Regulation of the Miniature Plasma Membrane Ca\(^{2+}\) Channel \(I_{\text{min}}\) by Inositol 1,4,5-Trisphosphate Receptors*

(Received for publication, June 22, 1999)

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\(I_{\text{min}}\) is a plasma membrane-located, Ca\(^{2+}\)-selective channel that is activated by store depletion and regulated by inositol 1,4,5-trisphosphate (IP\(_3\)). In the present work we examined the coupling between \(I_{\text{min}}\) and IP\(_3\) receptors in excised plasma membrane patches from A431 cells. \(I_{\text{min}}\) was recorded in cell-attached mode and the patches were excised into medium containing IP\(_3\). In about 50% of experiments excision caused the loss of \(I_{\text{min}}\). In the remaining patches activation of \(I_{\text{min}}\) by IP\(_3\) was lost upon extensive washes of the patch surface. The ability of IP\(_3\) to activate \(I_{\text{min}}\) was restored by treating the patches with rat cerebellar microsomes reach in IP\(_3\) receptors but not by control forebrain microsomes. The re-activated \(I_{\text{min}}\) had the same kinetic properties as \(I_{\text{min}}\) when it is activated by Ca\(^{2+}\)-mobilizing agonists in intact cells and by IP\(_3\) in excised plasma membrane patches and it was inhibited by the \(I_{\text{crac}}\) inhibitor SKF95365. We propose that \(I_{\text{min}}\) is a form of \(I_{\text{crac}}\) and is gated by IP\(_3\) receptors.

The Ca\(^{2+}\) signal evoked by agonists that stimulate phospholipase C is generated by Ca\(^{2+}\) release from intracellular stores and Ca\(^{2+}\) influx across the plasma membrane (1, 2). The two Ca\(^{2+}\) transporting events are linked through the regulation of Ca\(^{2+}\) influx by Ca\(^{2+}\) content in the stores, a gating behavior termed capacitative Ca\(^{2+}\) entry (CCE).1 In many cells CCE is manifested as Ca\(^{2+}\) release-activated Ca\(^{2+}\) current (\(I_{\text{crac}}\)). \(I_{\text{crac}}\) is distinguished by its low conductance and very high selectivity for Ca\(^{2+}\) (3–5). Store depletion by agonist or IP\(_3\)-mediated Ca\(^{2+}\) release (5), inhibition of SERCA pumps (6), and/or intracellular infusion of Ca\(^{2+}\)-chelating agents (4) can activate \(I_{\text{crac}}\).

The molecular identity of \(I_{\text{crac}}\) is not known. However, its functional characteristics began to emerge. An elegant single-channel recording of \(I_{\text{crac}}\) in Jurkat T cells estimated an \(I_{\text{crac}}\) single channel conductance of about 1 pS when transporting Ca\(^{2+}\) (7). This is remarkably similar to the conductance of a miniature, Ca\(^{2+}\)-selective channel (\(I_{\text{min}}\)) we described in several cell types (8, 9). Moreover, similar to \(I_{\text{crac}}\) \(I_{\text{min}}\) is highly selective for Ca\(^{2+}\) over K\(^{+}\) (3, 8, 9). The open probability, but not the conductance of both channels, is increased by membrane hyperpolarization (7–9). \(I_{\text{crac}}\) and \(I_{\text{min}}\) are activated by store depletion with Ca\(^{2+}\)-mobilizing agonists or thapsigargin in intact cells, and \(I_{\text{min}}\) is activated by IP\(_3\) in the same excised plasma membrane patch. Therefore studying the gating of \(I_{\text{min}}\) by IP\(_3\) and IP\(_3\)R may be relevant to understanding the gating of \(I_{\text{crac}}\).

Although widely documented (for review, see Refs. 1–3), the way \(I_{\text{min}}\) is gated by store Ca\(^{2+}\) content is not understood. The two leading hypotheses to explain gating of \(I_{\text{min}}\) by stored Ca\(^{2+}\) are the soluble messenger (10) and the conformational coupling hypothesis (2, 11). The former proposes generation of a soluble messenger, such as the Ca\(^{2+}\) influx factor (10), in response to store depletion that diffuses to the plasma membrane to activate \(I_{\text{crac}}\). The conformational coupling model proposes gating of \(I_{\text{crac}}\) by direct interaction with IP\(_3\)R. Ca\(^{2+}\) release from internal stores causes a conformational change in the IP\(_3\)R, which is transduced to and is sensed by the \(I_{\text{crac}}\) to regulate its activity (2). Additional suggestions include gating of \(I_{\text{crac}}\) by agonist-generated lipid mediators (12) or by vesicle fusion events (13). Recently we studied gating of the store-operated, human homologue of the Drosophila Trp channel, hTrp3 by IP\(_3\)R (14). We found that hTrp3 channels are gated by coupling to IP\(_3\)R (14). These findings were corroborated by studies reported as unpublished observations (15), which identified sequences in hTrp3 and IP\(_3\)R that interacts with each other to influence Ca\(^{2+}\) influx.

Building upon our studies of hTrp3 gating by IP\(_3\)R (14) and identification of \(I_{\text{min}}\) as an \(I_{\text{crac}}\)-like channel (8, 9), in the present work we studied regulation of \(I_{\text{min}}\) by IP\(_3\)R. We report that \(I_{\text{min}}\) in excised plasma membrane patches is indeed gated by IP\(_3\)R, further supporting the coupling hypothesis and the possible identity of \(I_{\text{min}}\) with \(I_{\text{crac}}\).

**MATERIALS AND METHODS**

**Cells**—Human carcinoma A431 cells (Cell Culture Collection, Institute of Cytology, St. Petersburg, Russia) were kept in culture as described elsewhere (8). For patch clamp experiments cells were seeded onto coverslips and maintained in culture for 1 to 3 days before use.

**Electrophysiology**—Single-channel currents were recorded using the inside-out and cell-attached modes of the patch clamp technique (16). Currents filtered at 500 Hz were recorded using a PC-501A patch clamp amplifier (Warner Instruments, Hamden, CT) with a conventional feedback resistance in the headstage (10 GΩ). During recording the currents were digitized at 2.5 kHz. For data analysis and presentation, currents were additionally digitally filtered. \(NP\) was determined using the following equation: \(NP\) = \(<I\>–<I\>_i\), where \(<I\>\) and \(<I\>_i\) are the mean channel current and unitary current amplitude, respectively. \(<I\>_i\) was estimated from the time integral of the current above the base line, and \(i\) was determined from current records and all-point amplitude histograms. Data were collected from 20–a current records after channel activity reached steady state.

Unless otherwise specified, all experiments were performed at standard conditions optimal for \(I_{\text{min}}\) activity. These include free Ca\(^{2+}\) buffered at pCa 7 and membrane potential of ~70 mV (8). Experiments were carried out at room temperature (22–24 °C). \(I_{\text{min}}\) was activated by 2.5 μM IP\(_3\).

**Solutions**—The pipette solution contained (in mM): 105 BaCl\(_2\) and 10 Tris/HC1 (pH 7.4). The standard intracellular solution contained (in...
mM: 140 potassium glutamate, 5 NaCl, 1 MgCl₂, 10 HEPES/KOH, 1.13 CaCl₂ and 2 EGTA/KOH (pCa 7, pH 7.4). In cell-attached experiments, the bath solution contained (in mM): 140 KCl, 5 NaCl, 10 HEPES/KOH, 1 MgCl₂, and 2 CaCl₂. Drugs were applied to the patches either by bath perfusion or by brief pressure ejection. In both cases, the time required for a complete change of solution around the patch was less than 1 s.

**Microsomes**—Cerebellar and forebrain microsomes were isolated from rat brains (Wistar 4–5 weeks old) as described (17) and stored at −70 °C. Microsomes were suspended at a protein concentration of 5 µg/µl. IP₃R content was determined by Western blot analysis with the use of polyclonal antibodies against type 1 IP₃R. As reported before (17) cerebellar microsomes contained at least 20-fold more IP₃R than forebrain microsomes at comparable microsomal protein content.

**Chemicals**—HEPES was from Sigma, EGTA was from Fluka Chemie AG (Buchs, Switzerland), and IP₃ and SKF96365 were from Calbiochem (Behring Diagnostics, La Jolla, CA).

Data are given as mean ± S.E. Error bars denoting S.E. are shown where they exceed the symbol size.

**RESULTS AND DISCUSSION**

In previous work we reported that IP₃ activates a Ca²⁺-selective channel in excised plasma membrane patches which we termed Iₘₐₓ (8, 9). However, activation of Iₘₐₓ by IP₃ was successful only in about 50% of excised patches. Furthermore, in these studies we noticed that the likelihood of Iₘₐₓ activation by IP₃ was decreased with increasing patch washes. This is reminiscent of a similar observation noted when activation of hTrp3 by IP₃ and IP₃R was studied (14). In the case of hTrp3, it was shown that membrane washes prevented hTrp3 activation by IP₃ due to removal of IP₃R associated with the patch. Activation of hTrp3 by IP₃ could be restored by addition of native or recombinant IP₃R1 to the patches (14).

Suspecting that the loss of Iₘₐₓ regulation by IP₃ was due to dissociation and loss of IP₃R attached to Iₘₐₓ in a manner similar to that reported for hTrp3, we tested whether native IP₃R can restore regulation of Iₘₐₓ by IP₃. For these experiments we used the following experimental protocol. The cell

**FIG. 1. Reconstitution of functional Iₘₐₓ/IP₃R complexes in excised plasma membrane patches.** A, a typical reconstitution experiment. Before patch excision channel activity was monitored in cell-attached mode to ensure the existence of an Iₘₐₓ channel in the patch. After excision the patch was exposed to 2.5 µM IP₃. When the patch showed no IP₃-induced channel activity, it was treated with cerebellar microsomes (cer.m/s). Removal of IP₃ abolished the activity of Iₘₐₓ in the presence of cerebellar microsomes. B, segments of current recording in A presented in expanded time scale. Filtering was at 100 Hz. C, a patch excised from intact cells showing Iₘₐₓ activity was exposed to cerebellar microsomes and IP₃. Forebrain microsomes were not able to re-activate Iₘₐₓ. D, frequency of Iₘₐₓ activation.

attached mode of the patch clamp technique was obtained in A431 cells. Only patches showing channel activity in cell-attached mode were used for further experiments. In most cases patch excision into IP₃-free bath solution abolished channel activity observed in the cell-attached mode (8, 9). When patches were excised into a bath solution containing IP₃, Iₘₐₓ was observed in about 50% of patches. If Iₘₐₓ activity in response to IP₃ application was observed, the patches were washed until activation by IP₃ was lost. If the patches showed no Iₘₐₓ activity upon excision, they were not further washed. Figs. 1, A and B, show an example of an excised patch that did not respond to bath application of IP₃. After the control period patches were incubated with IP₃ and microsomes prepared either from rat cerebellum (rich source of IP₃R, see Ref. 18) or rat forebrains (poor source of IP₃R (18)). The middle portion of Fig. 1A and the traces in Fig. 1B show that cerebellar microsomes restored the ability of IP₃ to activate Iₘₐₓ. Removal of IP₃ abolished channel activity, despite the continuous presence of microsomes. Hence, activation of IP₃R by IP₃ was needed to activate Iₘₐₓ. Fig. 1D shows that cerebellar microsomes restored activation of Iₘₐₓ by IP₃ in 26/41 (63%) experiments. On the other hand, no activation was observed in all 27 experiments with forebrain microsomes or in 6 experiments in which cerebellar microsomes without IP₃ were used.

To further establish the specificity of the effect of cerebellar
microsomes, their activity was compared with forebrain microsomes in the same patches and in patches showing weak activity of I_{min}. Patches from five different cells exposed to IP_{3} and microsomes prepared from forebrain were inactive. Subsequent exposure of the same patches to IP_{3} and cerebellar microsomes activated I_{min}. Application of microsomes together with IP_{3} to patches that show weak response to IP_{3} increased the activity of I_{min} by about 7-fold. Therefore, N_{P_{o}} increased from 0.22 ± 0.09 to 1.55 ± 0.38 (n = 7).

The I_{min} current restored by IP_{3}R had properties identical to those reported previously for I_{min} (8, 9). Several properties of the reconstituted I_{min} are illustrated in Fig. 2. Specifically, the single channel conductance measured in the presence of 105 mM Ba^{2+} in the pipette was 1 pS (Fig. 2B). In six experiments the extrapolated reversal potential of the restored channel was close to +60 mV (Fig. 2B), indicating high selectivity for Ca^{2+} (=Ba^{2+}) over K^{+}. Furthermore, I_{min} open probability was highly voltage-dependent with increased activity at potentials below −40 mV (Fig. 2C). The channel mean open time was about 7.7 ms (Fig. 2D).

SKF 96385 (SKF) is probably the best characterized I_{crac} inhibitor (19). This compound also inhibits Ca^{2+} influx and current mediated by the store-operated Trp family channels (20). Therefore, it was of interest to test the effect of SKF on the I_{min} activated by IP_{3}R. Fig. 3 shows that SKF strongly inhibited I_{min} activity after its restoration by IP_{3} and IP_{3}R. Similar results were obtained in eight experiments.

The mechanism of I_{crac} gating by store depletion remained a mystery for a long time due to a lack of adequate experimental systems to study this question. Two recent findings, however, allowed the reprobing of this question. The first is the demonstration of the regulation of the store-operated hTrp3 channel by IP_{3}R (14), and the second is the measurement of I_{crac} single channel conductance (7). Previous studies estimated single channel conductance of I_{crac} to be below the resolution of a standard excised patch technique (4, 5). However, isolation of I_{crac} single channel conductance in whole-cell recording revealed that I_{crac} conductance is about 1 pS when transporting divalent ions, a conductance that can be comfortably resolved in excised patches. In previous studies, we measured the conductance of I_{min} to be about 1 pS (8, 9). In this study, we determined the channel mean open time to be about 7.7 ms (Fig. 2D). Channel mean open time is the most characteristic of channel properties and is commonly used as channel fingerprints (21). In this respect, we note that I_{crac} mean open time was reported to be about 8 ms (7). The findings that I_{min} open probability is regulated by voltage in a manner similar to that of I_{crac} (7), both channels are highly selective for divalent ions (4, 5), and the two channels have almost identical mean open time suggest to us that I_{min} is a form of I_{crac}.

The similarity (and possible identity) of I_{min} and I_{crac}, and our ability to record I_{min} in excised plasma membrane patches provided us with the opportunity to study the gating of native I_{crac}-like channels by IP_{3}R. The present work shows that I_{min} is gated by interaction with IP_{3}R, that this protein-protein interaction is relatively loose, and that the gating required occupation of IP_{3}R by IP_{3}. All of these characteristics are identical to those we found for the regulation of the store-operated hTrp3 channel by IP_{3}R (14). Gating of store-operated channels by IP_{3}R may be mediated by direct interaction between the channels. Thus, recent findings reported without observations (15) indicated that IP_{3}R/hTrp complexes can be co-immunoprecipitated, and peptide sequences in IP_{3}R can bind to peptide sequences in hTrp3 in vitro. Interaction between I_{crac} and IP_{3}R may occur in vivo in microdomains rich in IP_{3}R. Such microdomains were reported in many cells (22) and probably facilitated excision of I_{min} with IP_{3}R attached to them in our experiments.

Acknowledgments—We thank Dr. A. Arnautov for helping with Western blot analysis.

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