Pflügers Arch – Eur J Physiol (1999) 437:305–314

ORIGINAL ARTICLE

Kirill I. Kiselyov · Svetlana B. Semyonova
Anton G. Mamin · Galina N. Mozhayeva

Miniature Ca^{2+} channels in excised plasma-membrane patches: activation by IP_3

Received: 16 June 1998 / Received after revision: / 24 August 1998 / Accepted: 1 September 1998

Abstract In the present work, we characterized the receptor properties and the conductive features of the inositol (1,4,5)-trisphosphate (IP_3)-activated Ca^{2+} channels present in excised plasma-membrane patches obtained from mouse macrophages and A431 cells. We found that the receptor properties of the channels tested were similar to those of the IP_3 receptor (IP_3R) expressed in the endoplasmic reticulum (ER) membrane. These properties include activation by IP_3, inhibition by heparin, time-dependent inactivation by high IP_3 concentrations, activation by guanosine 5′o-thiotriphosphate and regulation by arachidonic acid. On the other hand, in terms of conductive properties, the channel closely resembles Ca^{2+}-release-activated Ca^{2+} channels (I_{crac}). These conductive properties include extremely low conductance (≈1 pS), very high selectivity for Ca^{2+} over K^+ (P_{Ca}/P_K>1000), inactivation by high intracellular Ca^{2+} concentration and, importantly, strong inward rectification. Notably, the same channel was activated by: (1) agonists in the cell-attached mode of channel recording, and (2) cytosolic IP_3 after patch excision. Although the possibility cannot be completely excluded that a novel type of IP_3R is expressed exclusively in the plasma membrane, in their entirety our findings suggest that the plasma membrane of mouse macrophages and A431 cells contains I_{crac}-like Ca^{2+} channels coupled to an IP_3-responsive protein which displays properties similar to those of the IP_3R expressed in the ER membrane.

Key words Ca^{2+} channels · IP_3 · Ca^{2+}-release-activated Ca^{2+} channels · Excised plasma-membrane patches · IP_3 receptors · Mouse macrophages · A431 cells

K.I. Kiselyov · S.B. Semyonova · A.G. Mamin
G.N. Mozhayeva (✉)
Institute of Cytology, Russian, Academy of Sciences,
Tikhoretsky Ave. 4, 194064 St. Petersburg, Russia

Introduction

The increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) that results from activation of phospholipase C (PLC) and release of inositol (1,4,5)-trisphosphate (IP_3) into the cytosol leads to the activation of a Ca^{2+} influx mechanism in the plasma membrane. The IP_3-induced Ca^{2+} release from the internal stores and the properties of the IP_3-activated Ca^{2+} channels present in endoplasmic reticulum (ER) membranes have both been analysed in quite some detail. On the other hand, the nature of the Ca^{2+} influx pathway in the plasma membrane, and its regulation are poorly understood.

Ca^{2+} influx is mediated by a distinctive Ca^{2+} channel termed Ca^{2+}-release-activated Ca^{2+} channels (I_{crac}) [12, 13]. Whole-cell recordings have revealed that I_{crac} have an extremely low conductance and a high selectivity for divalent cations and that they are inhibited by high $[Ca^{2+}]_i$ and show inward rectification [12, 13, 20, 28]. Extensive work in many cells has revealed that Ca^{2+} release from the internal stores is both necessary and sufficient for I_{crac} activation (for reviews see [29, 30]). However, it is still not clear how the Ca^{2+} content of the stores is sensed and then transduced to the plasma membrane to regulate I_{crac} activity. Several messenger molecules have been proposed as candidates for the role of mediator of this activity but none could be confirmed to exist in all cells. An alternative mechanism, initially proposed by Irvine [14], is a direct coupling between the IP_3 receptor (IP_3R) and I_{crac} in a manner similar to that found between the dihydropyridine and ryanodine receptors present in skeletal muscle. In view of the complexity of the putative coupling mechanism and the low conductance of I_{crac}, obtaining evidence to support or refute such a hypothesis is quite difficult and technically very demanding.

Another hypothesis requires that IP_3 directly gates an IP_3R in the plasma membrane. Although biochemical studies have shown that IP_3R are preferentially located in the ER [33], a functionally distinct type of IP_3R has been found to be associated with the plasma membrane

of lymphocytes: this receptor differs from the microsomal IP_3R in its terminal sugar content, IP_3 affinity and sensitivity to heparin [15]. Later, the same authors identified IP_3R in the plasma membrane of Jurkat T-cells and demonstrated the co-capping of this receptor with the T-cell-receptor-CD3 complex during T-cell activation [16]. This hypothesis has also been supported by patch-clamp studies [6, 18, 23, 37] in which Ca^{2+} channels activated by IP_3 have been found in excised patches.

We reasoned that detailed characterization of these channels might help to clarify their nature and their relationship to I_{crac}.

In the present paper, we report the presence of miniature IP_3-activated Ca^{2+} channels in excised plasma-membrane patches obtained from mouse macrophages and A431 cells. In addition, we make a detailed comparison between these channels and those previously found in the plasma membrane of other cell types. All of the pharmacological tests we carried out showed that in terms of its activation and regulation, the channel is very similar to the IP_3R present in the ER membrane, yet the permeability properties of the channel closely resemble those reported for I_{crac}. These findings, together with reports of high levels of IP_3R in ER domains close to or associated with the plasma membrane [19, 34] lead us to speculate that the coupling model may be valid for the regulation of I_{crac}.

Materials and methods

Chemicals

ATP, guanosine 5′o-thiotriphosphate (GTPγS), heparin, bradykinin (BK), uridine 5′-triphosphate (UTP), mepacrine, arachidonic acid (AA), 4-bromophenacyl bromide (4-BPB), thapsigargin (Tg) and 4-(2-hydroxyethyl)-1-piperatzineethanesulphonic acid (HEPES) were all obtained from Sigma (Sigma, St. Louis, USA); ethyleneglycol bis(aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA) was from Fluka Chemie (Buchs, Switzerland) and IP_3 was from Calbiochem (Behring Diagnostics, La Jolla, USA).

Cells

For the preparation of macrophages, cells from the peritoneal cavity of CC57/W mice were placed on top of 5×5-mm glass coverslips which, in turn, were placed in plastic dishes. The cells were cultured in Dulbeccos modified Eagles medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 mg/ml streptomycin. The cells were kept in a 5% CO_2, humidified atmosphere at 37°C and were used for patch-clamp experiments 1–3 days after plating. Macrophages were identified using α-naphthyl acetate esterase staining. More than 98% of the cells in each monolayer were macrophages. Human carcinoma A431 cells (Cell Culture Collection, Institute of Cytology, Russia) were kept in culture as described elsewhere [23].

Electrophysiology

Single-channel currents were recorded using the inside-out and cell-attached modes of the patch-clamp technique [10]. Currents filtered at 200 Hz were recorded with the aid of a PC-501A patch-clamp amplifier (Warner, USA) with a conventional feedback resistance in the headstage (10 GΩ). During recording, the currents were digitized at 1–2 kHz. For data analysis and presentation, currents were filtered at 50–100 Hz. Channel activity was expressed in terms of NP_o (where P_o is the probability of a single channel being open and N is the number of channels present in a membrane patch). NP_o was determined using the following equation: $NP_o = \langle I \rangle / i$ where $\langle I \rangle$ and 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 i was determined from current records and all-point amplitude histograms.

Solutions

The pipette solution contained (in mM): 105 $BaCl_2$ or 100 $CaCl_2$ and 10 TRIS/HCl (pH 7.4). The standard intracellular solution contained (in mM): 140 K glutamate, 5 NaCl, 1 $MgCl_2$, 10 HEPES/KOH, 1.13 $CaCl_2$ and 2 EGTA/KOH (pCa 7, pH 7.4). When the Ca^{2+}-dependence of channel activity was to be measured, $[Ca^{2+}]_i$ was clamped with 10 mM EGTA and calculated using the algorithm of Fabiato and Fabiato [5]. In cell-attached experiments, the bath solution contained 140 mM KCl and 2 mM $CaCl_2$ to nullify the cell's resting potential. 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 <1 s. Experiments were carried out at room temperature (22–24°C). Data are given as mean ± SEM. Error bars denoting SEM are shown where they exceed the symbol size.

Results

IP_3 activates Ca^{2+}-permeable channels in excised plasma-membrane patches

Application of 0.2–15 μM IP_3 to the cytoplasmic surface of patches excised from the plasma membrane of mouse macrophages activated small-conductance inward currents in approximately 50% of the experiments (25 out of 49 attempts). Ca^{2+} currents were isolated by including 105 mM Ba^{2+} or 100 mM Ca^{2+} as the only permeant cation in the pipette solution.

Figure 1 illustrates several properties of these currents. Displaying sections of the current on an expanded time scale revealed the small amplitude of the current which resulted in a relatively poor signal-to-noise ratio despite the heavy filtration used to analyse the data. However, transient opening from a distinct baseline could be resolved visually (Fig. 1A,B) and by the use of all-point amplitude histograms (Fig. 1C).

An important and distinctive property of any channel is its ion selectivity. Figure 1B shows the currents carried by 105 mM Ba^{2+} (b1) and 100 mM Ca^{2+} (b2) at several holding potentials. Comparison of the records shows that at each potential tested the Ca^{2+} and Ba^{2+} currents have similar amplitudes. The current/voltage-relationship, obtained from a total of 12 experiments with Ba^{2+} (Fig. 1D), indicates that the channel has a conductance of about 1 pS and an extrapolated reversal potential exceeding +65 mV. These values are very similar to those reported for the channels present in the plasma membrane of A431 cells [17]. The P_{Ca}/P_K permeability ratio of a channel can be estimated from the extrapolated reversal potential value using a modification of the Goldman-Hodgkin-Katz equation [11]. The ratio obtained in this

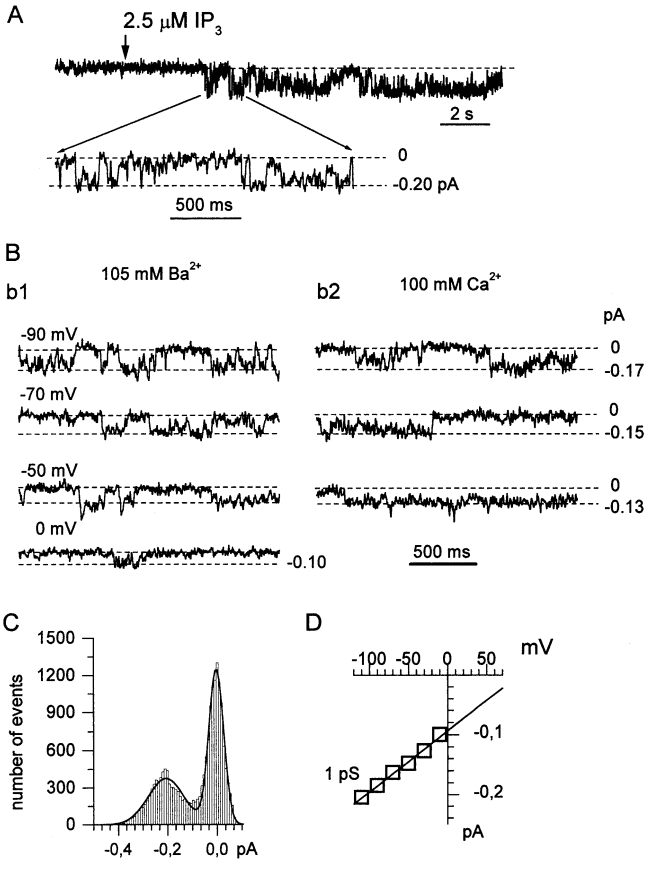

Fig. 1A–D Inositol (1,4,5)-trisphosphate (IP_3) activates miniature Ca^{2+} channels in inside-out plasma-membrane patches from mouse macrophages. **A** A plasma-membrane patch from a mouse macrophage was exposed to 2.5 μM IP_3. The pipette solution contained 105 mM Ba^{2+}. The membrane potential was –90 mV. In the expanded traces, in this and other Figures, the *broken horizontal lines* and the numbers to the right indicate current amplitude in pA. The currents were filtered at 100 Hz. **B** Examples of Ba^{2+} *(b1)* and Ca^{2+} *(b2)* currents recorded at different membrane potentials after application of 5 μM IP_3 to plasma-membrane patches. **C** An all-points amplitude histogram constructed from an 18-s recording obtained in an experiment with a macrophage membrane at a holding potential of –90 mV. **D** Plot of mean current amplitude as a function of membrane potential. The pipette solution contained 105 mM Ba^{2+}. Each point represents the average from three to seven experiments

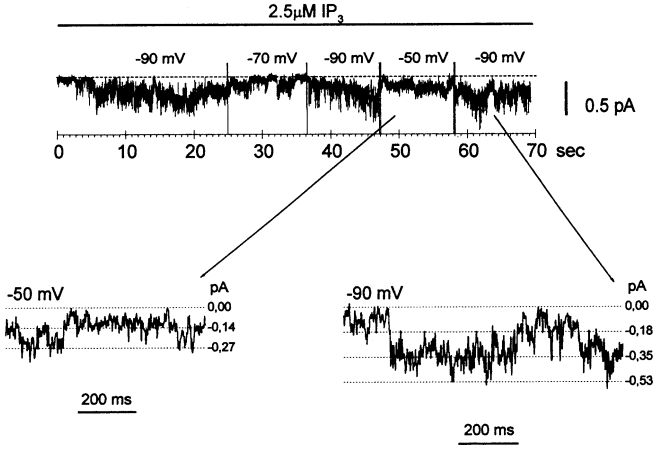

Fig. 2 The activity of IP_3-sensitive channels depends upon the holding membrane potential. This data is from a typical inside-out patch obtained from a mouse macrophage, and shows the effect of changing the holding potential on the channel activity evoked by application of 2.5 μM IP_3. The mean current is greater at –90 mV, apparently due to an increase in the number of active channels, their individual conductance being unchanged. The pipette solution contained 105 mM Ba^{2+}. The currents were filtered at 100 Hz

case was $P_{Ba}=P_{Ca}/P_K>1000$, indicating a very high selectivity for Ca^{2+} over K^+. This is very different from the selectivity reported for the IP_3R found in native ER membranes [21] or reconstituted into lipid bilayers [3].

Finally, as shown previously for the miniature IP_3-activated channels in A431 cells [17], channel activity (expressed in terms of NP_o) was very low at a holding potential of –30 mV, and increased upon hyperpolarization in the –30 mV to –100 mV range. Figure 2 shows the channel activity evoked by IP_3 in macrophages when several channels were present in the patch. The figure shows that shifting the holding potential from –50 to –90 mV caused an increase in NP_o that appeared to be due mainly to an increase in the number of active channels (since their individual conductance was unchanged). Time-dependent inactivation of the channels present in plasma membranes from macrophages (see below) precluded a more detailed characterization of the effect of membrane potential on channel NP_o.

Transient behaviour of channel activity

In the course of our study, we noted that in each experiment the IP_3-induced channel activity was transient: it faded away despite the continued presence of IP_3 in the cytoplasmic solution. This effect was probably responsible for the wide variation in channel open probability between patches. A similar transient behaviour of IP_3-evoked channel activity has been reported in plasma-membrane patches obtained from bovine aortic endothelial cells [39] and A431 cells [17].

In the next stage of our study, we attempted to determine the mechanism(s) responsible for channel inactivation. IP_3-dependent inactivation, particularly at high IP_3 concentrations, was most pronounced in experiments on membrane patches from macrophages. Figure 3 A shows the development of the channel activity evoked in plasma membrane patches from macrophages by the application of 2.5, 5 or 10 μM IP_3, averaged data from three experiments being shown for each concentration of IP_3. The results were normalized with respect to the maximal NP_o value obtained in a given experiment; hence, the curves do not reflect the real NP_o values but rather the pattern of channel activity. It can easily be seen that the activity induced by 10 μM IP_3 disappears almost completely within 30 s. After application of 5 μM IP_3, channel activity declined but could still be observed after 100 s of recording, whereas application of 2.5 μM IP_3 brought it to a quasi-steady level, with no decline apparent during the same recording period.

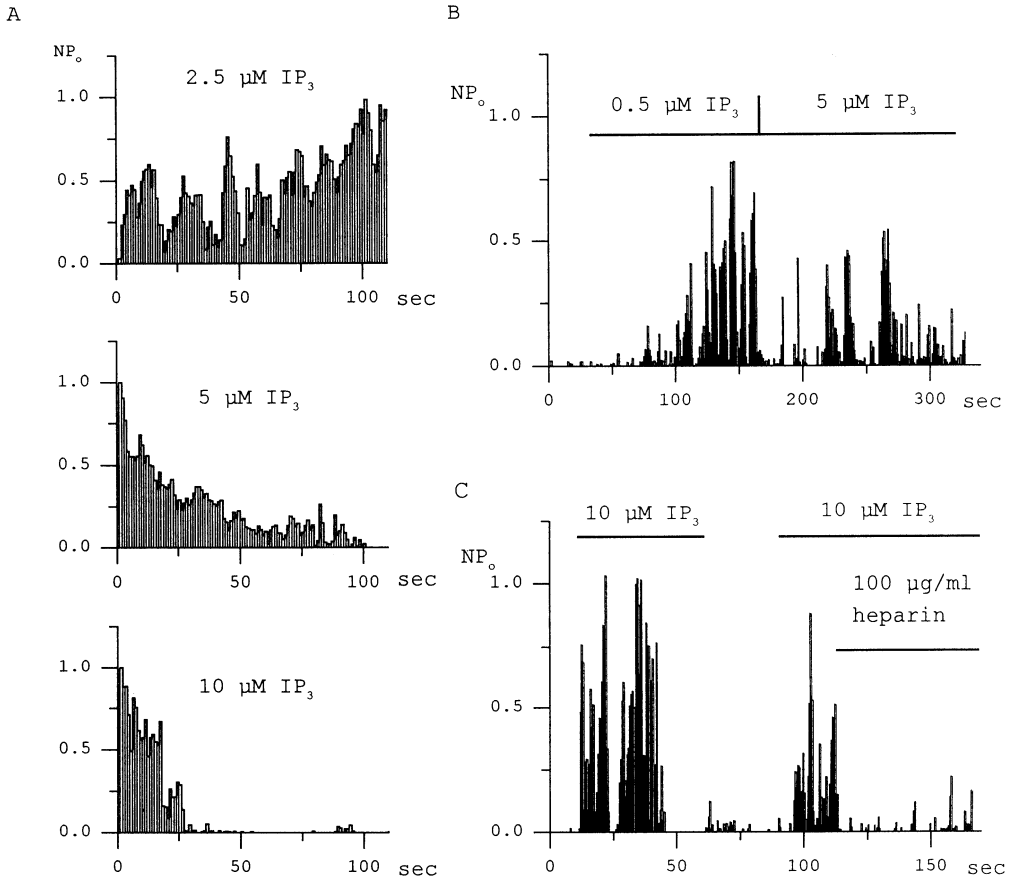

Fig. 3A–C IP_3-dependent channel inactivation. **A** Open-probability plots showing the development of channel activity following application of the indicated IP_3 concentration to patches from mouse macrophages. Each plot shows averaged data from three experiments. To facilitate comparison, the results were normalized before averaging with respect to the maximal NP_o value (where N is the number of channels present in a membrane ptach and P_o is the probability of a single channel being open) obtained during each experiment. The pipette solution contained 105 mM Ba^{2+}. The membrane potential was –70 mV. **B** NP_o plot illustrating the inactivation by IP_3 of IP_3-activated plasma-membrane Ca^{2+} channels. The addition of a high concentration of IP_3 to a patch already exposed to a low concentration of IP_3 causes a decrease in NP_o. **C** Activity evoked by the application of 10 μM IP_3 returns to the control level within 1 min. Subsequent removal and re-addition of 10 μM IP_3 restores channel activity. Addition of heparin almost abolishes channel activity

IP_3-dependent inactivation of IP_3-mediated Ca^{2+} release has been reported before in hepatocytes [8] and was attributed to channel desensitization. We believe that the same phenomenon is responsible for the inactivation reported here for the following reasons. First, channel activity did not increase significantly on application of a high IP_3 concentration (5 or 10 μM) to patches that were already being treated with a low IP_3 concentration (0.5 or 2.5 μM). Figure 3B shows a typical experiment in which we initially exposed the channel to 0.5 μM IP_3, then after channel activity had reached a steady state, subsequent application of 5 μM IP_3 resulted in a reduction in channel NP_o. Second, the loss of channel activity was reversible. As can be seen in Fig. 3C, removal of IP_3 for 60 s or less was sufficient to restore channel responsiveness to IP_3. Just as in our previous study of A431 cells [17], in the present study heparin (100–500 μg/ml) was effective in inhibiting the IP_3-induced channel activity in macrophages(n=5; Fig. 3C).

Dependence of channel open probability on IP_3 concentration

The channel desensitization described above prevented us from testing the effect of different IP_3 concentrations on a single patch. For this reason, a concentration-dependency curve was constructed from the initial response in a number of patches following the application of various concentrations of IP_3. At each IP_3 concentration channel activity was allowed to reach a steady state prior to data collection; this steady-state period was longer than 10 s to reduce the effect of activity fluctuations, but shorter than 40 s to avoid the influence of time-dependent channel inactivation. This set of experiments was performed at –70 mV and with Ca^{2+} buffered at pCa 7, optimal conditions for the activity of these channels.

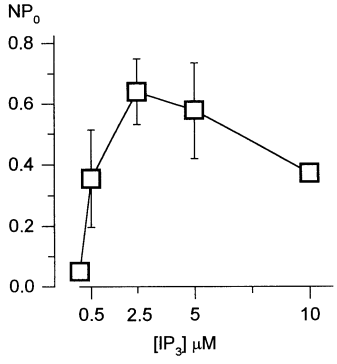

Fig. 4 Concentration-dependency of IP_3-induced channel activity. The dependence of channel activity on IP_3 concentration is expressed in terms of NP_o. These results were obtained from macrophages. Each point represents data from two to seven experiments. Results were obtained from 20 patches. The pipette solution contained 105 mM Ba^{2+}. The membrane potential was –70 mV

The dependence of the open probability on IP_3 concentration is illustrated in Fig. 4. NP_o increased with increasing IP_3 concentrations up to 2 μM. In contrast to the situation in A431 cells [17], we found that in macrophages (in which the IP_3-induced channel inactivation was more pronounced) channel activity decreased at high IP_3 concentrations. In both cell types, half-maximal NP_o was achieved at approximately 0.2–0.5 μM IP_3, which is very close to the value reported for the IP_3R present in ER membranes [27]. Large variations in NP_o between patches, which were the main source of the large standard deviations, prevented the accurate determination of the apparent binding constant and the possible existence of cooperativity in IP_3 interaction.

Regulation of channel activity

The presence of channel inactivation suggested that additional factor(s) play a role in the stabilization of IP_3-mediated Ca^{2+} influx in intact cells, and that these are lost after patch-excision. Indeed, as detailed below, we found evidence that the miniature IP_3-activated Ca^{2+} channels present in macrophages and A431 cells are each regulated by two separate mechanisms. Both mechanisms are expressed in each of these cell types, but they display cell-specific behaviour.

Modulation of channel activity by AA

In several cells-types, the inhibition of AA release has been shown to both increase the activity of the IP_3R and prevent channel inactivation [22, 35]. Moreover, AA itself has been shown to block IP_3R [38]. We therefore tested the effect of the phospholipase A_2 (PLA_2) inhibitors 4-BPB and mepacrine on channel activity. For these experiments, macrophages were incubated with 5 μM mepacrine for 3 min. Then, patches were excised and channel activity was evaluated following application of IP_3. Pretreatment with mepacrine substantially increased channel activity; indeed, in five experiments with 10 μM IP_3, the NP_o value was at least three times that seen in the absence of mepacrine (compare Fig. 5A,B). Mepacrine was also effective when applied after patch excision. Figure 5B shows that application of mepacrine to plasma-membrane patches incubated with IP_3 not only restored, but actually increased and prolonged channel activity (n=5). In the absence of IP_3, mepacrine had no effect on channel activity (n=4). Another PLA_2 inhibitor, 4-BPB, affected channel activity in a manner similar to that shown by mepacrine (data not shown). In fact, these two inhibitors act by different mechanisms: mepacrine competes with PLA_2 for the substrate, while 4-BPB is a histidine-alkylating agent. Hence, the effects of the compounds on channel activity are not likely to be due to nonspecific effects. Finally, as expected from the effect of inhibitors of AA metabolism, AA itself inhibited the activity of the IP_3-sensitive channels (Fig. 5C; n=4).

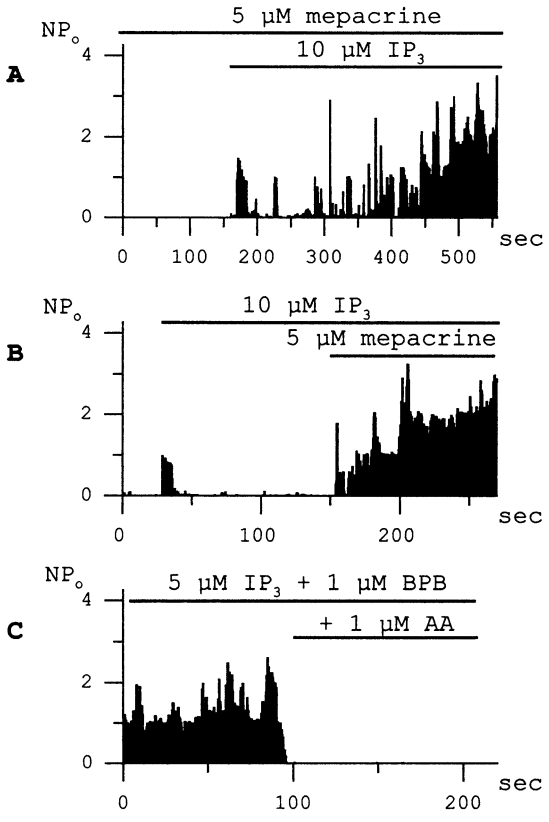

Fig. 5A–C Arachidonic acid promotes, and mepacrine prevents channel inactivation. **A** The cell was pretreated with 5 μM mepacrine for 3 min prior to patch excision. Subsequently, the patch was excised into a solution containing the same concentration of mepacrine. Addition of 10 μM IP_3 evoked channel activity (expressed as NP_o) that displayed no marked rundown throughout the experiment during many minutes of recording. **B** The channel activity induced by the application of 10 μM IP_3 to an untreated membrane patch disappeared within seconds. Addition of 5 μM mepacrine restored and substantially increased the channel activity. The pipette solution contained 105 mM Ba^{2+}. The membrane potential was –70 mV. **C** NP_o plot presenting results of a typical experiment in which arachidonic acid abolished the stable channel activity induced by IP_3 plus 4-bromophenacyl bromide

A guanosine triphosphate-dependent step is involved in channel activation

A guanosine triphosphate (GTP)-dependent step has been shown to facilitate Ca^{2+} release from the ER [41] and to increase the open probability of IP_3-activated channels in the plasma membrane [23]. We tested for the presence of such a mechanism in our experimental system by examining the effect of GTPγS, a nonhydrolysable GTP analogue, in experiments on patches from A431 cells. As shown in Fig. 6A, GTPγS potentiated the action of IP_3 (increasing channel NP_o) whether Ba^{2+} or Ca^{2+} was used as the charge carrier (n=11). With 105 mM Ba^{2+} and 2 μM IP_3, 100 μM GTPγS increased NP_o from 0.53±0.19 (n=7) to 0.82±0.13 (n=3). Figure 6 shows that the channel activity induced by 15 μM IP_3 inactivated rapidly. Channel activity was re-

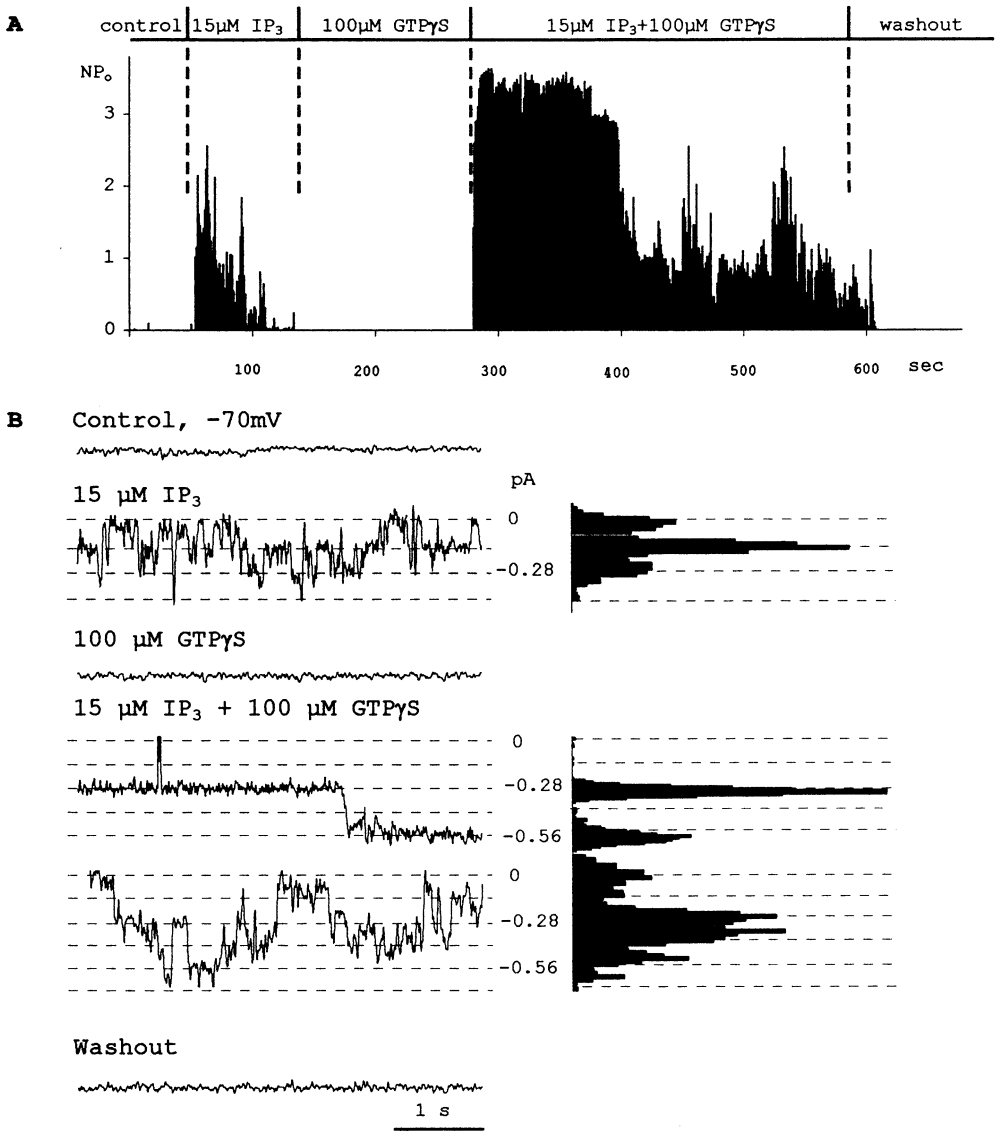

Fig. 6A, B Effect of guanosine 5′o-thiotriphosphate on IP_3-induced Ca^{2+} channel activity. The data is from a typical inside-out patch obtained from an A431 cell. The pipette solution contained 105 mM Ba^{2+}; the bath solution had a pCa of 7. The membrane potential was –70 mV. **A** Open-probability plot illustrating the time course of induced changes in channel activity (conditions are indicated at the top of the panel). **B** Current records from the experiment illustrated in **A** together with all-point amplitude histograms constructed from the corresponding current records

stored and NP_o substantially increased by the addition of GTPγS together with the same concentration of IP_3. Moreover, in the presence of GTPγS, the IP_3-evoked channel activity lasted for several minutes. GTPγS alone had no effect (n=6). In Fig. 6B, it is evident from the current traces presented at an expanded time scale and from the all-points amplitude histograms that IP_3 alone and a mixture of IP_3 and GTPγS activated the same channel. In contrast to the situation in macrophages, in A431 cells, a rather high frequency of duplicate openings has been reported [17]. The addition of the IP_3/GTPγS mixture increased the frequency of duplicate openings in the present study, indicating some influence of GTPγS on channel gating.

In general, the data presented here argue strongly for the involvement of G-proteins in the modulation of channel activity. In this, our findings are in agreement with recent studies on the ER IP_3R [41]. However, clarification of this mechanism will require further detailed studies.

Ca^{2+}-mobilizing agonists and IP_3 activate one and the same channel

If IP_3 indeed activates Ca^{2+}-permeable channels directly, we should expect any agonists that increase the IP_3 concentration in the cytoplasm to cause activation of channels whose properties are the same as those of the channels activated by IP_3 in excised patches. The strategy we used to test this possibility was to record single-channel activity following stimulation by Ca^{2+}-mobilizing agonists using the cell-attached mode, then, after patch excision, to determine whether addition of IP_3 activated the same type of channel. In most of the experiments (using A431 cells) no basal channel activity was observed, and in those few experiments in which such activity was present, its open probability did not exceed 0.1. Activation of G_q-coupled receptors by addition of either 100 μM UTP or 10 μM BK to an extracellular solution containing 140 mM K^+ and 2 mM Ca^{2+} resulted in the appearance of channel activity. Patch excision always led to the dis-

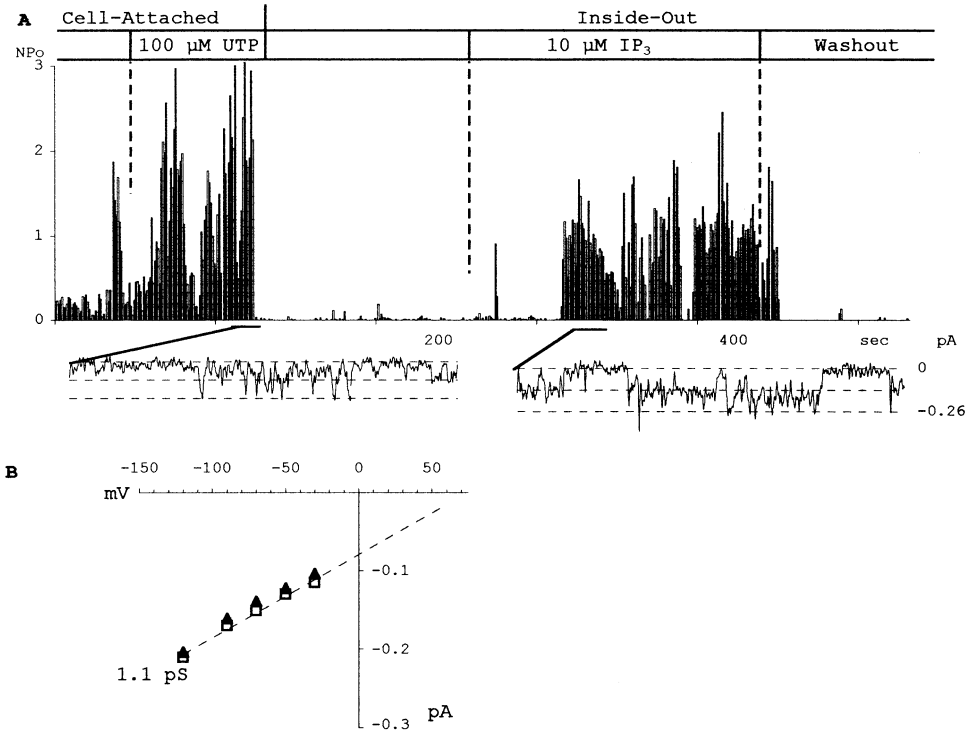

Fig. 7A, B Ca^{2+}-mobilizing agonists and IP_3 activate the same plasma-membrane Ca^{2+} channel. **A** Open-probability plot illustrating induced changes in channel activity following application of uridine 5′-triphosphate (current was recorded using the cell-attached mode). After patch excision (control period), channel activity disappeared. Application of 10 μM IP_3 to the inside-out patch restored channel activity. This experiment was carried out using an A431 cell. The pipette solution contained 105 mM Ba^{2+}. The 2-s traces below the plot show currents recorded at –50 mV. Filtering was performed at 100 Hz. **B** Current/voltage relationships obtained in cell-attached (*squares*) and inside-out (*triangles*) configurations. The pipette solution contained 105 mM Ba^{2+}

appearance of this activity, which resumed upon subsequent application of IP_3 (n=9).

Figure 7A illustrates the time course of a typical experiment in which channel activity (expressed in terms of NP_o) was enhanced in the cell-attached mode by the application of 100 μM UTP, and in the inside-out configuration by the application of IP_3. This figure also shows periods of current recording at an expanded time scale. Figure 7B shows the mean current/voltage-curves. The results show clearly that the amplitude and the extrapolated reversal potential of the currents evoked by UTP and IP_3 are indistinguishable. Much the same results were obtained when 10 μM BK was used (n=4). The NP_o values for the channels evoked by 100 μM UTP and 10 μM BK in cell-attached experiments were 0.99±0.08 (n=17) and 0.71±0.11 (n=8), respectively. An additional point to note is that the channels activated by Ca^{2+}-mobilizing agonists in intact cells, and subsequently by IP_3 in excised patches, exhibited inward rectification (not shown). Thus, the overall similarities in channel properties allow us to conclude with confidence that in intact cells UTP and BK activated the channel that was activated by IP_3 application in excised patches.

The activation of the channel induced by agonists may have been due to depletion of Ca^{2+} stores as a result of an increase in intracellular IP_3 concentration. To provide evidence for or against the involvement of store depletion, we attempted to activate the channel in intact cells by inhibition of SERCA pumps using Tg. It has been firmly established that depletion of stores by Tg has no detectable effect on the cellular levels of IP_3 [30]. To facilitate store depletion, cells were incubated in a solution containing 10 mM EGTA and 10 μM Tg. Using the

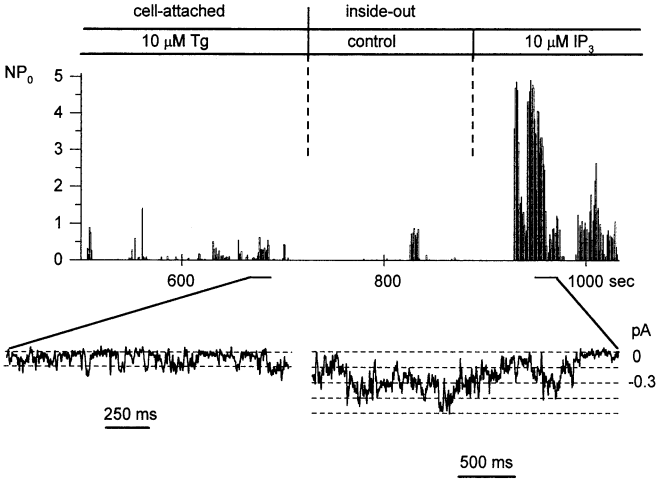

Fig. 8 Thapsigargin (Tg) treatment of the intact cell and IP_3 application to excised patches activates the same plasma-membrane Ca^{2+} channel. Open-probability plot illustrating activation of the same miniature Ca^{2+} channels in the plasma membrane of an intact cell by Tg and, after patch excision, by IP_3. This experiment was carried out using an A431 cell. The pipette solution contained 105 mM Ba^{2+}. The 2-s traces below the plot show currents recorded at –50 mV. Filtering was performed at 100 Hz

cell-attached configuration, we observed that channel activation by Tg was not as efficient as activation by agonists. Nonetheless, in 11 out of 27 experiments, channel activity was observed. Figure 8 shows some of the properties of the channel activated by Tg in the cell-attached mode and subsequently by IP_3 in the same patch after excision. The currents evoked by Tg and IP_3 had the same amplitude. In experiments with Tg, the channel NP_o

(average 0.08±0.06 (n=11) was lower than that evoked by agonists. To determine with certainty which channel was activated by Tg, the above NP_o value was calculated using data only from the 11 experiments in which channel activity was completely absent prior to the application of Tg. Thus, although the NP_o of the channel activated by Tg in intact cells is low, it is significant.

Furthermore, the ability of Tg to reactivate the channel after patch excision was verified. In fact, there is evidence [36] that Ca^{2+}-containing organelles in the closest proximity to the plasma membrane could be excised together with the membrane fragment. In this case, IP_3 can act indirectly through the depletion of the attached calciosomes. In the present study, no channel activity was observed upon patch treatment with Tg (1 μM) for 3–5 min (after 3–10 min exposure of the membrane fragment to the solution containing 10 mM EGTA). Such a treatment did not abolish the ability of IP_3 to activate the channels; subsequent IP_3 application evoked channel activity (n=7).

Discussion

The simplest explanation of our experimental data is that IP_3 directly gates an IP_3R expressed in the plasma membrane of mouse macrophages and A431 cells. Data from biochemical studies suggest that although IP_3R are principally located in the ER [33], a functionally distinct type of IP_3R is associated with the plasma membrane of lymphocytes [15]. An IP_3R has been found in the plasma membrane of Jurkat T-cells and its co-capping with the T-cell-receptor-CD3 complex during T-cell activation has been demonstrated [16]. IP_3 -activated channels have also been identified in excised plasma-membrane patches [6, 18, 23, 39]. These various channels have all been reported to be selective for divalent cations and not to exhibit inward rectification, but they display a wide range of conductance values (from 4 to 30 pS). Their conductance value seems to be tissue-specific.

In our study, we noticed that the IP_3-sensitive channels had an exceedingly low conductance by comparison with those of the IP_3R reported so far [3, 27]. Although because of our experimental approach we cannot exclude completely the possibility that we recorded the activity of a novel IP_3R that is exclusively expressed in the plasma membrane, we consider it to be quite unlikely. Indeed, the cloning of all known IP_3R has revealed a high degree of similarity among the receptors, in particular in the putative transmembrane domains. This makes it difficult to believe that the miniature channels studied here are novel IP_3R. However, a complementary molecular approach would be needed to resolve this issue.

Of all the components of the Ca^{2+} signal, the activation and regulation of I_{crac} continue to be the most difficult to understand. The most fundamental aspect of I_{crac} regulation is the sensing and responding to the Ca^{2+} content of the internal stores [29, 30], a property that was formulated into the capacitative model of Ca^{2+} influx [29]. Over the years, several products of the phosphoinositide pathway, a GTPγS inhibitor [7] or other as yet undefined molecules have been proposed as the soluble mediator that activates I_{crac} [25, 31]. However, none of these has been proven to be the common activator of I_{crac} that is present in all cells. An alternative model, originally proposed by Irvine [14] and extended upon by Berridge [1], involves a coupling mechanism equivalent to the coupling between the dihydropyridine and ryanodine receptors found in skeletal muscle. In this model, a population of IP_3R in close proximity to the plasma membrane interacts with I_{crac} channels to regulate their activity. The major advantages of this model are that it can explain both how I_{crac} communicates with the ER Ca^{2+} stores and also the intimate relationship between the degree of store depletion and I_{crac} activity. Unfortunately, obtaining direct evidence to either support or refute the coupling model is quite difficult in view of the complexity arising from the required protein-protein interaction and the fact that the two channel proteins reside in two separate cellular membranes. Immunolocalization of IP_3R in a growing number of cell types has led to the identification of subplasmalemmal domains with high levels of IP_3R expression [9, 19, 37], and in some cells IP_3R could be localized to caveolae [4], a site known to contain several signalling proteins and/or complexes [40]. Such an arrangement would seem to fulfil the structural requirements of the coupling model.

A direct prediction of the coupling model is that all of the receptor properties of the channel will be similar to those of IP_3R and that both the conductance and selectivity of the channel will be similar to those of I_{crac}.

The results obtained in our study are summarized in Table 1. An analysis of Table 1 makes it difficult to escape the conclusion that the receptor properties of the

Table 1 Summary of the receptor and permeability properties of inositol (1,4,5)-trisphosphate receptors (IP_3R) Ca^{2+}-release-activated Ca^{2+} channels (I_{crac}) and "miniature Ca^{2+} channels". (*ER* Endoplasmic reticulum, *IP*$_3$ inositol (1,4,5)-triphosphate, *GTPγS* guanosine 5′o-thiotriphosphate, *AA* arachidonic acid, *[Ca*$^{2+}$*]*$_i$ intracellular free Ca^{2+} concentration)

Property	ER IP_3R	I_{crac}	Miniature Ca^{2+} channels
Activation by IP_3	Yes	No [12]	Yes
Sensitivity to heparin	Yes [2]	No [12]	Yes
IP_3-dependent inactivation	Yes [8]	No	Yes
GTPγS-dependence	Activation [41]	Inhibition [7]	Activation
Sensitivity to AA	Inhibition [38]	?	Inhibition
$[Ca^{2+}]_i$-dependence	Bell-shaped [2]	Inactivation [13]	Asymmetric, bell-shaped [17]
Conductance	20–80 pS [3, 6]	<1 pS [13]	1 pS
Selectivity Ca^{2+}/K^+	Low [3, 27]	High [13, 20, 28]	High
Inward rectification	No	Yes [13, 20, 28]	Yes

plasma-membrane miniature Ca^{2+} channel closely resemble those of IP_3R. These include activation by IP_3, inhibition by heparin, IP_3-dependent inactivation, stimulation (rather than inhibition) by GTPγS, and sensitivity to AA. On the other hand, the conductive properties of the miniature channel are very similar to those previously described for I_{crac}. The most notable of these properties are the low conductance, the high Ca^{2+} selectivity, and the regulation of channel activity by voltage. It will be instructive to deal with these properties in turn.

The conductance of the miniature Ca^{2+} channel studied here is close to the conductance values calculated for I_{crac} [12, 13], although much lower single channel conductances have been found in T-cells [42] using whole-cell current noise analysis. Such a difference is not very surprising when one takes into account that: (1) lower current carrier concentrations were used in the whole-cell experiments [13], (2) conductance may be overestimated when multiconductance channel activity which cannot be resolved is recorded in the cell-attached mode, and (3) the channel conductance may be underestimated by use of the whole-cell current noise analysis procedure, since this detects mainly the transitions between the conductance sublevels [24].

No channel other than I_{crac} [12, 13, 20, 28, 42] displays a Ca^{2+}/K^+ selectivity greater than 1000. Even the voltage-operated Ca^{2+} channels in excitable cells exhibit a lower selectivity [11], while IP_3R and the ryanodine receptor are both almost equally permeable to Ca^{2+} and K^+ [3].

Regulation by voltage is among the most distinctive features of an ion channel [11]. Strong inward rectification has been reported for I_{crac} (and for no other known Ca^{2+} channel) in virtually all of the cells tested so far. Recording the whole-cell current precludes determination of whether membrane voltage regulates the conductance or NP_o of I_{crac}. However, our studies show that the NP_o of the plasma-membrane Ca^{2+} channels is regulated by the membrane potential.

It has been demonstrated that the $[Ca^{2+}]_i$-dependence of the miniature Ca^{2+} channels follows an asymmetric bell-shaped curve [17], a relationship different both from that reported for IP_3R [2] and from that reported for I_{crac} [12, 13, 42]. Such a unusual $[Ca^{2+}]$-dependence raised the possibility that $[Ca^{2+}]_i$ was influencing not IP_3R directly, but rather a channel that is regulated by the IP_3R.

The hallmark of I_{crac} is regulation by store depletion. We have demonstrated that the miniature plasma-membrane Ca^{2+} channel was induced by an agonist (Fig. 7) or by Tg (Fig. 8). A somewhat disturbing observation was that the channel was more readily activated on agonist stimulation than on application of Tg, although in most cell-types Tg appears to be as effective as the agonists in causing activation of Ca^{2+} influx and I_{crac} current [13,28,42]. Our data tempts us to suggest that in plasma-membrane patches the spatiotemporal relationship favours channel activation by agonist rather than by Tg. Indeed, recent studies have provided multiple functional evidence for the compartmentalization of Ca^{2+} signalling [9, 19]. The initial sites of the Ca^{2+} wave always reside at a region of high levels of IP_3R. In pancreatic acinar cells, IP_3-mediated intracellular Ca^{2+} mobilization may occur primarily from Tg-insensitive secretory granules (which apparently have no Ca^{2+} pump) rather than from the ER [9]. The microsomal fraction that carries IP_3 binding properties has been shown to be associated with the plasma membrane, and to be disaggregated by cytochalasin [34]. Such a disruption of the cytoskeleton has been show to block agonist-elicited Ca^{2+} mobilization, and does not block Tg-induced Ca^{2+} mobilization [32]. A dissociation between the amount of Ca^{2+} released and subsequent activation of Ca^{2+} influx has been established [26]. A supposition was made that Tg was less effective than IP_3 in activating I_{crac}, which indicates that Ca^{2+}-release-activated Ca^{2+} (CRAC) stores are either less leaky or they are relatively Tg insensitive. On the other hand, the ability of Tg to induce channel activity in experiments on intact cells could be explained in terms of the Ca^{2+} dependence of PLC. It can be activated by a rise in $[Ca]_i$, so it is conceivable that Tg does modestly elevate IP_3 through the slow rise in Ca^{2+} that accompanies pump inhibition.

In summary, we emphasize that such properties as conductance, selectivity and inward rectification (expressed as the elevation of NP_o caused by hyperpolarization) are of special importance for the identification of the present miniature Ca^{2+} channels as being some kind of CRAC-like channel. It should be noted, however, that the very different experimental conditions used for single-channel recording in excised patches and for I_{crac} measurements hinders a detailed comparison of the regulation of these channels.

In the present work, we used functional fingerprints to characterize the nature and regulation of the miniature Ca^{2+} channels present in excised plasma membrane patches from two types of cell, A431 cells and peritoneal macrophages. Taken together, our results do not rule out the possibility that these channels present a novel type of IP_3R that is expressed in the plasma membrane. However, the most reasonable view of our data is that they provide evidence in support of the assumption that the plasma membrane contains an I_{crac}-like channel coupled to an IP_3-responsive protein, which displays properties of IP_3R expressed in the ER membrane. We believe that our data should encourage further experimental probing of this model.

Acknowledgements The authors are deeply grateful to Prof. S. Muallem for encouragement and kind assistance in preparing the manuscript for publication; we are also thankful to Prof. B. Hille, Prof. B. Nilius and Prof. I. Bezprozvanny for their critical comments and useful advice. This work was supported by the Russian Basic Research Foundation (grants # 98-04-49512, 96-15-50737).

References

1. Berridge MJ (1995) Capacitative calcium entry. Biochem J 312:1–11
2. Bezprozvanny I, Ehrlich B (1991) Bell-shaped calcium response curves of Ins(1,4,5)P_3 and calcium-gated channels from endoplasmic reticulum of cerebellum. Nature 351:751–754

3. Bezprozvanny I, Ehrlich B (1994) Inositol (1,4,5)-trisphosphate ($InsP_3$)-gated Ca channels from cerebellum: conduction properties for divalent cations and regulation by intraluminal calcium. J Gen Phys 104:821–568
4. Bush KT, Stuart RQ, Li SH, Moure LA, Sharp AH, Ross CA, Nigam SK (1994) Epithelial inositol 1,4,5-trisphosphate receptors. Multiplicity of localization, solubility, and isoforms. J Biol Chem 269:23694–23699
5. Fabiato A, Fabiato F (1979) Calculator programs for computing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells. J Physiol (Paris) 75:463–505
6. Fadool DA, Ache BW (1992) Plasma membrane inositol 1,4,5-trisphosphate-activated channels mediate signal transduction in Lobster olfactory receptor neurons. Neuron 9:907–918
7. Fasolato C, Hoth M, Penner R (1993) A GTP-dependent step in the activation mechanism of capacitative calcium influx. J Biol Chem 268:20737–20740
8. Hajnoczky G, Thomas AP (1994) The inositol trisphosphate calcium channel is inactivated by inositol-trisphosphate. Nature 370:474–477
9. Gerasimenko OV, Gerasimenko JV, Belan PV, Petersen O (1996) Inositol trisphosphate and cyclic ADP-ribose-mediated release of Ca^{2+} from single isolated pancreatic zimogen granules. Cell 84:473–480
10. Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflügers Arch 391:85–100
11. Hille B (1992) Ionic channels of excitable membranes. Sinauer Associates Sunderland, Mass.
12. Hoth M, Penner R (1992) Depletion of intracellular calcium stores activates a calcium current in mast cells. Nature 355: 353–356
13. Hoth M, Penner R (1993) Calcium release-activated calcium current in rat mast cells. J Physiol (Lond) 465:359–386
14. Irvine RF (1990) 'Quantal' Ca^{2+} release and the control of Ca^{2+} entry by inositol phosphates – a possible mechanism. FEBS Lett 263:5–9
15. Khan AA, Steiner JP, Snyder SH (1992) Plasma membrane inositol 1,4,5-trisphosphate receptor at lymphocytes: selective enrichment in sialic acid and unique binding specificity. Proc Natl Acad Sci USA 89:2849–2853
16. Khan AA, Steiner JP, Klein MG, Schneider MF, Snyder SH (1992) IP_3 receptor: localization to plasma membrane of T cells and cocapping with the T cell receptor. Science 257:815–818
17. Kiselyov KI, Mamin AG, Semyonova SB, Mozhayeva GN (1997) Low-conductance high selective inositol (1,4,5)-trisphosphate activated Ca^{2+} channels in plasma membrane of A431 cells. FEBS Lett 407:309–312
18. Kuno M, Gardner P (1987) Ionic channels activated by IP_3 in plasma membrane of T-lymphocytes. Nature 326:301–304
19. Lee MG, Xu X, Zend W, Diaz J, Wojcikiewicz R, Kuo TH, Wuytack F, Racymaekers L, Muallem S (1997) Polarized expression of Ca^{2+} channels in pancreatic and salivary cells. Correlation with initiation and propagation of $[Ca^{2+}]_i$ waves. J Biol Chem 272:15765–15770
20. Lewis RS, Cahalan M (1989) Mitogen-induced oscillations of cytoplasmic Ca^{2+} and transmembrane Ca^{2+} currents in human leukemic T cells. Cell Regul 1:99–112
21. Mak DD, Foskett JK (1994) Single-channel inositol 1,4,5-trisphosphate receptor currents revealed by patch clamp of isolated *Xenopus* oocyte nuclei. J Biol Chem 269:29375–29378
22. Maruyama Y (1993) Control of inositol polyphosphate-mediated calcium mobilization by arachidonic acid in pancreatic acinar cells of rat. J Physiol (Lond) 463:729–746
23. Mozhayeva GN, Naumov AP, Kuryshev YA (1990) Inositol 1,4,5-trisphosphate activates two types of calcium permeable channels in human carcinoma cells. FEBS Lett 277:233–234
24. Naumov AP, Kaznacheyeva EV, Kiselyov KI, Kuryshev YA, Mamin AG, Mozhayeva GN (1995) ATP-activated inward current and calcium-permeable channels in rat macrophage plasma membrane. J Physiol (Lond) 486:323–337
25. Parekh AB, Terlau H, Stuhmer W (1993) Depletion of $InsP_3$ stores activates a Ca^{2+} and K^+ current by means of a phosphatase and a diffusible messenger. Nature 364:814–818
26. Parekh AB, Fleig A, Penner R (1997) The store-operated calcium current I_{crac}: nonlinear activation by $InsP_3$ and dissociation from calcium release. Cell 89:973–980
27. Parys JB, Bezprozvanny I (1995) The inositol trisphosphate receptor of *Xenopus* oocytes. Cell Calcium 19:353–363
28. Premack BA, McDonald TV, Gardner P (1994) Activation of Ca^{2+} current in Jurkat T cells following the depletion of Ca2+ stores by microsomal Ca^{2+}-ATP-ase inhibitors. J Immunol 152:5226–5240
29. Putney JWJ (1990) Capacitative calcium entry revisited. Cell Calcium 11:611–624
30. Putney JWJ, Bird GSJ (1993) The inositol phosphate-calcium signaling system in nonexcitable cells. Endocr Rev 14(5): 610–631
31. Randriamampita C, Tsien RY (1993) Emptying of intracellular Ca^{2+} stores releases a novel small messenger that stimulates Ca^{2+} influx. Nature 364:809–814
32. Ribeiro CMP, Reece J, Putney JWJ (1997) Role of the cytoskeleton in calcium signaling in NIH 3T3 cells. An intact cytoskeleton is required for agonist-induced $[Ca^{2+}]_i$ signaling, but not for capacitative calcium entry. J Biol Chem 272:26555–26561
33. Ross CA, Meldolesi J, Milner TA, Satoh T, Supattapone S, Snyder SH (1989) Inositol 1,4,5-trisphosphate receptor localized to endoplasmic reticulum in cerebellar Purkinje neurons. Nature 339:468–470
34. Rossier MF, Bird GS, Putney JWJ (1991) Subcellular distrubution of the calcium-storing inositol 1,4,5-trisphosphate-sensitive organelle in rat liver. Possible linkage to the plasma membrane through the actin microfilaments. Biochem J 274: 643–650
35. Rowles S, Gallacher DV (1996) $Ins(1,3,4,5)P_4$ is effective in mobilizing Ca^{2+} in mouse exocrine pancreatic acinar cells if phospholipase A2 is inhibited. Biochem J 319:913–918
36. Ruknudin A, Song MJ, Sachs F (1991) The ultrastructure of patch-clamped membranes: a study using high voltage electron microscopy. J Cell Biol 112:125–134
37. Sasaki T, Shimura M, Wakui M, Ohkawara Y, Takishima T, Mikoshiba K (1994) Apically localized IP_3 receptors control chloride current in airway gland acinar cells. Am J Physiol 267:L152–L158
38. Strigoff F, Ehrlich B (1997) Regulation of intracellular calcium release channel function by arachidonic acid and leucotriene B4. Biochem Biophys Res Commun 237:413–418
39. Vaca L, Kunze DL (1995) IP_3-activated Ca^{2+} channels in the plasma membrane of cultured vascular endothelial cells. Am J Physiol 269:c733-c738
40. Wu C, Bute S, Yang Y, Anderson RG (1997) Tyrosine kinase receptors concentrated in caveolae-like domains from neuronal plasma membrane. J Biol Chem 272:3554–3559
41. Xu X, Zeng W, Muallem S (1996) Regulation of the IP_3-activated Ca^{2+} channel by activation of G proteins. J Biol Chem 271:11737–11744
42. Zweifach A, Lewis RS (1993) Mitogen-regulated Ca^{2+} current of T lymphocytes is activated by depletion of intracellular Ca^{2+} stores. Proc Natl Acad Sci USA 90:6295–6299