a and c). After patch excision in bath solution containing 2.5 μM IP3, moderate activity of Imin in i/o patches was observed with NPomax30 equal to 0.86 ± 0.2 (n = 12) (Fig. 1 a and c). Similar behavior of Imin channels in c/a and i/o configurations has been described (10–12, 17). As we previously reported, addition of 100 μM UTP to 10 μM bradykinin to the solution bathing A431 cells leads to activation of PLC-coupled receptors and an increase in Imin activity in c/a patches to NPomax30 of 0.7–1.0 (12). When 100 μM UTP was included in the pipette solution, significantly higher Imin channel activity was observed with NPomax30 equal to 1.5 ± 0.17 (n = 33) (Fig. 1b). With either bath (12) or pipette (Fig. 1b) UTP application, activity of Imin was transient and resulted in channel inactivation within several minutes. After patch excision into intracellular solution containing 2.5 μM IP3, very high levels of Imin channel activity were observed (Fig. 1b). On an average, Imin channel NPomax30 increased from 1.31 ± 0.17 (c/a) to 2.91 ± 0.23 (i/o) in this series of experiments (n = 9) following patch excision (Fig. 1c).

To gain insight into the mechanism responsible for the unusually high activity of Imin channels in i/o recordings observed in the experiments with UTP in the pipette (Fig. 1 b and c), we determined the sensitivity of Imin activation by IP3 when 100 μM of UTP was included in the pipette solution. In all experiments of this series, we waited until Imin activity in c/a patches subsided before the patch excision. A single IP3 concentration in the 0.05–2.5 μM range was tested in each experiment to avoid IP3-induced Imin desensitization (12). Fitting the Hill equation to the data (Fig. 1d, a) yielded an apparent affinity (Kapp) of 0.15 μM IP3, maximal NPomax30 (NPomax) of 3.33, and a Hill coefficient (nH) of 0.83 (Fig. 1d, curve). When similar experiments were performed in control recording conditions, sensitivity of Imin to IP3 activation was much lower (Kapp = 0.51 μM IP3, NPomax = 0.87, nH = 1 of 1.05) (17) (Fig. 1d, c, and dashed line on the right). The dramatic increase in Imin apparent affinity for IP3 and in NPomax induced by exposure to UTP in the pipette

![Fig. 1. Sensitization of Imin to IP3 by extracellular UTP. (a) Plot of Imin open channel probability (NPo) in cell-attached (c/a) patch recorded in control conditions and in i/o patch from the same cell in the presence of 2.5 μM IP3. The NPo was averaged over 1-s intervals and plotted vs. time in the experiment. Mean NPomax30 was 0.08 in c/a and 0.88 in i/o for the experiment shown. Data are representative of 12 experiments. (b) Same plot as in a for the experiment with 100 μM UTP in the pipette. Mean NPomax30 was 1.5 in c/a and 3.41 in i/o for the experiment shown. Data are representative of nine experiments. (c) The summary plot of Imin open channel probability in c/a (open bars) and i/o (closed bars) recordings performed in control conditions (n = 12, left) or in the presence of 100 μM UTP in the pipette (n = 9, right). Imin activity is represented as NPomax30 (mean ± SEM). (d) NPomax of Imin channels in i/o experiments at IP3 concentrations as indicated measured with 100 μM UTP in the pipette (a), in the presence of PiP2Ab (b) and in control conditions (c). Average data at each IP3 concentration are shown as mean ± SEM (n ≥ 6). Smooth curve, best fit the data obtained with UTP in the pipette by using the equation NPomax30 = (NPomax)max [IP3]MHi/[IP3]MHi + Kapp, the values of parameters are in the text. Dashed lines, fit to the similar data obtained in control conditions (curve on the right) and in the presence of PiP2Ab (curve on the left). The data for control conditions and in the presence of PiP2Ab are taken from ref. 17.](https://example.com/figure1.png)
channels by Ca\(^{2+}\) store depletion. Does depletion of Ca\(^{2+}\) stores activate the same channel as activation of PLC? To answer this question, we evaluated effects of Tg on Ca\(^{2+}\) channel activity in patch–clamp experiments. As in our previous studies (12), addition of 1 \(\mu\)M Tg to the bath had only minimal effect on \(I_{\text{min}}\) activity when compared with control conditions, with \(NP_{\text{max},30}\) equal to 0.11 ± 0.03 (\(n = 9\)) (Fig. 2a; also see Fig. 4a and h). In contrast to these results, if 1 \(\mu\)M Tg was included in the pipette, active \(I_{\text{min}}\) channels were observed following a short delay after patch formation, with \(NP_{\text{max},30}\) equal to 1.7 ± 0.24 (\(n = 18\)) (Figs. 2b and 4b and h). We interpret this delay as the time needed for depletion of submembrane Ca\(^{2+}\) stores by Tg entering the cell from the pipette.

One potential explanation of different effects caused by bath and pipette applications of Tg is Ca\(^{2+}\)-induced inactivation of \(I_{\text{min}}\). From comparison of \(I_{\text{min}}\) rundown kinetic with Ca\(^{2+}\) as a current carrier, we previously concluded that \(I_{\text{min}}\) is likely to undergo Ca\(^{2+}\)-induced inactivation process (11). Massive Ca\(^{2+}\) release from the stores resulting from bath application of Tg may quickly inactivate \(I_{\text{min}}\), but if Tg is included only in the pipette, Ca\(^{2+}\) leak is much slower, and \(I_{\text{min}}\) inactivation may be reduced or decelerated. To test this hypothesis, we clamped Ca\(^{2+}\) concentration in A431 cells by loading them with the membrane-permeable Ca\(^{2+}\) chelator BAPTA-AM. Bath application of 0.1 mM BAPTA-AM by itself resulted in \(I_{\text{min}}\) activity in 9 of 15 experiments. In six remaining experiments, application of Tg to BAPTA-loaded cells evoked \(I_{\text{min}}\) channel activity. To simplify experimental procedure, we combined application of Tg and BAPTA-AM to the bath, which resulted in \(I_{\text{min}}\) channel activity in 7 of 10 experiments (Figs. 2c and 4c). From these results, we concluded that the low potency of Tg in the bath to activate \(I_{\text{min}}\) in our previous studies (12), (a) mostly likely results from Ca\(^{2+}\)-dependent inactivation of \(I_{\text{min}}\).

Activation of \(I_{\text{min}}\) by depletion of intracellular Ca\(^{2+}\) stores with

![Diagram](image-url)

**Fig. 2.** Activation of \(I_{\text{min}}\) channels by Tg. (a) Ca\(^{2+}\) channel current traces in c/a patches recorded in the presence of 1 \(\mu\)M Tg in the bath solution. The fragments of current records are shown on the bottom on expanded time scale. The unitary current amplitude in used recording conditions (−70 mV membrane resting potential) is −0.18 pA. (b) Same as (a) with 1 \(\mu\)M Tg in the pipette. (c) Same as in a with 100 \(\mu\)M BAPTA-AM and 1 \(\mu\)M Tg in the bath.

Quantitatively matches with the effects exerted by PIP\(_2\)Ab on \(I_{\text{min}}\) (\(K_{\text{app}} = 0.08 \text{ \mu M}\) IP\(_3\), \(NP_{\text{max}} = 3.21, n_{\text{H}} = 0.8\) ) (17) (Fig. 1d, e, and dashed line on the left) and on the IP\(_3\)R (26). We reasoned that synergistic actions of extracellular UTP and intracellular IP\(_3\) in our experiments (Fig. 1) result from UTP receptor stimulation of PLC which decreases PIP\(_2\) levels in the patch. Reduction of PIP\(_2\) levels leads to an increase in the apparent affinity of IP\(_3\)R for IP\(_3\) (26) and in the potency of IP\(_3\) to activate \(I_{\text{min}}\).

**I\(_{\text{min}}\) Is the I\(_{\text{CRAC}}\) Channel Activated by Depletion of Intracellular Ca\(^{2+}\) Stores.** I\(_{\text{CRAC}}\) currents can be activated in cells without PLC activation as a result of intracellular Ca\(^{2+}\) store depletion following exposure to Ca\(^{2+}\)-ATPase inhibitor Tg or intracellular Ca\(^{2+}\) chelators BAPTA and EGTA (4, 5). The experiments described in the previous section support the \(I_{\text{min}}\)-IP\(_3\)-R-PIP\(_2\) coupling model (17). This model explains activation of \(I_{\text{min}}\) channels by direct action of PLC but not the activation of I\(_{\text{CRAC}}\) channels by Ca\(^{2+}\) store depletion.

**Fig. 3.** Conductance properties of store-operated channels in A431 cells. (a) Store-operated channels in A431 cells, activated by the mixture of 100 \(\mu\)M BAPTA-AM and 1 \(\mu\)M Tg in the bath solution, were recorded in c/a mode with 105 mM Ba\(^{2+}\) (Left), 105 mM Ca\(^{2+}\) (Center), and 140 mM Na\(^{+}\) (Right) in the pipette solution at membrane potential as indicated. (b) Fit to the unitary current-voltage relationship of store-operated channels with Ba\(^{2+}\) and Ba\(^{2+}\) as a current carrier were averaged at each membrane potential (\(V\)) (b) and (c) The average values are shown as mean ± SEM, unless the size of the error bars is smaller than the size of the symbols.

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Tg and BAPTA-AM (Fig. 2 b and c) reinforces the idea that \( I_{\text{min}} \) and \( I_{\text{CRAC}} \) may in fact be the same channels (17). To test this idea further and in the absence of molecular information and specific blockers, we resorted to comparison of \( I_{\text{min}} \) and \( I_{\text{CRAC}} \) single-channel properties. The divalent single-channel conductance of \( I_{\text{CRAC}} \) channels in Jurkat T cells has been estimated to be 24 fS from the noise analysis (6), and the monovalent single-channel conductance has been measured at 40 pS with \( \text{Na}^+ \) as a current carrier (7). It has also been demonstrated that the permeability of \( I_{\text{CRAC}} \) to \( \text{Ca}^{2+} \) is higher than for \( \text{Ba}^{2+} \) (6, 27). With 105 mM divalent cations in the pipette, the store-operated channels in A431 cells were equally permeable to \( \text{Ca}^{2+} \) and \( \text{Ba}^{2+} \) (Fig. 3), displayed a single-channel current amplitude of \(-0.18\) pA at \(-70\) mV membrane potential (Figs. 2 and 3) and a single-channel conductance of about 1 pS (Fig. 3). Thus, conductance properties of these channels are identical to the properties of \( I_{\text{min}} \) channels activated by UTP (in c/a) or by IP\(_3\) (in i/o) (12). We also demonstrated that the open probability of store-operated channels in A431 cells is strongly dependent on the membrane potential (Fig. 3), in line with the properties of \( I_{\text{min}} \) (12). Using 140 mM \( \text{Na}^+ \) as a current carrier, we determined that store-depletion activated channels in A431 cells displayed the main conductance level of \(-0.56\) pA at \(-70\) mV membrane potential and the corresponding single channel conductance of 6 pS (Fig. 3), which is several-fold smaller than conductance of \( I_{\text{CRAC}} \) channels in Jurkat T cells in similar ionic conditions (7). From these results, we concluded that the store-depletion activated \( \text{Ca}^{2+} \) current in A431 is carried by \( I_{\text{CRAC}} \)-like (\( I_{\text{CRACL}} \)) channels, which are identical to the previously described \( I_{\text{min}} \) channels (12). In the remaining section of the paper, these channels will be referred to simply as \( I_{\text{CRACL}} \).

**PIP2 Is a Modulator of \( I_{\text{CRACL}} \).** When activated by UTP (Fig. 1b) or Tg (Fig. 4b), \( I_{\text{CRACL}} \) channel activity was transient, with channels typically lasting between 2 and 5 min. Loading of A431 cells with BAPTA-AM dramatically extended the period of Tg-induced

![Fig. 4. Role of PIP2 in \( I_{\text{CRACL}} \) modulation. (a) Plot of \( I_{\text{CRACL}} \) open channel probability (NPo) in c/a patch recorded with 1 \( \mu \text{M} \) Tg in the bath and in i/o patch from the same cell in the presence of 2.5 \( \mu \text{M} \) IP\(_3\). The NPo was averaged over 1-s intervals and plotted vs. time in the experiment. Mean N Po max 30 was 0.07 in c/a and 0.66 in i/o for the experiment shown. Data are representative of nine experiments. (b) Same plot as in a for the experiment with 1 \( \mu \text{M} \) Tg in the pipette. Mean N Po max 30 was 1.62 in c/a and 0.09 in i/o for the experiment shown. Data are representative of 20 experiments. (c) Same plot as in a for the experiment with 100 \( \mu \text{M} \) BAPTA-AM and 1 \( \mu \text{M} \) Tg in the bath. Mean N Po max 30 was 0.48 in c/a and 0 in i/o for the experiment shown. Data are representative of nine experiments. (d) Same as in b, but with patch excision within 30 s after \( I_{\text{CRACL}} \) activation. Data are representative of four experiments. (e) \( I_{\text{CRACL}} \) channel current traces in c/a patches recorded in the presence of 1 \( \mu \text{M} \) Tg in the pipette solution followed by i/o current recordings in the presence of 2.5 \( \mu \text{M} \) IP\(_3\) and PIP2Ab as shown. Data are representative of seven experiments. (f) Same as in e with the order of PIP2Ab and IP\(_3\) additions to i/o patch reversed. Data are representative of five experiments. (h) The summary plot of \( I_{\text{CRACL}} \) open channel probability in c/a (open bars) and i/o (closed bars) recordings performed in the presence of 1 \( \mu \text{M} \) Tg in the bath (n = 9, left) or in the presence of 1 \( \mu \text{M} \) Tg in the pipette (n = 20, right). \( I_{\text{CRACL}} \) activity is represented as N Po max 30 (mean ± SEM).
ICRACL activity, effectively preventing ICRACL inactivation (Fig. 4c). Thus, we concluded that the Ca^{2+}-dependent mechanism plays a major role in ICRACL inactivation, similar to the previous studies of ICRACL (27, 28). To get additional insight into the mechanisms of ICRACL inactivation, we evaluated responses of ICRACL channels to IP₃ in i/o patches. With 1 μM Tg in the bath, normal activation of ICRACL channels by 2.5 μM IP₃ was observed in i/o patches (Fig. 4a and h), similar to control experiments (Fig. 1d). However, exposure to 1 μM Tg in the pipette, which initially resulted in ICRACL activation, eventually led to channel inactivation and greatly diminished activity of IP₃-gated ICRACL channels in i/o mode (Fig. 4b). On average, ICRACL channel activity in i/o patches with Tg in the pipette was reduced to Nₚₒ-max⁴ₐₜ equal to 0.11 ± 0.03 (n = 20) (Fig. 4b). Tg-induced loss of ICRACL channel sensitivity to activation by IP₃ developed in time. Indeed, when patches were excised within 30 s from the initial channel activation, substantial ICRACL channel activity in i/o patches was initially observed in the presence of 2.5 μM IP₃ in 1 of 4 experiments (Fig. 4d). Although loading the cells with BAPTA-AM almost completely removed ICRACL inactivation in c/a mode (Fig. 4e), the channels in these experiments were also unresponsive to IP₃ in i/o mode (Fig. 4c). Thus, following exposure to Tg, patch excision lead to a loss of ICRACL responsiveness to IP₃, even in the absence of Ca^{2+}-dependent inactivation.

Inclusion of UTP in the pipette resulted in sensitization of ICRACL channels to IP₃ (Fig. 1c and d), which we concluded was related to PLC-dependent reduction in IP₃ levels in the patch (see above). What if depletion of Ca^{2+} stores, which leads to a loss of ICRACL sensitivity to IP₃ in i/o patches (Fig. 4b–d and h), increases the fraction of IP₃-tethered IP₃R-ICRACL complexes? To test this hypothesis, we analyzed the effect of PIP2Ab on ICRACL in i/o patches taken from cells exposed to Tg in the pipette or to the BAPTA-AM/Tg mixture in the bath. Although ICRACL was rendered sensitive to IP₃ as a result of prolonged patch exposure to Tg, addition of PIP2Ab restored ICRACL channel activity (Fig. 4e), with Nₚₒ-max⁴ₐₜ = 2.73 ± 0.3 (n = 7) (Fig. 4h). Similar results were obtained in the experiments (n = 4) where ICRACL channels were initially activated by a BAPTA-AM/Tg mixture in the bath (Fig. 4f). The observed effect was specific for PIP2Ab, as addition of PIP2Ab had no effect on ICRACL channel activity in control experiments (n = 5). Similar to our previous results (17), PIP2Ab alone did not induce channel activity in these conditions, but instead greatly potentiated the ability of IP₃ to activate the ICRACL (Fig. 4g). The experiments with PIP2Ab support the hypothesis that, following exposure to Tg and store-depletion, all ICRACL-IP₃R complexes in the patch are shifted to the IP₃-tethered state. In the absence of Ca^{2+}-induced inactivation, ICRACL channels in ICRACL-IP₃R-PIP2 complexes remain active as long as store is depleted but do not respond to IP₃.

Discussion

PLC-Dependent and Store-Operated Pathways of ICRACL Activation. Our results lead us to conclude that both PLC-linked and Ca^{2+}-store-operated Ca^{2+} entry pathways in A431 cells are in fact supported by the same Ca^{2+}-channel, with single-channel properties identical to the properties of the previously described Imin channel (12). Similar to Imin, the store-operated channels in A431 cells are equally permeable to Ca^{2+} and Ba^{2+} and display a divalent single channel conductance of 1 pS. Monovalent single-channel conductance of these channels is 5.5–6 pS with 140 mM Na^{+} as a current carrier, which is several-fold smaller than single-channel conductance of ICRACL channels in Jurkat T cells measured in similar ionic conditions (4μM pS) (7). To account for the observed differences in conductance and selectivity properties, we called the store-operated channel in A431 cells ICRACL (ICRACL-like). Ca^{2+} channels activated by depletion of intracellular stores in A431 cells were previously described (29). However, these channels are clearly distinct from ICRACL as they display higher permeability to Ba^{2+} than to Ca^{2+} (16 pS at 160 mM Ba^{2+} and 2 pS at 200 mM Ca^{2+}), not permeable to Na^{+}, not voltage-dependent, and do not respond to IP₃ in i/o patches (29). Therefore, these channels constitute an alternative depletion-activated Ca^{2+} influx pathway in A431 cells. We have not observed channels described by Luckhoff and Clapham (29) in our experiments, most likely because of variability between different A431 clones or effects of culture conditions on channel expression. In some patches on A431 cells, we observed nonselective cation permeable channels with large conductance, which were clearly distinct from the ICRACL. These channels did not respond to IP₃ or Tg, and the patches containing these channels were discarded.

What is a mechanism of ICRACL activation? From the present results and from our previous work on Imin, we conclude that ICRACL channels in A431 cells are conformationally coupled to intracellular IP₃R and can be activated: (i) by changes in the IP₃R receptor conformation on IP₃ binding (16); (ii) by direct cleavage of ICRACL-IP₃R-tethered PI/P by PLC (17); and (iii) by the store-operated mechanism as in the conformational coupling mechanism originally proposed by Irvine (ref. 30) (present results). Gating of ICRACL-IP₃R complexes by IP₃ probably accounts for the low background channel activity in resting cells (Fig. 1a) (endogenous IP₃ level is estimated at 40–100 nM in unstimulated cells; ref. 31), and for the substantial activity of ICRACL channels in excised patches in the

Fig. 5. Model of ICRACL, conformational coupling to IP₃R and modulation by PIP2. ICRACL-IP₃R and ICRACL-IP₃R-PIP2 complexes exist in equilibrium in resting cells (Left). Background ICRACL channel activity in cell-attached patches in resting cells results from endogenous IP₃ (40–100 nM) (31) activating ICRACL-IP₃R complexes. Exposure of patches excised from the resting cells to 2.5 μM IP₃ leads to elevated ICRACL channel activity in i/o configuration (Right). Exposure to UTP in the pipette triggers cleavage of IP₃R-tethered PIP2 and direct activation of ICRACL-IP₃R-PIP2 complexes in cell-attached patches as previously proposed (17). The shift from ICRACL-IP₃R-PIP2 to ICRACL-IP₃R complexes (Top) explains high activity of ICRACL channels in i/o patches in the presence of 2.5 μM IP₃ (Top Right). Exposure to Tg in the pipette or to BAPTA-AM/Tg in the bath causes depletion of local Ca^{2+} stores and activation of ICRACL channels by means of conformational coupling mechanism (30). Depletion of Ca^{2+} stores by some unknown mechanism promotes formation of ICRACL-IP₃R-PIP2 complexes (Bottom), which leads to the loss of ICRACL sensitivity to activation by 2.5 μM IP₃ in excised patches (Bottom Right). Despite loss of sensitivity to activation by IP₃, ICRACL channels in ICRACL-IP₃R-PIP2 complexes remain active as long as stores are depleted and ICRACL inactivation is prevented by chelating Ca^{2+} with BAPTA. The model drawing is adapted from ref. 47.
presence of 2.5 μM IP$_3$ (Fig. 1a). Cleavage of IP$_3$-R-tethered PIP$_2$ by PLC is likely to be responsible for activation of ICRACL by UTP in the pipette in our experiments (Fig. 1b). The activation of ICRACL by UTP in the pipette (Fig. 2b) and by BAPTA-AM/Tg in the bath (Fig. 2c) results from the IP$_3$R conformational changes on intracellular Ca$^{2+}$ store depletion. In physiological conditions, stimulation of cells by agonist leads to PLC activation, increase in IP$_3$ levels, and depletion of Ca$^{2+}$ stores. Therefore, an additive or even synergistic action of three different pathways of ICRACL activation in cells is expected in response to application of agonist in situ. Similar to ICRACL (27, 28), ICRACL channels are under strong negative inhibitory control by cytosolic Ca$^{2+}$, which normally leads to a transient nature of ICRACL activity (Figs. 1b and 4b). Loading A431 cells with BAPTA removes Ca$^{2+}$-dependent inactivation and dramatically increases the duration of ICRACL activity (Fig. 4c).

Role of PIP$_2$ as a Modulator of ICRACL Channels. Our data also suggest that PIP$_2$ may play a role of ICRACL modulator by regulating a dynamic equilibrium between ICRACL-IP$_3$R and ICRACL-IP$_3$R-PIP$_2$ complexes (Fig. 5 Left). Following exposure to UTP, activation of PLC and cleavage of PIP$_2$ in the patch, the majority of ICRACL channels are shifted to PIP$_2$-free ICRACL-IP$_3$R state (Fig. 5 Top), as manifested by NP$_{max,30}$ = 3 in i/o patches with 2.5 μM IP$_3$ in these experiments (Fig. 5 Top Right) compared with NP$_{max,30}$ = 0.86 in control patches (Fig. 5 Right). Depletion of the stores with Tg or BAPTA appears to shift the equilibrium in the opposite direction, with all of ICRACL channels driven to ICRACL-IP$_3$R-PIP$_2$ complexes (Fig. 5 Bottom). ICRACL channels in these experiments were unresponsive to IP$_3$ in i/o patches with NP$_{max,30}$ = 0.1 (Fig. 5 Bottom Right) but responded essentially at the maximal level (NP$_{max,30}$ = 2.7) to a combination of 2.5 μM IP$_3$ and PIP$_2$Ab (Fig. 4d). Despite loss of sensitivity to activation by IP$_3$$_3$, ICRACL channels in ICRACL-IP$_3$R-PIP$_2$ complexes remain active in c/o mode (but not in i/o mode, for reasons that need to be further investigated) as long as stores are depleted and ICRACL inactivation is prevented by chelating Ca$^{2+}$ (Fig. 4c). Possible mechanisms responsible for the store-dependent shift toward a PIP$_2$-occupied state of the IP$_3$R may include physical rearrangement of mobile Ca$^{2+}$ stores (32), changes in local IP$_3$ levels in the patch (33), or an increase in IP$_3$R affinity for PIP$_2$ following Ca$^{2+}$ stores depletion. Additional experiments will be needed to discriminate between these possibilities.

Conformational Coupling Model of ICRACL Activation. ICRACL-IP$_3$R association is likely to involve direct binding of the IP$_3$R amino-terminal region to the ICRACL protein, similar to mTrp-IP$_3$R association (21). Interestingly, the same amino-terminal region of IP$_3$R also includes specific IP$_3$ (34, 35) and PIP$_2$ (36) binding sites. Thus, ligand-induced conformational changes of the IP$_3$R amino-terminal region can be transmitted directly to the ICRACL channel. The store-operated ICRACL activation is likely to involve an IP$_3$R-associated endoplasmic reticulum resident Ca$^{2+}$-binding protein, such as calreticulin (37–39), which serves as a sensor of intraluminal Ca$^{2+}$. Additional signaling components are likely to be recruited to the ICRACL-IP$_3$R complex via actions of a modular adaptor protein, such as mGlur1/IP$_3$R-binding protein Homer in neuronal cells (40), the Syk/Btk/Grb2/PLC- binding protein BLNK in B lymphocytes (41), or the trp/PKC/PLC-binding protein inad in Drosophila photoreceptors (42). The actin cytoskeleton may also play an important role in correct spatial arrangement of required signaling components (43–45). In chicken B lymphocytes, removal of all three IP$_3$R isoforms by genetic means had no effect on Tg-induced Ca$^{2+}$ influx (46), in apparent conflict with the conformational coupling model of ICRACL activation in A431 cells (Fig. 5). From these results, we conclude that the B lymphocytes must have an additional or alternative Ca$^{2+}$ influx pathway, coupled to Ca$^{2+}$ store depletion by means of IP$_3$R-independent mechanism that may involve a global “diffusible messenger.” Additional functional studies with B lymphocytes will be required for its detailed characterization.

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