Low-conductance high selective inositol (1,4,5)-trisphosphate activated Ca^{2+} channels in plasma membrane of A431 carcinoma cells

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Abstract In many cells, activation of receptors coupled to PIP_2 turnover results in Ca^{2+} release from the intracellular stores accompanied by Ca^{2+} influx across the PM. It is not well established yet whether Ca^{2+} influx is activated by IP_3 or by an unknown signal generated upon Ca^{2+} store depletion. We report here a single-channel study of low-conductance IP_3-activated channels of very high selectivity for Ca^{2+} in the PM of A431 carcinoma cells. The channels are strongly potential dependent and sensitive to $[Ca^{2+}]_i$ within the physiological range. The data obtained argues for IP_3 acting directly on plasma membrane Ca^{2+} channels.

Key words: Inositol (1,4,5)-trisphosphate; Plasma membrane; Calcium channel; A431 cell

1. Introduction

In many non-excitable cells, activation of receptors coupled to PIP_2 metabolism evokes a biphasic rise in $[Ca^{2+}]_i$. This rise is due to both Ca^{2+} release from the intracellular stores and to Ca^{2+} influx across the PM. The fact that IP_3 releases stored Ca^{2+} via IP_3-activated Ca^{2+} channels is widely documented [1,2], while the data on the mechanisms of Ca^{2+} influx are controversial. The data available have led to two hypotheses: (i) IP_3 directly activates PM Ca^{2+} channels (IP_3-hypothesis) or (ii) Ca^{2+} influx is triggered by a not well characterized signal generated upon depletion of Ca^{2+} stores [3] (depletion-hypothesis). The IP_3-hypothesis has received a direct support from patch clamp [4–6] and biochemical studies [7]. The IP_3-activated Ca^{2+} channels in PM are reported to be voltage insensitive and selective to divalent cations with a conductance of 4–30 pS. On the other hand, Ca^{2+} conductances have been observed [8–10] when intracellular Ca^{2+} stores are emptied by $[Ca^{2+}]_i$ buffering, application of IP_3 or microsomal ATPase inhibitors, providing support for the depletion-hypothesis. In the present study we used a conventional patch clamp technique to demonstrate the existence of low-conductance IP_3-sensitive Ca^{2+} channels in the plasma membrane of A431 carcinoma cell, to investigate their properties and mechanisms of regulation.

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Abbreviations: IP_3, inositol (1,4,5)-trisphosphate; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; PM, plasma membrane; I_{IP3}, IP_3-activated Ca^{2+} currents; I_{crac}, calcium release-activated Ca^{2+} currents; IP_3R, IP_3 receptor; NP_o, channel's open probability; MP, membrane potential; PIP_2, phosphatidylinositol 4,5-bisphosphate; ER, endoplasmic reticulum; EGTA, ethylenebis(oxonitrilo)tetraacetate

2. Materials and methods

Human carcinoma A431 cells (Cell Culture Collection, Institute of Cytology, Russia) were kept in culture as described elsewhere [6] and were seeded onto coverslips for patch clamp experiments [11]. Currents filtered at 300 Hz were recorded using a PC 501A patch clamp amplifier (Warner Instr.) and digitized at 1–2 kHz. Pipette solutions contained (in mM): 105 $BaCl_2$ or 100 $CaCl_2$, 10 Tris-HCl (pH 7.4). Intracellular solution contained (in mM): 140 KCl or K glutamate, 5 NaCl, 1 $MgCl_2$, 10 HEPES/KOH, 1.13 $CaCl_2$ and 2 EGTA/KOH (pCa 7, pH 7.4). When Ca^{2-}-dependence of the channel activity was investigated, $[Ca^{2-}]_i$ was buffered by 10 mM EGTA and calculated using the algorithm of Fabiato and Fabiato [12]. No difference in the IP_3-induced activity was detected irrespective of whether Cl^- or glutamate was used. NP_o was determined from the following equation: $NP_o = <I>/i$ where $<I>$ and i are the mean channel current and unitary current amplitude, respectively. $<I>$ was estimated as the time integral of the patch current above the base line and i was determined from current records; N-number of permeable units present in the patch. Experiments were carried out at room temperature (22–24°C). Data are given as mean ± S.E.M. (number of experiments). Error bars denoting S.E.M. are shown where they exceed the symbol size.

3. Results and discussion

We used the inside-out mode of the patch clamp technique to test the effect of IP_3 on the PM of human carcinoma A431 cells. Addition of 0.2–10 μM IP_3 to the cytoplasmic surface of excised patches in 119 out of 228 attempts induced inward currents (I_{IP3}) of a rather small amplitude which could be carried by Ba^{2+} (105 mM) or Ca^{2+} (100 mM). Usually, no basal activity was observed after patch excision, except for some cases when rare inward current events were detected. Typically, it took a matter of seconds for activity to appear. The delay averaged 20.77 ± 6.61 s ($n = 13$) and 21.60 ± 9.46 s ($n = 5$) for 5 μM and 0.2 μM IP_3, respectively. This latency is similar to that reported in experiments on the effect of IP_3 on excised plasma membrane patches from non-excitable cells, such as T lymphocytes [4] and vascular endothelial cells [5].

Fig. 1A shows current record at a compressed time scale at different holding potentials prior to, and after application of 10 μM IP_3 in an experiment with 105 mM Ba^{2+} as the current carrier. In all experiments IP_3 was applied at holding potentials either −70 or −90 mV. The IP_3 removal abolished the activity completely ($n = 12$). As seen from the expanded fragments of the current records (Fig. 1B), channel openings are represented by current substates that are apparently multiple; single current transitions coexist with the duplicate ones. Corresponding all-points amplitude histograms (Fig. 1C) confirmed this observation. In most cases, the records expressed two current sublevels (see also Fig. 2). Rather high frequency of duplicate openings, coexistence of events exhibiting both amplitudes and direct transitions between the two types of

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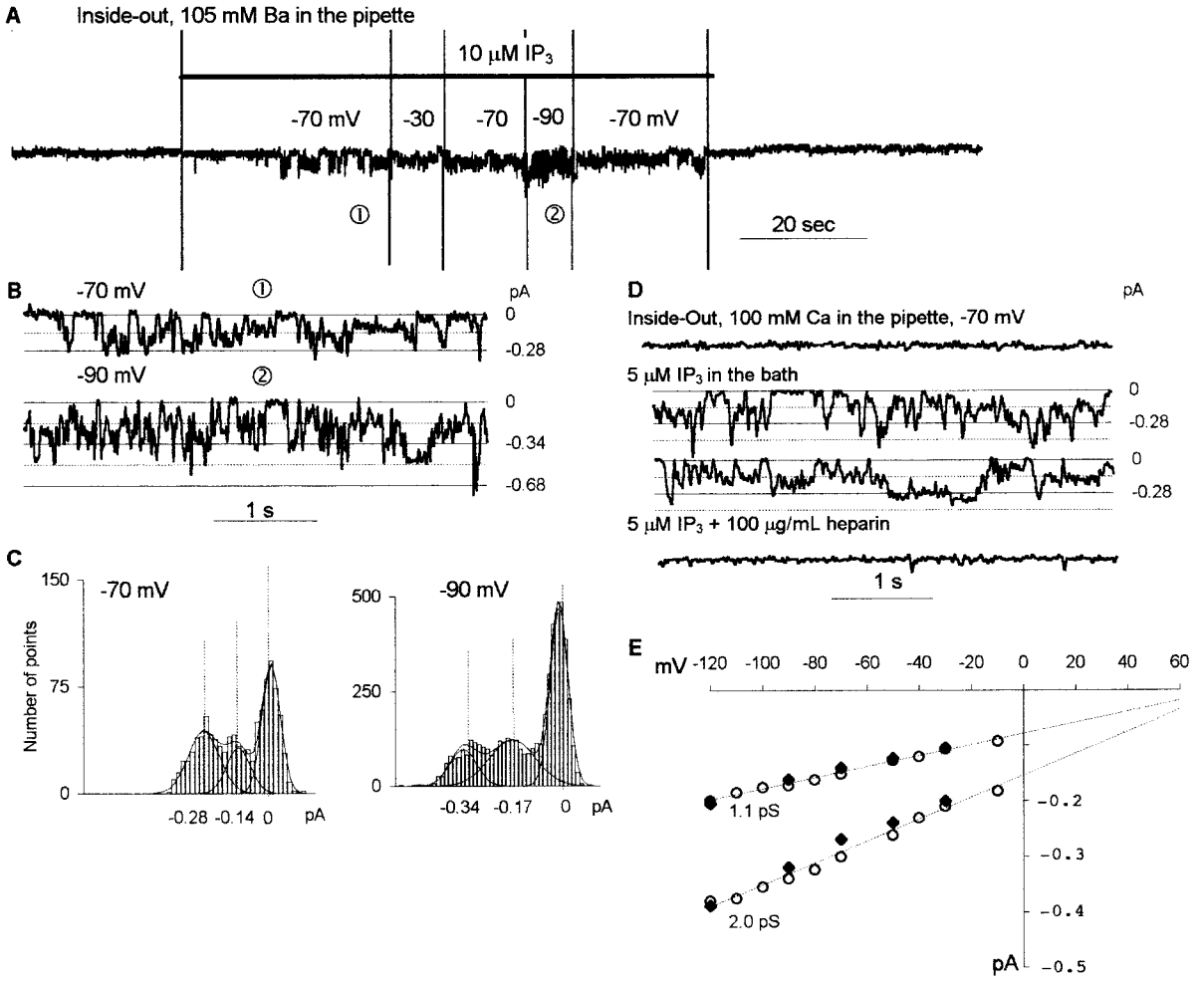

Fig. 1. Ba^{2+} and Ca^{2+} currents through IP_3-activated Ca^{2+}-permeable channels in inside-out patches of A431 cells plasma membrane. A: current trace recorded at different holding potentials prior to, and after application of 10 μM IP_3. 105 mM Ba^{2+} is in the pipette. Duration of IP_3 application is shown by solid line above the trace. Filter 50 Hz. B: current traces from different parts (indicated by figures) of the experiment presented in A. Horizontal lines pass through the current substates and figures on the right indicate current amplitude in pA. Filter 80 Hz. C: all points amplitude histograms constructed from traces in B and fitted by the sum of Gaussian distributions. Bin width 0.02 pA. D: current traces prior to and after application of 5 μM IP_3. 100 mM Ca^{2+} in the pipette. The bottom trace illustrates effect of heparin. E: mean current-voltage relations for two conductance substates of IP_3-induced channels with 105 mM Ba^{2+} (♦) ($n=16$) and 100 mM Ca^{2+} (○) ($n=7$) as permeant cation. Each point was derived from 3–7 experiments.

events have led us to suppose that IP_3 gates one channel with two current substates, rather than two different channels.

Surprisingly, the amplitudes of the currents with Ca^{2+} as the only permeant cation (Fig. 1D) were very close to those observed in experiments with Ba^{2+} in the pipette. Addition of heparin (100–500 μg/ml) known to block IP_3-binding sites [13] to the intracellular solution suppressed the IP_3-induced activity ($n=5$). Introduction of ATP (0.1 or 1 mM) in the solution containing IP_3 was not found to affect channel activity ($n=11$). The channels have equal permeabilities for Ba^{2+} and Ca^{2+} with slope conductances of about 1.1 and 2 pS for single and duplicate substates, respectively (Fig. 1E). Extrapolated reversal potentials were not less than +70 mV indicating very high selectivity of the channels for the divalent cations over potassium.

It should be noted that, in all experiments, the IP_3-induced channel activity was transient. This observation could be explained in terms of the recent discovery of the ability of IP_3 to inactivate IP_3-R in the ER [14]. On the other hand, in all experiments with Ca^{2+} as the current carrier, the transience of the IP_3-induced activity was much more evident. With Ca^{2+}, it lasted usually only 2–4 min, while with Ba^{2+} in the pipette it could be observed for up to 10 min in some experiments. The NP_o values were also much lower when Ca^{2+} was used as the permeant cation. They averaged 0.32 ± 0.20 ($n=4$) with Ca^{2+} and 0.61 ± 0.19 ($n=14$) with Ba^{2+} in the pipette solution for channels activated by 5 μM IP_3. These findings provide evidence for the involvement of Ca^{2+}-dependent inactivation in the channel rundown. Involvement of some factors modulating channel activity can not be ruled out either.

In a number of experiments, with Ba^{2+} as a charge carrier, the activity lasted long enough to perform several solution exchanges and short-term MP changes to investigate channel properties in detail. Experiments in which channel activity could not be restored to initial value after a return to original experimental conditions were discarded.

The dependence of the open probability of the I_{IP3} on IP_3 concentration is illustrated in Fig. 2A. The channel NP_o rose

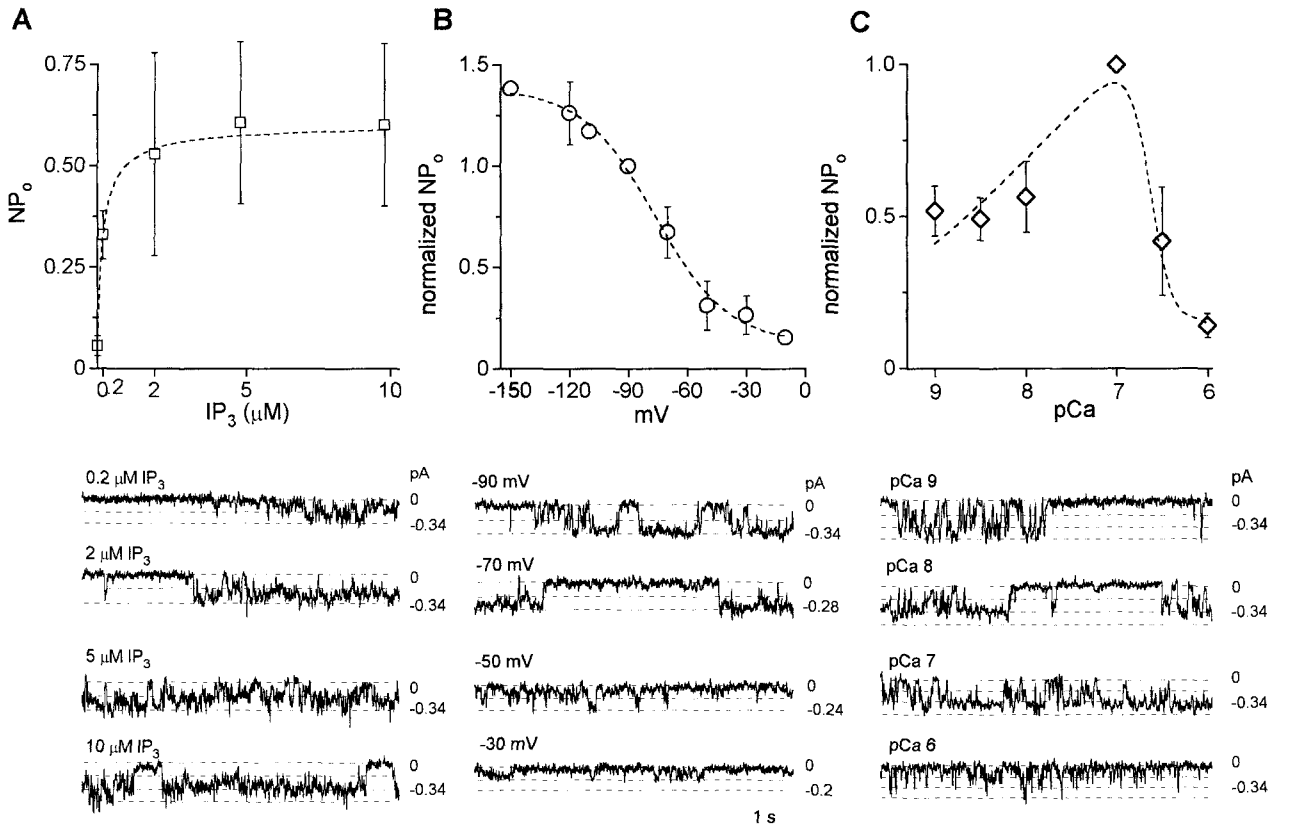

Fig. 2. Effect of membrane potential, IP_3 concentration and $[Ca^{2+}]_i$ on the open probability of IP_3-activated channels. A: dose-response curve for I_{IP3} activity expressed in NP_o. To calculate the NP_o the amplitude of the minimal current substrate was used in all cases. Bottom panel: traces of current induced by different IP_3 concentrations. B: plot of channels NP_o vs. membrane potential (data from 16 inside-out experiments). NP_o values were normalized with respect to the activity achieved at −90 mV. Bottom panel: traces of IP_3 (5 μM) induced currents at different MP. C: dependence of IP_3-induced channels NP_o on $[Ca^{2+}]_i$ expressed in pCa. Channel activity was monitored in the presence of 5 μM IP_3 at MP = −90 mV. Bottom panel: traces of currents at different pCa. 105 mM Ba^{2+} in the pipette. Filter 80 Hz. Horizontal lines pass through current sublevels which amplitudes are indicated in pA.

with increasing IP_3 concentration in the range 0.2–10 μM. Data were obtained from 25 patches under different IP_3 concentrations, though not all concentrations were tested on all patches. After the activity had reached steady state, data were collected from traces longer than 10 s to reduce the effect of activity fluctuations, but shorter than 40 s to avoid the influence of the time-dependent activity loss. Apparent half-maximal value of NP_o was attained at IP_3 concentration of about 0.2 μM. This set of experiments was performed at a holding potentials of either −70 or −90 mV, being kept unchanged throughout the entire experiment. Although it is shown below that the channel NP_o depends strongly on MP, it is NP_o variation from patch to patch rather than I_{IP3} channel voltage-dependence which is the main source of a large data deviation which disallows us to present the exact value of apparent binding constant and cooperativity of IP_3 binding. Traces of currents induced by different IP_3 concentrations are shown at expanded time scale on the bottom panel of Fig. 2A.

The channel NP_o decreased markedly upon depolarization (Fig. 2B); a half-maximal NP_o was achieved at a membrane potential of about −73 mV. It seems to us extremely important that the zone of maximal voltage-sensitivity covered both the resting membrane potential and the range of MP levels that occur during the hyperpolarization, induced by agonists under physiological conditions [15].

To clarify the involvement of Ca^{2+}-dependent inactivation in channel rundown, we performed a set of experiments in which effect of changes in the $[Ca^{2+}]_i$ on channel activity induced by 5 μM IP_3 was tested. $[Ca^{2+}]_i$ was buffered by 10 mM EGTA, and 105 mM Ba^{2+} was used as the charge carrier. Current traces under the curve in Fig. 2C demonstrate that use of solutions of different $[Ca^{2+}]_i$ gave rise to marked changes in channel activity. Data were collected from 10–20 s current records when the activity reached steady state. Within a given experiment the NP_o values were averaged and normalized with respect to the NP_o value obtained at pCa 7; these values are plotted in Fig. 2C vs. $[Ca^{2+}]_i$ expressed in pCa. As seen in the figure, the dependence of NP_o on $[Ca^{2+}]_i$ is bell-shaped but does not coincide exactly with the bell-shaped curve reported for the IP_3R from the ER [16]. Moreover, it cannot be explained in terms of a simple negative feedback mechanism of the type reported at the single-channel level for ATP-activated Ca^{2+} channels from the PM of rat peritoneal macrophages [17].

Attention should be drawn to the similarity of some specific properties of channels described in the present study to that of I_{crac}. Among them are high selectivity for Ca^{2+}, very low conductance of the channels, their strong voltage-dependence and activity inhibition by high $[Ca^{2+}]_i$. All that is mentioned above as well as the evidence [18] that the patch can contain

some organelles closely attached to PM allow one to explain our findings in terms of depletion-hypothesis. Indeed, IP_3 application would release Ca^{2+} from the stores attached to the patch and thus induce store depletion followed by I_{crac} activation. However, the following ideas and observations are inconsistent with the above supposition: (i) A diffusible messenger would not be effective, due to dilution, in experiments on an excised patch. If to suppose that the messenger traffic is strictly localized or that a mechanical link reports the state of the stores to the PM Ca^{2+} channels, then the patch exposure to ATP-free solution with Ca^{2+} buffered at a low level (with 10 mM of EGTA) would be expected to mimic the effect of microsomal ATPases inhibitors and induce store depletion. However, in our study no activity had developed within 10 min under these conditions. (ii) The IP_3 washout or heparin addition abolished the activity. This would not occur if there were a depletion-induced signal. (iii) The IP_3-R from the ER has been shown to have its open probability increased on ATP addition [2]. This means that store depletion and therefore the NP_o of the channel we have recorded would be enhanced by ATP if the channel is indeed activated by store depletion. In our case this was not true – the NP_o was not enhanced by ATP.

Taken together, the above evidences effectively rule out the possibility that the channel activity described could be attributed to the store depletion. Results of the present study provide a strong support for the notion that it is a direct effect of IP_3 on PM Ca^{2+} channels which is responsible for PIP_2 turnover-associated Ca^{2+} influx.

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