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}) and intracellular inositol 1,4,5-bisphosphate (PIP_{2}). The observed channels display the same properties as I_{min} channels, but are not available to activation by IP_{3} or anti-PIP_{2} 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 is activated by the combined action of IP_{3} and anti-PIP_{2} 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)-triphosphate (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} appear 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_{3R}) (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 activation of I_{min} in i/o patches is potentiated by addition of IP_{3}-enriched microsomes as predicted by an I_{min}-IP_{3} conformational coupling model (16). More recently, we discovered that anti-PIP_{2} antibody (PIP_{2}Ab) sensitizes I_{min} to IP_{3} activation and proposed an I_{min}-IP_{3}-PIP_{2} functional coupling model based on these findings (17). In parallel with our results, a potential role of PIP_{2} in trp-like (trpl) channel activation has been recently demonstrated in Sf9 cells (23). The I_{min}-IP_{3}-PIP_{2} 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 A341 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 PIP_{2} 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,

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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_{3R}, IP_{3} receptor; PIP_{2}, phosphatidylinositol (1,4,5)-bisphosphate; 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.

NP_{\text{o}} was determined by using the following equation: NP_{\text{o}} = \langle I \rangle / i, where \langle I \rangle and \text{i} are the mean channel current and unitary current amplitude, respectively. \langle I \rangle was estimated from the time integral of the current above the baseline, and \text{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 NP_{\text{o}} collected during 30 s of maximal activity (NP_{\text{o,max}}^{30}) as a standard way to compare open channel probability among different experiments. Average NP_{\text{o,max}}^{30} 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 BaCl_{2}, 105 CaCl_{2} or 140 NaCl as indicated, and 10 Tris/HCl (pH 7.4). In cell-attached experiments, the bath solution contained 140 mM KCl and 2 mM CaCl_{2} to nullify the cell’s resting potential. For BAPTA-AM experiments, the bath solution contained 140 mM KCl and 2 mM CaCl_{2} to nullify the cell’s resting potential. For BAPTA-AM loading, 100 μM BAPTA-AM and 1 μM Tg were added to the bath solution containing (in mM): 140 KCl, 5 NaCl, 10 Hepes/KOH, and 2 EGTA (pH 7.4). For i/o experiments, patches were excised into the standard intracellular solution containing (in mM): 140 K glutamate, 5 NaCl, 1 MgCl_{2}, 10 Hepes/KOH, 1.13 CaCl_{2}, and 2 EGTA (pCa 7, pH 7.4), with or without IP_{3} 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-PIP_{2} antibody (PIP_{2}Ab) (25) was from PerSeptive Biosystems (Framingham, MA), and monoclonal anti-PIP antibody (PIPAb) was from Assay Designs (Ann Arbor, MI). PIP_{2}Ab 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). IP_{3} and BAPTA-AM were from Calbiochem.

Results

Exposure to Extracellular UTP Sensitizes I_{\text{min}} to IP_{3} Activation. When cell-attached (c/a) recordings of I_{\text{min}} in A431 cells were performed in control recording conditions, the channel activity was very low with NP_{\text{o,max}}^{30} equal to 0.08 ± 0.06 (n = 12) (Fig. 1 a and c). After patch excision in bath solution containing 2.5 μM IP_{3}, moderate activity of I_{\text{min}} in i/o patches was observed with NP_{\text{o,max}}^{30} equal to 0.86 ± 0.2 (n = 12) (Fig. 1 a and c). Similar behavior of I_{\text{min}} channels in c/a and i/o configurations has been described (10–12, 17). As we previously reported, addition of 100 μM UTP or 10 μM bradykinin to the solution bathing A431 cells leads to activation of PLC-coupled receptors and an increase in I_{\text{min}} activity in control recording conditions, sensitivity of I_{\text{min}} activation by IP_{3} when 100 μM of UTP was included in the pipette solution. In all experiments of this series, we waited until I_{\text{min}} activity in c/a patches subsided before the patch excision. A single IP_{3} concentration in the 0.05–2.5 μM range was tested in each experiment to avoid IP_{3}-induced I_{\text{min}} desensitization (12). Fitting the Hill equation to the data (Fig. 1d, ▲) yielded an apparent affinity (K_{app}) of 0.15 μM IP_{3}, maximal NP_{\text{o,max}}^{30} (NP_{\text{o,max}}) of 3.33, and a Hill coefficient (n_{H}) of 0.83 (Fig. 1d, curve). When similar experiments were performed in control recording conditions, sensitivity of I_{\text{min}} to IP_{3} activation was much lower (K_{app} = 0.51 μM IP_{3}, NP_{\text{o,max}} = 0.87, n_{H} = 0.95) (17) (Fig. 1d, ▼, and dashed line on the right). The dramatic increase in I_{\text{min}} apparent affinity for IP_{3} and in NP_{\text{o,max}} induced by exposure to UTP in the pipette

Kaznacheyeva et al.  
PNAS | January 2, 2001 | vol. 98 | no. 1 | 149
Ca$_2$P2 levels leads to an increase in the apparent affinity of IP$_3$R by direct action of PLC but not the activation of ICRAC coupling model (17). This model explains activation of I$_{\text{min}}$ as a result of intracellular Ca$_2$.

Activation of I$_{\text{min}}$ by depletion of intracellular Ca$_2$. 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 mM Tg to the bath had only minimal effect on I$_{\text{min}}$ activity when compared with control conditions, with NP$_{\text{max at 0}}$ equal to 0.11 ± 0.03 (n = 9) (Fig. 2a; also see Fig. 4a and h). In contrast to these results, if 1 mM Tg was included in the pipette, active I$_{\text{min}}$ channels were observed following a short delay after patch formation, with NP$_{\text{max at 0}}$ 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$_2$-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) 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

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**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 mM 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 in a with 1 mM Tg in the pipette. (c) Same as in a with 100 μM BAPTA-AM and 1 mM Tg in the bath.

Quantitatively matches with the effects exerted by PIP$_2$Ab on I$_{\text{min}}$ (K$_{\text{app}}$ = 0.08 μM IP$_3$, NP$_{\text{max at 0}}$ = 3.21, n$_H$ = of 0.8) (17) (Fig. 1d, o, 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 ICRAC 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. 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 μM Tg to the bath had only minimal effect on I$_{\text{min}}$ activity when compared with control conditions, with NP$_{\text{max at 0}}$ equal to 0.11 ± 0.03 (n = 9) (Fig. 2a; also see Fig. 4a and h). In contrast to these results, if 1 μM Tg was included in the pipette, active I$_{\text{min}}$ channels were observed following a short delay after patch formation, with NP$_{\text{max at 0}}$ 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.

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Activation of I$_{\text{min}}$ by depletion of intracellular Ca$_2$ stores with

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**Fig. 3.** Conductance properties of store-operated channels in A431 cells. (a) Store-operated channels in A431 cells, activated by the mixture of 100 μM BAPTA-AM and 1 mM 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$_{2+}$ (v, n = 4–6), Ca$_{2+}$ (u, n = 4), Na$_{2+}$ (w, n = 3) yielded slope single-channel conductance of 1 pS for Ca$_{2+}$ and Ba$_{2+}$ and 6 pS for Na$. (c) Open channel probability of store-operated channels (NP$_{\text{max at 0}}$) expressed as a function of membrane potential. Data from six independent experiments in c/a mode with 105 mM Ba$^{2+}$ as a current carrier were averaged at each membrane potential (w). (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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Kaznacheyeva et al.
Tg and BAPTA-AM (Fig. 2 b and c) reinforces the idea that Imin and ICRAC 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 Imin and ICRAC single-channel properties. The divalent single-channel conductance of ICRAC 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 Na\(^{+}\) as a current carrier (7). It has also been demonstrated that the permeability of ICRAC to Ca\(^{2+}\) is higher than for Ba\(^{2+}\) (6, 27). With 105 mM divalent cations in the pipette, the store-operated channels in A431 cells were equally permeable to Ca\(^{2+}\) and 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 Imin channels activated by UTP (in c) or by IP\(_3\) (in i) (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 Imin (12). Using 140 mM 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 ICRAC channels in Jurkat T cells in similar ionic conditions (7). From these results, we concluded that the store-depletion activated Ca\(^{2+}\) current in A431 is carried by ICRAC-like (ICRACL) channels, which are identical to the previously described Imin channels (12). In the remaining section of the paper, these channels will be referred to simply as ICRACL.

**PPIP2 Is a Modulator of ICRACL.** When activated by UTP (Fig. 1 b) or Tg (Fig. 4 b), ICRACL 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 activity of ICRACL. (a) Plot of ICRACL open channel probability (NP\(_{\text{max}}\)) in c/a patch recorded with 1 \(\mu\)M Tg in the bath and in i/o patch from the same cell in the presence of 2.5 \(\mu\)M IP\(_3\). The NP\(_{\text{max}}\) was averaged over 1-s intervals and plotted vs. time in the experiment. Mean NP\(_{\text{max}}\) 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 \(\mu\)M Tg in the pipette. Mean NP\(_{\text{max}}\) 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 \(\mu\)M BAPTA-AM and 1 \(\mu\)M Tg in the bath. Mean NP\(_{\text{max}}\) was 0.48 in c/a and 0 in i/o for the experiment shown. Data are representative of nine experiments. (d) Same plot as in b, but with patch excision within 30 s after ICRACL activation. Data are representative of four experiments. (e) ICRACL channel current traces in c/a patches recorded in the presence of 1 \(\mu\)M Tg in the pipette solution followed by i/o current recordings in the presence of 2.5 \(\mu\)M IP\(_3\) and PIP\(_2\)-Ab as shown. Data are representative of seven experiments. (f) Same as in e with the order of PIP\(_2\)-Ab and IP\(_3\) additions to i/o patch reversed. Data are representative of five experiments. (g) The summary plot of ICRACL open channel probability in c/a (open bars) and i/o (closed bars) recordings performed in the presence of 1 \(\mu\)M Tg in the bath (n = 9, left) or in the presence of 1 \(\mu\)M Tg in the pipette (n = 20, right). ICRACL activity is represented as NP\(_{\text{max}}\) (mean ± SEM).
ICRACL activity, effectively preventing ICRACL inactivation (Fig. 4c). Thus, we concluded that the \( \text{Ca}^{2+} \)-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 \( \mu \text{M} \) Tg in the bath, normal activation of ICRACL channels by 2.5 \( \mu \text{M} \) IP3 was observed in i/o patches (Fig. 4a and h), similar to control experiments (Fig. 1a). However, exposure to 1 \( \mu \text{M} \) Tg in the pipette, which initially resulted in ICRACL activation, eventually led to channel inactivation and greatly diminished activity of IP3-transfected 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 \( \text{NP}_{\text{max}}^{\text{o}} \) equal to 0.11 \( \pm \) 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 \( \mu \text{M} \) 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. 4c), 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 \( \text{Ca}^{2+} \)-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 IP2 levels in the patch (see above). What if depletion of \( \text{Ca}^{2+} \) stores, which leads to a loss of ICRACL sensitivity to IP3 in i/o patches (Fig. 4b–d and h), increases the fraction of IP2-tethered IP3R-ICRACL complexes? To test this hypothesis, we analyzed the effect of IP2Ab 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 IP2Ab restored ICRACL channel activity (Fig. 4e), with \( \text{NP}_{\text{max}}^{\text{o}} \) equal to 2.73 \( \pm \) 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 IP2Ab, as addition of IP2Ab had no effect on ICRACL channel activity in control experiments (\( n = 5 \)). Similar to our previous results (17), IP3RAb 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 IP2Ab 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 \( \text{Ca}^{2+} \)-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 \( \text{Ca}^{2+} \)-store-operated \( \text{Ca}^{2+} \) entry pathways in A431 cells are in fact supported by the same \( \text{Ca}^{2+} \) 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 \( \text{Ca}^{2+} \) and \( \text{Ba}^{2+} \) 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 (4f pS) (7). To account for the observed differences in conductance and selectivity properties, we called the store-operated channel in A431 cells ICRACL (Imin-like). \( \text{Ca}^{2+} \) 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 \( \text{Ba}^{2+} \) than to \( \text{Ca}^{2+} \) (16 pS at 160 mM \( \text{Ba}^{2+} \) and 2 pS at 200 mM \( \text{Ca}^{2+} \)). 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 \( \text{Ca}^{2+} \) 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 PIP2 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...
presence of 2.5 μM IP$_3$ (Fig. 1a). Cleavage of IP$_3$-tethered PIP$_2$ by PLC is likely to be responsible for activation of ICRACL channels by UTP in the pipette in our experiments (Fig. 1b). The activation of ICRACL channels by Tg 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$_3$, 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 PIP$_2$ levels in the patch (33), or an increase in IP$_3$,R affinity for IP$_3$ 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/PLCγ-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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