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

Elena Kaznacheyeva*, Alexander Zubov*, Konstantin Gusev*, Ilya Bezprozvanny†, and Galina N. Mozhaeva*‡

*Institute of Cytology RAS, 4 Tikhoretsky Avenue, St. Petersburg 194064, Russia; and †Department of Physiology, University of Texas Southwestern Medical Center, Dallas, TX 75390

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Activation of phospholipase C in nonexcitable cells causes the release of calcium (Ca²⁺) from intracellular stores and activation of Ca²⁺ influx by means of Ca²⁺ release-activated channels (ICRAC) 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 Ca²⁺ channels in human carcinoma A431 cells, which can be activated by extracellular UTP, by depletion of intracellular Ca²⁺ stores after exposure to the Ca²⁺-pump inhibitor thapsigargin, or by loading the cells with Ca²⁺ 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 ICRAC channels. Thus, we concluded that the depletion-activated Ca²⁺ current in A431 cells is supported by I CRAC-like (ICRAC) channels, identical to I min. We further demonstrated synergism in activation of ICRAC Ca²⁺ channels by extracellular UTP and intracellular inositol (1,4,5)-triphosphate (IP₃), apparently because of reduction in phosphatidylinositol 4,5-bisphosphate (PIP₂) levels in the patch. Prolonged exposure of patches to thapsigargin renders ICRAC Ca²⁺ channels unresponsive to IP₃ but still available to activation by the combined action of IP₃ and anti-PIP₂ antibody. Based on these data, we concluded that phospholipase C-mediated and store-operated Ca²⁺ influx pathways in A431 cells converge on the same ICRAC Ca²⁺ channel, which can be modulated by PIP₂.

Activation of phospholipase C (PLC)-mediated signaling pathways in nonexcitable cells causes the release of Ca²⁺ from intracellular Ca²⁺ stores and activation of Ca²⁺ influx across the plasma membrane by means of capacitative Ca²⁺ entry or store-operated Ca²⁺ entry processes (1–3). These processes are mediated by plasma membrane Ca²⁺ channels termed “Ca²⁺ release activated channels” (ICRAC) (4–7). The molecular identity of ICRAC 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 ICRAC, 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 Ca²⁺ 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 (IP₃) to excised inside-out (i/o) patches (10–12). IP₃-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 ICRAC channels (12, 16). Thus, we previously suggested that I min and ICRAC may in fact be the same channels (17).

The mechanism of ICRAC activation remains similarly controversial (1–3). When studied in a heterologous expression system, activation of mTrp channels by IP₃ 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 IP₃ receptor (IP₃R) (18–21). However, whether mTrp can serve as an appropriate model system for understanding ICRAC 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 IP₃R-enriched microsomes as predicted by an I min-IP₃R conformational coupling model (16). More recently, we discovered that anti-PIP₂ antibody (PIP₂Ab) sensitizes I min to IP₃ activation and proposed an I min-IP₃-R-PIP₂ functional coupling model based on these findings (17). In parallel with our results, a potential role of PIP₂ in trp-like (trpl) channel activation has been recently demonstrated in Sf9 cells (23). The I min-IP₃-R-PIP₂ coupling model can adequately explain activation of I min channels by direct action of PLC but not the activation of ICRAC channels resulting from Ca²⁺ store depletion (4–6).

A number of critical questions related to a depletion-activated Ca²⁺ 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, Ca²⁺ pump inhibitor thapsigargin (Tg), and Ca²⁺ chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'′-tetraacetate (BAPTA) on plasma membrane Ca²⁺ channel activity in patch-clamp experiments performed with human carcinoma A431 cells. We conclude that PLC activation and depletion of intracellular Ca²⁺ stores activate the same Ca²⁺ 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 ICRAC channels described in Jurkat T cells (5–7). Thus, we will refer to store-operated channels in A431 cells as I CRAC (“crac-like”). We also concluded that PIP₂ plays a role in modulation of ICRAC 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; ICRAC, Ca²⁺ release-activated channel; ICRAC-like; IP₃, inositol 1,4,5-triphosphate; IP₃R, IP₃ receptor; PIP₂, 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.

To whom reprint requests should be addressed. E-mail: gnmozh@link.cytspb.rssi.ru.

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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 \( N_{\text{Pomax30}} \) values of 0.08 ± 0.06 (n = 12) (Fig. 1 a and c). After patch excision in bath solution containing 2.5 \( \mu \)M IP\(_3\), moderate activity of \( I_{\text{min}} \) in i/o patches was observed with \( N_{\text{Pomax30}} \) 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 \( \mu \)M UTP or 10 \( \mu \)M Bradykinin to the bath solution bathing A431 cells leads to activation of PLC-coupled receptors and an increase in \( I_{\text{min}} \) activity in c/a patches to \( N_{\text{Pomax30}} \) of 0.7–1.0 (12). When 100 \( \mu \)M UTP was included in the pipette solution, significantly higher \( I_{\text{min}} \) channel activity was observed with \( N_{\text{Pomax30}} \) equal to 1.5 ± 0.17 (n = 33) (Fig. 1b). With either bath (12) or pipette (Fig. 1b) UTP application, activity of \( I_{\text{min}} \) was transient and resulted in channel inactivation within several minutes. After patch excision into intracellular solution containing 2.5 \( \mu \)M IP\(_3\), very high levels of \( I_{\text{min}} \) channel activity were observed (Fig. 1b). On an average, \( I_{\text{min}} \) channel \( N_{\text{Pomax30}} \) 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 \( I_{\text{min}} \) channels in i/o recordings observed in the experiments with UTP in the pipette (Fig. 1 b and c), we determined the sensitivity of \( I_{\text{min}} \) activation by IP\(_3\) when 100 \( \mu \)M 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 \( \mu \)M 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, a) yielded an apparent affinity (\( K_{\text{app}} \)) of 0.15 \( \mu \)M IP\(_3\), maximal \( N_{\text{Pomax30}} \) 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 (\( K_{\text{app}} \) = 0.51 \( \mu \)M IP\(_3\)), \( N_{\text{Pomax}} \) = 0.87, n\(_H\) = 1.05 (17) (Fig. 1d, c, and dashed line on the right). The dramatic increase in \( I_{\text{min}} \) apparent affinity for IP\(_3\) and in \( N_{\text{Pomax}} \) induced by exposure to UTP in the pipette.

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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$_{\text{max},30}$ equal to 0.11 ± 0.03 (n = 9) (Fig. 2a; also see Fig. 4 a 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$_{\text{max},30}$ equal to 1.7 ± 0.24 (n = 18) (Figs. 2b and 4 b 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) 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

quantitatively matches with the effects exerted by PIP$_2$Ab on I$\text{min}$ ($K_{\text{app}} = 0.08$ μM IP$_3$, NP$_{\text{max}} = 3.21, n_H = 0.8$) (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 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$\text{CRAC}$

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

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 μM 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$^{2+}$ (•, n = 4–6), Ca$^{2+}$ (○, n = 4–6), Na$^+$ (□, 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$_{\text{max},30}$) 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.
Tg and BAPTA-AM (Fig. 2 b and c) reinforces the idea that I_{min} and I_{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_{min} and I_{CRAC} single-channel properties. The divalent single-channel conductance of I_{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 Na\(^{+}\) as a current carrier (7). It has also been demonstrated that the permeability of I_{CRAC} 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 I_{min} channels activated by UTP (in c\(\alpha\)) or by IP\(_{3}\) (in i\(\alpha\)) (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_{min} (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 I_{CRAC} 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 I_{CRAC}-like (I_{CRACL}) channels, which are identical to the previously described I_{min} channels (12). In the remaining section of the paper, these channels will be referred to simply as I_{CRACL}.

**PIP\(_{2}\) Is a Modulator of I_{CRACL}**. When activated by UTP (Fig. 1b) or Tg (Fig. 4b), I_{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

![Image](https://example.com/image.png)

**Fig. 4.** Role of PIP\(_{2}\) in I_{CRACL} modulation. (a) Plot of I_{CRACL} open channel probability (NP_0) 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_0 was averaged over 1-s intervals and plotted vs. time in the experiment. Mean NP_{0,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_{0,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_{0,max} was 0.48 in c/a and 0.05 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 I_{CRACL} activation. Data are representative of four experiments. (e) I_{CRACL} 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 I_{CRACL} 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). I_{CRACL} activity is represented as NP_{0,max} (mean \\pm SEM).

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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. 4 a and h), similar to control experiments (Fig. 1a). 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 NP$$^{\text{max}}_{i/o}$ equal to 0.11 ± 0.03 (n = 20) (Fig. 4b). Tg-induced loss of ICRACL channel sensitivity to activation by IP3 developed in time. Indeed, when patches were excised within 30 s from the initial channel activation, substantial ICRACL channel activity in i/o patches was initially observed in the presence of 2.5 μM IP3 in 1 of 4 experiments (Fig. 4d). Although loading the cells with BAPTA-AM almost completely removed ICRACL inactivation in c/a mode (Fig. 4e), the channels in these experiments were also unresponsive to IP3 in i/o mode (Fig. 4f). Thus, following exposure to Tg, patch excision led to a loss of ICRACL responsiveness to IP3, even in the absence of Ca$$^{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 IP2 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 IP2-tethered IP3-R-ICRACL complexes? To test this hypothesis, we analyzed the effect of PIPAb 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 PIPAb restored ICRACL channel activity (Fig. 4e), with NP$$^{\text{max}}_{i/o}$ = 2.73 ± 0.3 (n = 7) (Fig. 4h). Similar results were obtained in the experiments (n = 4) where ICRACL channels were initially activated by a BAPTA-AM/Tg mixture in the bath (Fig. 4f). The observed effect was specific for PIPAb, as addition of PIPAb had no effect on ICRACL channel activity in control experiments (n = 5). Similar to our previous results (17), PIPAb alone did not induce channel activity in these conditions, but instead greatly potentiated the ability of IP3 to activate the ICRACL (Fig. 4g). The experiments with PIPAb support the hypothesis that, following exposure to Tg and store-depletion, all ICRACL-IP3 complexes in the patch are shifted to the IP3-tethered state. In the absence of Ca$$^{2+}$$-induced inactivation, ICRACL channels in ICRACL-IP3-R-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 (40 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 nonelective 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 IP3-R receptor conformation on IP3 binding (16); (ii) by direct cleavage of ICRACL-IP3R-tethered IP2 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 IP3 (Fig. 1a). Cleavage of IP3-R-tethered PIP2 by PLC is likely to be responsible for activation of ICRACL channels by UTP in the pipette (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 IP3-R conformational changes on intracellular Ca2+ store depletion. In physiological conditions, stimulation of cells by agonist leads to PLC activation, increase in IP3 levels, and depletion of Ca2+ 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 Ca2+, which normally leads to a transient nature of ICRACL activity (Figs. 1b and 4b). Loading A431 cells with BAPTA removes Ca2+-dependent inactivation and dramatically increases the duration of ICRACL activity (Fig. 4c).

Role of PIP2 as a Modulator of ICRACL Channels. Our data also suggest that PIP2 may play a role of ICRACL modulator by regulating a dynamic equilibrium between ICRACL-IP3R and ICRACL-IP3-PIP2 complexes (Fig. 5 Left). Following exposure to UTP, activation of PLC and cleavage of PIP2 in the patch, the majority of ICRACL channels are shifted to PIP2-free ICRACL-IP3R state (Fig. 5 Top), as manifested by NP30 = 3 in i/o patches with 2.5 μM IP3 in these experiments (Fig. 5 Top Right) compared with NP30 = 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-IP3-PIP2 complexes (Fig. 5 Bottom). ICRACL channels in these experiments were unresponsive to IP3 in i/o patches with NP30 = 0.1 (Fig. 5 Bottom Right) but responded essentially at the maximal level (NP30 = 2.7) to a combination of 2.5 μM IP3 and PIP2Ab (Fig. 4d). Despite loss of sensitivity to activation by IP3, ICRACL channels in ICRACL-IP3-PIP2 complexes remain active in c/a 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 Ca2+ (Fig. 4c). Possible mechanisms responsible for the store-dependent shift toward a PIP2-occupied state of the IP3R may include physical rearrangement of mobile Ca2+ stores (32, changes in local PIP2 levels in the patch (33), or an increase in IP3-R affinity for PIP2 following Ca2+ stores depletion. Additional experiments will be needed to discriminate between these possibilities.

Conformational Coupling Model of ICRACL Activation. ICRACL-IP3R association is likely to involve direct binding of the IP3-R amino-terminal region to the ICRACL protein, similar to mTrp-IP3R association (21). Interestingly, the same amino-terminal region of IP3-R also includes specific IP3 (34, 35) and PIP2 (36) binding sites. Thus, ligand-induced conformational changes of the IP3-R amino-terminal region can be transmitted directly to the ICRACL channel. The store-operated ICRACL activation is likely to involve an IP3-R-associated endoplasmic reticulum resident Ca2+-binding protein, such as calreticulin (37–39), which serves as a sensor of intraluminal Ca2+. Additional signaling components are likely to be recruited to the ICRACL-IP3R complex via actions of a modular adapter protein, such as mGluR1/IP3-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 IP3R isoforms by genetic means had no effect on Tg-induced Ca2+ 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 Ca2+ influx pathway, coupled to Ca2+ store depletion by means of IP3-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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