The effects of free [Ca^{2+}] on the cytosolic face of the inositol (1,4,5)-trisphosphate receptor at the single channel level

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Cytosolic free Ca^{2+} has been shown to have both activating and inhibitory effects upon the inositol (1,4,5) trisphosphate receptor ($InsP_3R$) during intracellular Ca^{2+} release. The effects of cytosolic free Ca^{2+} on the $InsP_3R$ have already been monitored using cerebellar microsomes (containing $InsP_3R$) incorporated into planar lipid bilayers [Bezprozvanny, Watras and Ehrlich (1991) Nature (London) **351**, 751–754]. In these experiments the open probability of the channel exhibited a 'bell-shaped Ca^{2+} dependence'. However, this has only been seen when the receptor is in the presence of its native membrane (e.g. microsomal vesicles). Using solubilized, purified $InsP_3R$ incorporated into planar lipid bilayers using the 'tip-dip' technique, investigations were carried out to see if the same effect was seen in the absence of the native membrane. Channel activity was observed in the presence of 4 μM $InsP_3$ and 200 nM free Ca^{2+}. Mean single channel current was 2.69 pA and more than one population of lifetimes was observed. Two populations had mean open times of approx. 9 and 97 ms. Upon increasing the free [Ca^{2+}] to 2 μM, the mean single channel current decreased slightly to 2.39 pA, and the lifetimes increased to 30 and 230 ms. Elevation of free [Ca^{2+}] to 4 μM resulted in a further decrease in mean single channel current to 1.97 pA as well as a decrease in lifetime to approx. 8 and 194 ms. At 10 μM free [Ca^{2+}] no channel activity was observed. Thus, with purified receptor in artificial bilayers, free [Ca^{2+}] on the cytosolic face of the receptor has major effects on channel behaviour, particularly on channel closure, although inhibition of channel activity is not seen until very high free [Ca^{2+}] is reached.

INTRODUCTION

Free [Ca^{2+}] can have both stimulatory and inhibitory effects on Ins(1,4,5)P_3-stimulated Ca^{2+} release, suggesting that Ca^{2+} released from the $InsP_3R$ channel exerts both positive and negative feedback on the channel activity [1–14].

Initial studies using rat cerebellar tissue [3] revealed that Ins(1,4,5)P_3 binding to the $InsP_3R$ was reversibly inhibited at 300 nM Ca^{2+} in particulate fractions and detergent-solubilized membranes. However, free [Ca^{2+}] as high as 1.5 mM was unable to inhibit binding to purified $InsP_3R$, and inhibition was only restored when detergent-solubilized cerebellar membranes were added back to the preparation. In permeabilized smooth muscle cells a biphasic effect of free [Ca^{2+}] on Ins(1,4,5)P_3-induced Ca^{2+} release was observed with a maximal rate of release at 300 nM [4]. Other studies, involving synaptosome-derived microsomal vesicles [5], have focused on the sub-second kinetics and regulation of Ins(1,4,5)P_3-induced Ca^{2+} release (IICR), determined using a rapid superfusion system. Extravesicular Ca^{2+} was found to act as a co-agonist, potentiating IICR with a maximum amount of Ca^{2+} being released at 500 nM free Ca^{2+}. Extravesicular Ca^{2+} levels greater than this produced a more slowly developing, reversible inhibition of release measured with a time constant of 580 ms at 10 μM free Ca^{2+} [5]. Further experiments, again using rapid filtration methods [13–15], have confirmed these earlier results [5] and have also demonstrated the occurrence of reversible Ca^{2+}-dependent desensitization of the channel.

The possible biphasic effects of cytosolic free [Ca^{2+}] on single channel events, using $InsP_3R$ in cerebellar microsomes incorporated into planar lipid bilayers, were studied by Bezprozvanny et al. [6]. As free [Ca^{2+}] was elevated from 10 to 250 nM open probability of the channel increased and then subsequently decreased at [Ca^{2+}] greater than 250 nM (the so-called 'bell-shaped' curve). Both activating and inhibitory effects of free [Ca^{2+}] were found to be reversible. All the evidence thus far has indicated that cytosolic [Ca^{2+}] regulates $InsP_3R$ function in a biphasic manner with maximum channel activity being seen at about 200–300 nM free [Ca^{2+}]; the regulation is reversible. The question therefore arises as to whether free Ca^{2+} is interacting directly with the receptor or via an associated Ca^{2+}-binding protein.

The purified $InsP_3R$ binds Ca^{2+} [16] and at least one putative Ca^{2+} binding site has been located in the coupling domain [17,18], indicating a direct interaction of Ca^{2+} with the receptor. In cerebellum, however, the majority of evidence suggests that an associated integral membrane protein (calmedin) is involved [16]. In order to determine whether an associated Ca^{2+}-binding protein plays a role in regulation of the $InsP_3R$ by cytosolic Ca^{2+}, or whether Ca^{2+} exerts its effects directly, partially purified $InsP_3R$ from rat cerebellum was incorporated into planar lipid bilayers. The method used was the 'tip-dip' technique which has the advantage of producing low noise single channel recordings. By using purified receptor, the potential role of an accessory protein in Ca^{2+} regulation is probably precluded, thus any effects observed should be as a result of a direct interaction of Ca^{2+} with the receptor itself.

METHODS

Purification of the $InsP_3R$

$InsP_3R$ was purified as described previously [19]. Briefly, $InsP_3R$ was solubilized in 1 % Triton-X100 and purified from rat cerebellum [3], using a two-step column purification procedure, the latter stage consisting of heparin affinity chromatography.

The heparin–agarose column was washed with 60 ml of 50 mM Tris/HCl pH 8.3, 1 mM EDTA, 1 mM β-mercaptoethanol,

Abbreviations used: $InsP_3R$, Ins(1,4,5)P_3 receptor; IICR, Ins(1,4,5)P_3-induced Ca^{2+} release; CICR, Ca^{2+}-induced Ca^{2+} release; ER, endoplasmic reticulum; PEG, poly(ethylene glycol); HEDTA, *N*-hydroxyethyl-EDTA.

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0.25 M NaCl and 0.1 % octylglucoside followed by elution with a minimum of 20 ml Tris/HCl pH 7.7, 1 mM 2-mercaptoethanol, 0.1 % octylglucoside and 0.5 M NaCl. The purified receptor was collected as 0.5 ml fractions. Binding activity was determined by the poly(ethylene glycol) (PEG) precipitation assay [19].

Binding assays to determine the effects of free [Ca^{2+}] on Ins(1,4,5)P_3 binding to partially purified InsP_3R and cerebellar membranes (both crude and detergent-solubilized)

Binding activity of purified receptor and cerebellar membranes was carried out as described [3]. The effects of 1 mM EDTA (the chelator present in the standard binding assay buffer), 1 mM *N*-hydroxyethyl-EDTA (HEDTA) (the concentration of chelator present in bilayer studies), and 30 mM HEDTA (the concentration of chelator necessary to achieve the appropriate free [Ca^{2+}] in subsequent binding assays) were initially determined using the PEG assay [19].

Standard PEG-precipitation radioligand binding assays were carried out as described [19] on samples of purified InsP_3R, detergent-solubilized membranes and crude cerebellar membranes in the presence of 200 nM, 2 μM, 5 μM and 10 μM free Ca^{2+}. Non-specific binding was determined in the presence of 1 μM Ins(1,4,5)P_3.

The 'tip-dip' method

The 'tip-dip' method was used as described previously [19]. The micropipettes used in this technique were made from borosilicate glass capillaries (Clark Electromedical Instruments, GC150-15) using a KOPF vertical pipette puller. As before [19] the pipette acted as the *trans* (luminal) compartment, containing 25 mM KCl, 53 mM $Ba(OH)_2$/Hepes, pH 7.35. The buffer used in the *cis* (cytoplasmic) chamber was 25 mM KCl, 250 mM Hepes-KOH, 1 mM HEDTA pH 7.35.

The lipids used consisted of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine in the ratio 7:3 (Avanti Polar Lipids)

The apparatus was set up as described [19]. Briefly, the lipid monolayer was spread over the surface of the buffer in the *cis* chamber. The micropipette tip was immersed in the buffer, withdrawn, and then immersed again creating a bilayer. Single channel events were observed following addition of InsP_3R protein, 150 μM $CaCl_2$ (giving a free [Ca^{2+}] of 200 nM) and 4 μM Ins(1,4,5)P_3 to the *cis* side. Recordings were made using a microcomputer together with an interface (CED 1401, Cambridge Electronic Design) and software PAT V6.1 for single channel analysis supplied by J. Dempster, University of Strathclyde, and Mathcad [20]. Since Ins(1,4,5)P_3 is only present on the *cis* side of the bilayer we assume that channel activity will only be seen from channel molecules incorporated with the Ins(1,4,5)P_3-binding domain oriented on that side of the bilayer. The concentration of free Ca^{2+} on the *cis* side was subsequently increased via stepwise additions to 2, 4 and 10 μM (free [Ca^{2+}] determined by the method described in Birch-Machin and Dawson [27]) and any changes in single channel activity monitored. Channel activity was abolished by the addition of heparin (15 μg/ml), an InsP_3R-specific antagonist, to the *cis* side.

RESULTS

Effects of free [Ca^{2+}] on Ins(1,4,5)P_3 binding to partially purified InsP_3R and cerebellar membranes (both crude and detergent-solubilized)

As mentioned in the Introduction, Ins(1,4,5)P_3 binding to InsP_3R is reversibly inhibited at 300 nM free [Ca^{2+}] in particulate fractions and detergent-solubilized membranes, although not in purified receptor preparations [3]. Upon restoration of detergent-solubilized membranes to purified preparations, inhibition of Ins(1,4,5)P_3 binding by Ca^{2+} is restored. Before proceeding with planar lipid bilayer experiments, it was necessary to verify these observations and to determine whether the Ca^{2+} chelators used in bilayer studies actually affected Ins(1,4,5)P_3 binding [28]. In addition, it was important to establish that the partially purified receptor used in these experiments was effectively free of the influence of calmedin on Ins(1,4,5)P_3 binding. Non-specific

Table 1 Effects of Ca^{2+} chelators on [^{3}H]Ins(1,4,5)P_3 binding to purified Ins(1,4,5)P_3 receptor

Between 5 and 10 μg receptor/assay sample was used.

Assay buffer	[^{3}H]Ins(1,4,5)P_3 binding (c.p.m.)
50 mM Tris/HCl (pH 8.3)/ 1 mM EDTA	3032 ± 141
50 mM Tris/HCl (pH 8.3)/ 1 mM HEDTA	2912 ± 169
50 mM Tris/HCl (pH 8.3)/ 30 mM HEDTA	3029 ± 223

Table 2 Effects of increasing free [Ca^{2+}] on [^{3}H]Ins(1,4,5)P_3 binding to purified Ins(1,4,5)P_3 receptor, crude cerebellar membranes and Triton X-100-solubilized cerebellar membranes

Concentration of receptor/assay sample is 5–10 μg. Concentration of cerebellar membranes and Triton X-100-solubilized cerebellar membranes used/assay sample is approx. 0.2 mg. Assay buffer is 50 mM Tris/HCl (pH 8.3)/1 mM HEDTA.

	[^{3}H]Ins(1,4,5)P_3 binding (c.p.m.)		
Free [Ca^{2+}]	Purified Ins(1,4,5)P_3 receptor	Crude cerebellar membranes	Triton X-100 cerebellar membranes
0	1191 ± 42	1430 ± 41	1073 ± 153
200 nM	1460 ± 48	840 ± 8	366 ± 44
2 μM	1131 ± 92	51 ± 14	21 ± 6
5 μM	1217 ± 57	19 ± 4	21 ± 7
10 μM	1461 ± 73	–	–

Table 3 Effects of increasing free [Ca^{2+}] on [^{3}H]Ins(1,4,5)P_3 binding to purified Ins(1,4,5)P_3 receptor (InsP_3R) in the presence of heparin flow-through (i.e. ER membrane components)

Concentration of receptor/assay sample is between 5 and 10 μg. Assay buffer is 50 mM Tris/HCl, pH 8.3, 1 mM HEDTA. Protein concentration of heparin flow-through is approximately 1 mg/ml. Concentration of NaCl in heparin flow-through is 0.25 M.

Sample	Free [Ca^{2+}]	[^{3}H]Ins(1,4,5)P_3 binding (c.p.m.)
InsP_3R	0	2280 ± 18
InsP_3R + heparin flow-through	0	170 ± 45
InsP_3R + heparin flow-through; NaCl removed	0	428 ± 29
InsP_3R + heparin flow-through; NaCl removed	200 nM	230 ± 39
InsP_3R + heparin flow-through; NaCl removed	2 μM	24 ± 17
InsP_3R + heparin flow-through; NaCl removed	5 μM	33 ± 24

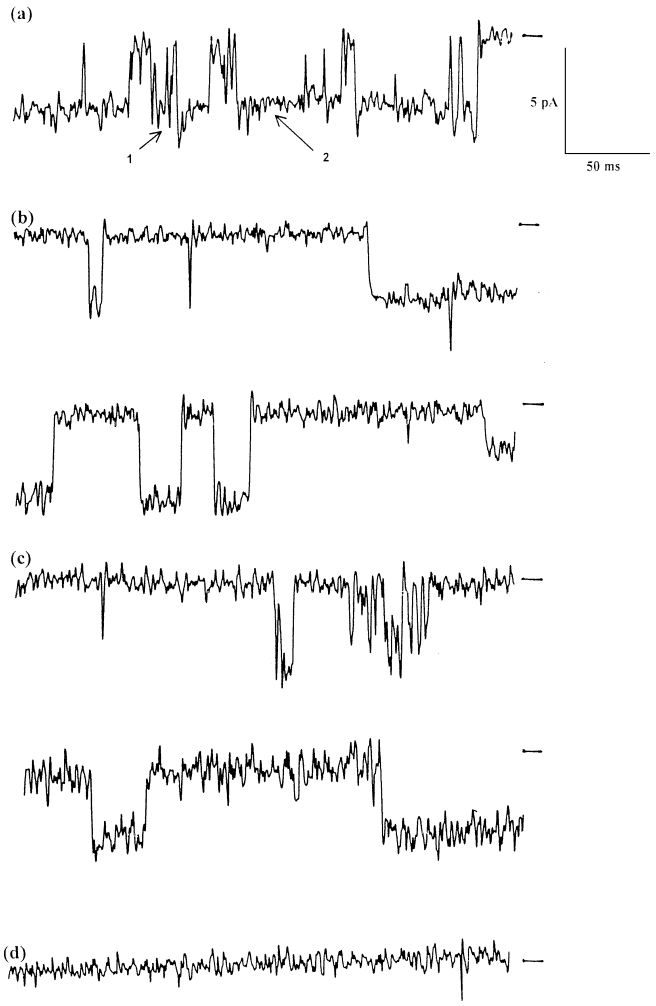

Figure 1 Single channel recordings of the Ins(1,4,5)P_3 receptor

Single channel recordings of the Ins(1,4,5)P_3R in the presence of 4 μM Ins(1,4,5)P_3 and (**a**) 200 nM free Ca^{2+}; (**b**) 2 μM free Ca^{2+}; (**c**) 4 μM free Ca^{2+}; and (**d**) 10 μM free Ca^{2+} at 0 mV applied potential difference. Openings are defined as downward deflections from the baseline (the closed state denoted as —). Note the short (denoted as 1) and long openings (denoted as 2).

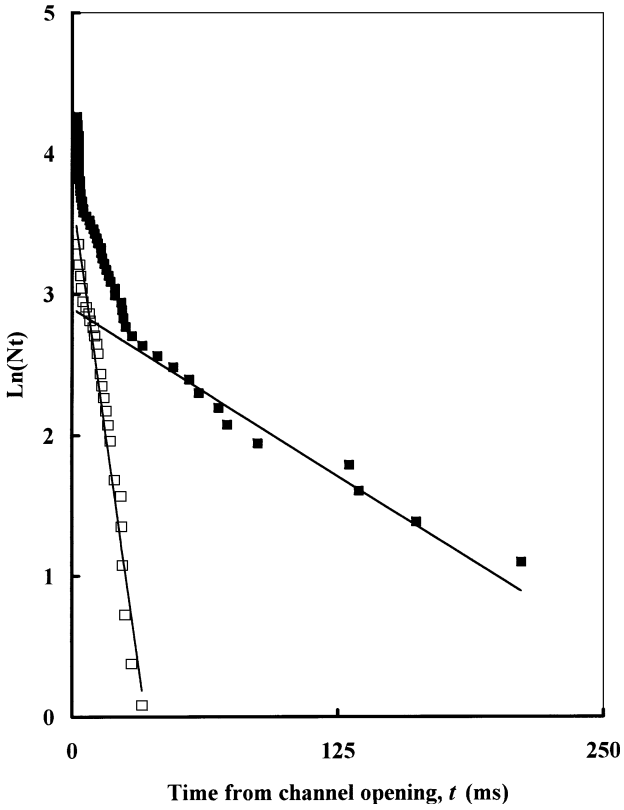

Figure 2 Determination of mean open time(s) for the Ins(1,4,5)P_3 receptor in the presence of 200 nM free Ca^{2+}

Current records like those in Figure 1(a) were analysed. Plotted against t is ln (N_t) where N_t is the number of channels with open time $\geqslant t$ (denoted by ■). Where the plot was linear, the gradient m was used to estimate mean open time τ by the relation $\tau = 1/m$. A linear fit was also applied to the initial few points on the curve, after removal of the longer open times, to yield another population of mean open times. This portion of the curve has been plotted separately (denoted by □). Two mean open time values were obtained: 9.39 ± 0.75 ms (S.E.M.) and 97.37 ± 11.05 ms (S.E.M.).

binding was determined in the presence of 1 μM Ins(1,4,5)P_3 and varying free [Ca^{2+}] (e.g. 200 nM, 2 μM, 5 μM, 10 μM). Experiments were carried out in triplicate.

As can be seen from Table 1 differences in chelators used in the binding buffers had little, if any, effect. The initial buffer, 50 mM Tris/HCl, pH 8.3, 1 mM EDTA, is a standard Ins(1,4,5)P_3 binding buffer although, for the purposes of these binding studies in addition to bilayer measurements, having EDTA as the chelator produces difficulties in finely controlling the range of free [Ca^{2+}] needed (e.g. 200 nM, 2 μM, 5 μM, 10 μM). It is for this reason that the lower affinity chelator HEDTA is used instead. HEDTA did not affect binding at either 1 or 30 mM.

Binding assays were then carried out, as before, to determine the effects of free [Ca^{2+}] on purified InsP_3R, crude cerebellar membranes and detergent-solubilized membranes. As can be seen from Table 2, increasing free [Ca^{2+}] has virtually no effect on [^{3}H]Ins(1,4,5)P_3 binding to purified receptor whereas it inhibits [^{3}H]Ins(1,4,5)P_3 binding in crude cerebellar membranes and detergent-solubilized membranes. From other studies [3] adding back the flow-through from the heparin column (which contains detergent-solubilized ER membrane components) should restore Ca^{2+}-sensitivity to the purified InsP_3R preparation. However, in these experiments, under control conditions (i.e. in the absence of free Ca^{2+} and in the presence of heparin flow-through), [^{3}H]Ins(1,4,5)P_3 binding was greatly reduced (see Table 3, rows 1 and 2) from 2280 to 170 c.p.m. A possible explanation for this is that 0.25 M NaCl present in the flow-through may in some way affect the binding, so the flow-through was desalted by dialysis against 50 mM Tris/HCl, pH 7.7, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 1% Triton X-100. The number of specific counts detected using the desalted fraction was increased although still far removed from the original control value (cf. rows 1 and 3 of Table 3). Nevertheless, a sufficient number of counts were available to demonstrate that a component in the heparin flow-through had indeed restored Ca^{2+}-sensitivity to the purified receptor preparation (see Table 3, rows 3–6). Testing just the flow-through alone showed that there was no specific [^{3}H]Ins(1,4,5)P_3 binding present in this fraction.

InsP_3R channel activity in the presence of 200 nM free [Ca^{2+}]

After addition of 4 μM Ins(1,4,5)P_3 and 1–8 μg of partially purified InsP_3R (specific binding approx. 400 pmol/mg of

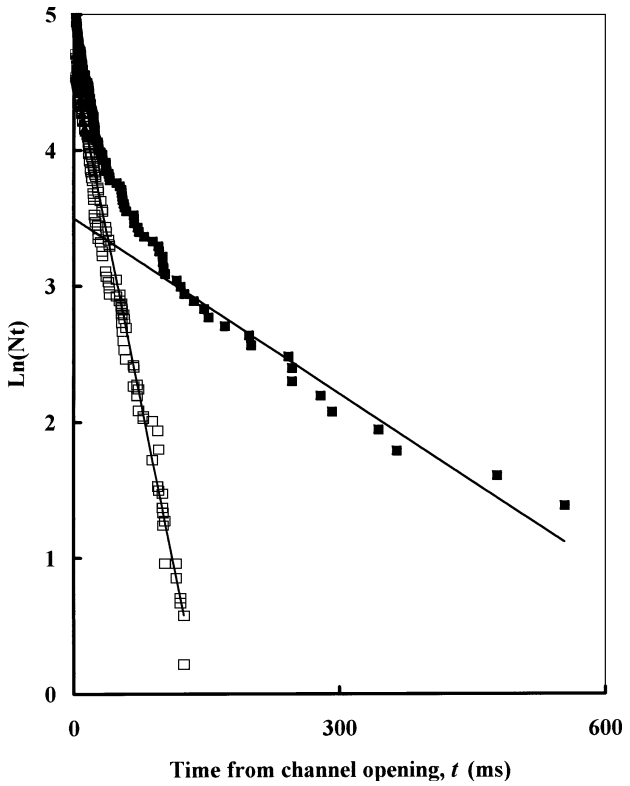

Figure 3 Determination of mean open time(s) for the Ins(1,4,5)P_3 receptor in the presence of 2 μM free Ca^{2+}

Current records like those in Figure 1(b) were analysed, as before. Symbols used (■, □) have the same meanings. Again, two mean open time values were found only they had increased to 30.92 ± 1.19 ms (S.E.M.) and 231.64 ± 24.04 ms (S.E.M.).

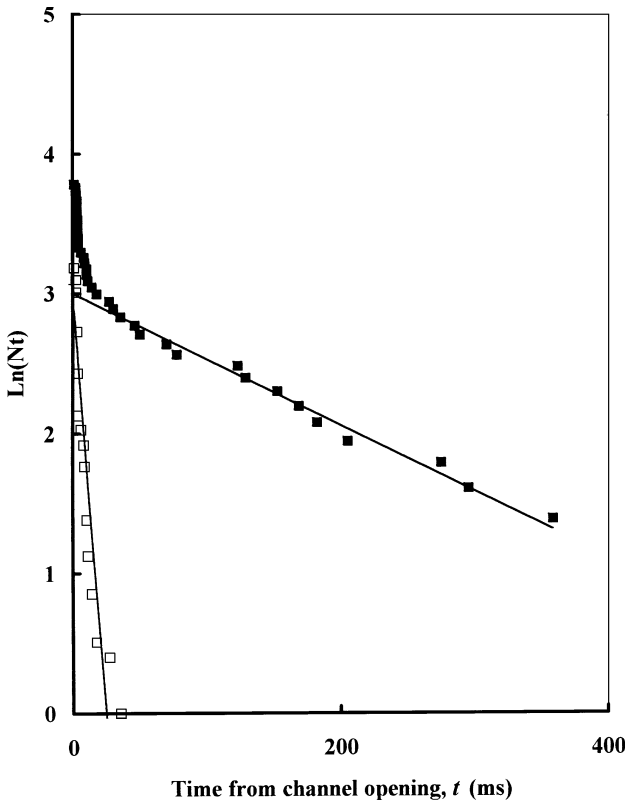

Figure 4 Determination of mean open time(s) for the Ins(1,4,5)P_3 receptor in the presence of 4 μM free Ca^{2+}

Data was derived from records similar to those shown in Figure 1(c). Analysis was as for Figures 2 and 3, the symbols (■) representing the complete data set with the linear fit shown, and (□) representing the open events, with long open times removed, and linear fit shown. Again, two mean open time values were found: 8.26 ± 1.27 ms (S.E.M.) and 194.33 ± 13.26 ms (S.E.M.).

protein, showing one major band on SDS/PAGE gels, see [19] for additional details) to the *cis* chamber, in the presence of 200 nM free Ca^{2+}, single channel events were recorded (see Figure 1a). The experiment shown is typical of four similar separate recordings and the trace represents one sixty-fourth of the total data record. The mean single channel current step was 2.69 ± 0.18 pA (S.E.M., $n = 45$) at zero applied potential difference (PD).

The mean open time(s) of the channel were determined, as described previously [19], by plotting $\ln(N_t)$ (where N_t is the number of channels with a lifetime $\geqslant$ t) against time (ms). Where this plot was linear, the gradient, m, was used to estimate mean open time τ by use of the relation $\tau = 1/m$. Figure 1 shows the data from four separate sets of records of channel openings plotted in this way. Analysis of the total population of channels gives a nonlinear plot, indicating that there is more than one mean channel open time. The linear portion of the curve shown in Figure 2 has a slope indicating a population of channels with a mean open time of 97.4 ± 11.0 ms (S.E.M.). When this population was stripped out of the data, the remaining channel lifetimes fitted on a single straight line and had a much shorter mean open time of 9.4 ± 0.8 ms (S.E.M.). Thus, the channel can apparently open in two states with different mean open times. This behaviour can be seen by inspection of the channel records (Figure 1a), where both long and short events are present. Approximately 75% of the total number of open events are short and the other 25% are long events.

Analysis of closed times at 200 nM free $[Ca^{2+}]$ was carried out using the same method as for open times and a double-exponential fit was obtained, indicating two populations of closed times (data not shown). The values were 72.5 ± 4.8 and 1150 ± 180 ms.

InsP_3R channel activity in the presence of 2 μM free $[Ca^{2+}]$

Further recordings were carried out after increasing free $[Ca^{2+}]$ to 2 μM, and again, single channel events were recorded (see Figure 1b). The experiment shown is typical of four similar separate recordings. The mean single channel current step was 2.39 ± 0.11 pA (S.E.M., $n = 109$) at zero applied potential difference.

Again, by plotting $\ln(N_t)$ against time (ms), the mean open time(s) of the channels were determined. Analysis of the total population of channels gave a nonlinear plot, again indicating more than one channel half-life. The linear region describing the long open time events (shown in Figure 3) had a slope yielding a mean open time of 231.6 ± 24.0 ms (S.E.M.). Stripping out the long open time events left another straight line, giving a shorter mean open time of 30.9 ± 1.2 ms (S.E.M.). The channel once again opens in two states with different mean open times although, compared with data at 200 nM free $[Ca^{2+}]$, both are markedly increased. Again, approx. 75% of the total number of open events are short and the other 25% are long events.

Closed times were calculated as before and again, two populations of closed time were detected (data not shown). The values were 51.3 ± 2.4 and 1120 ± 50 ms.

InsP_3R channel activity in the presence of 4 μM free [Ca^{2+}]

Similar experiments, in the presence of 4 μM free [Ca^{2+}], were carried out. Single channel events were recorded (see Figure 1c). The experiment shown is typical of two similar separate recordings. The mean single channel current step was 1.97 ± 0.1 pA (S.E.M., $n = 44$) at zero applied potential difference.

A plot of $\ln(N_t)$ against time (ms) gave a nonlinear plot. Manipulation of the data as previously described, gave a long mean open time of 194.3 ± 13.3 ms (S.E.M.) (see Figure 4). The shorter mean open time was 8.3 ± 1.3 ms (S.E.M.). The channel opened, again in two states with different mean open times, but in the presence of 4 μM free [Ca^{2+}] a decrease was seen. In this instance approx. 50 % of the total number of open events are short and 50 % are long.

Two populations of closed times were seen (data not shown) with values of 102 ± 10 and 4900 ± 2100 ms.

InsP_3R channel activity in the presence of 10 μM free Ca^{2+}

In the presence of 10 μM free Ca^{2+} with conditions as described previously, no channel activity was seen. This is typical of three similar separate experiments.

Theoretically, it is possible to add back chelator to the *cis* compartment to lower the free [Ca^{2+}] thus demonstrating the reversibility of this effect. Unfortunately, this proved technically too difficult to achieve using the 'tip-dip' method. In addition, it would be ideal to add back membrane components (e.g. detergent-solubilized or heparin flow-through) to the *cis* compartment and demonstrate restored Ca^{2+} sensitivity upon the receptor. However, the high levels of Triton X-100 present in these components would completely disrupt the bilayer, thus rendering this line of experimentation impossible.

DISCUSSION

From the data it can be seen that increasing free [Ca^{2+}] on the *cis* side tends to decrease the single channel current, suggesting inhibition of receptor activity. At 200 nM free [Ca^{2+}] the current is 2.69 ± 0.18 pA and this decreases as the free [Ca^{2+}] increases into the micromolar range (e.g. 2.39 ± 0.11 pA at 2 μM free Ca^{2+} and 1.97 ± 0.16 pA at 4 μM free Ca^{2+}). By carrying out a two-tailed t test, P-values were obtained to determine whether there was any significant difference between these reductions in current. Comparison of single channel current at 200 nM free [Ca^{2+}] with that at 2 μM gave a P-value of 0.16, indicating no significant difference. Comparison of current at 2 and 4 μM free [Ca^{2+}] gave a P-value < 0.05. For the current values obtained at 200 nM and 4 μM free [Ca^{2+}], however, $P < 0.01$, indicating a highly significant decrease in mean single channel current as free [Ca^{2+}] increases from submicromolar to micromolar concentrations. This observation was confirmed by using analysis of variance (Microsoft Excel 5.0, statistic option) which compared all three sets of data to give a P-value of 0.02, verifying the decrease in current. However, there is also an increase in channel lifetime going from 200 nM to 2 μM free [Ca^{2+}] and since the closed time remains similar this would suggest an activation of the channel. In order to determine whether channel activation is really taking place it would be necessary to observe channel activity over the range of 10–200 nM free [Ca^{2+}], although an underlying difficulty in this case is that a sufficient concentration of Ca^{2+} is required for artificial bilayer formation using these lipids, thus making this line of investigation difficult. The increase in lifetime is reversed by increasing free [Ca^{2+}] from 2 to 4 μM, again consistent with inhibition of channel activity at higher free [Ca^{2+}]. No channels were observed at a free [Ca^{2+}] of 10 μM. At all Ca^{2+} concentrations studied two different open times were observed, with proportionally slightly fewer short events at higher free [Ca^{2+}]. Two different open times imply two different open states of the channel (in this case with the same conductance), and this is clearly of potential importance in control of Ca^{2+} flux. It may also relate to the commonly observed biphasic kinetics of Ca^{2+} release but, as discussed below, we do not have sufficient temporal resolution to monitor effects immediately following Ins(1,4,5)P_3 addition.

It is apparent from this data and that of others [3–6] that InsP_3R has a Ca^{2+}-dependence and the question arises as to how Ca^{2+} exerts its stimulatory/inhibitory effects. As mentioned earlier, purified InsP_3R has been shown to bind Ca^{2+} [3] and in the coupling domain of the receptor, at least one Ca^{2+}-binding site has been identified [17,18]. This implies a direct interaction of Ca^{2+} with the receptor and therefore possibly direct control. However, addition of detergent-solubilized cerebellar membrane preparations to purified InsP_3R restores the inhibitory effect of Ca^{2+} on Ins(1,4,5)P_3 binding, indicating that perhaps the InsP_3R, in intact membranes, is closely associated with a controlling Ca^{2+}-binding protein [3]. One such protein called calmedin, which was referred to earlier, has been identified [16] as a result of these observations. In addition, experiments carried out using purified InsP_3R from *Xenopus* oocytes have indicated the involvement of an associated protein which mediates the Ca^{2+}-induced inhibition of the InsP_3R [21]. It is not yet clear whether all cell types expressing type I receptor also express calmedin. Therefore, while in neuronal tissues the effects of calmedin will predominate, the direct effects observed here on channel lifetime and conductance may be important in other cell types.

Another possible mode of action is that Ca^{2+} desensitizes the receptor, either in conjunction with a Ca^{2+}-binding protein or by a direct effect on the channel, rather than (or as well as) decreasing Ins(1,4,5)P_3 binding. Some studies, using human InsP_3R type I, overexpressed in insect ovary Sf9 cells, have shown that as free [Ca^{2+}] increases from 3 nM to 1.4 μM the affinity for Ins(1,4,5)P_3 decreases while there is no overall change in the number of Ins(1,4,5)P_3 binding sites [22]. Thus it is apparent that several options exist as to how Ca^{2+} may mediate its regulatory effects, ranging from the involvement of accessory proteins, to desensitization, to decreases in binding affinity.

The results from this study suggest that the isolated InsP_3R is indeed Ca^{2+}-activated up to a micromolar range and inhibition occurs in the 4–10 μM region although the changes in channel parameters are not very large. Experiments from Hajnóczky and Thomas [15] and Finch et al. [23], suggest that there are time-dependent activation/inactivation effects of Ca^{2+} and Ins(1,4,5)P_3 on the receptor. The static bilayer experiments described in this study would not be suitable for detecting transient changes on a rapid time scale. It is quite possible that the effects of Ca^{2+} observed reflect much greater changes which are lost during long-term adaptation (see, for example, Gyorke and Fill [24], with respect to the ryanodine receptor). As well as temporal resolution, a further difference with this work and previous data is that it is carried out using purified receptor, therefore neither native membrane (and hence calmedin) or kinases/phosphatases should be present in these experiments. Others have put forward the idea of Ca^{2+}-activated protein kinases and phosphatases (protein kinase C and calcineurin respectively) altering the phosphorylation state of the receptor and hence its Ca^{2+}-flux properties [25], and although recent studies have shown that calcineurin is still closely associated with purified receptor [25], no ATP is present in the experimental system used, therefore it is unlikely that these influences will be present.

The major implication from this study is that with isolated

receptor, much less pronounced effects of cytosolic Ca^{2+} are seen than in those studies using intact ER membranes. It therefore seems likely that accessory proteins are involved in sensitization to Ca^{2+}. Notably, however, the inhibition of channel current by 4 μM free [Ca^{2+}] has not been previously reported. In the studies of Bezprozvanny et al. [6], because of the presence of calmedin, inhibition of channel opening at these higher free [Ca^{2+}] may have obscured any decrease in channel conductance. Work carried out by Kaftan et al. [26] showed that the width of the 'bell-shaped' Ca^{2+}-dependence increased as Ins(1,4,5)P_3 concentrations increased from 0.02 to 2 μM. However, complete inhibition of Ins(1,4,5)P_3 binding was not seen, even up to concentrations as high as 100 μM Ca^{2+}, whereas Ins(1,4,5)P_3-gated channel activity could be inhibited by 5 μM Ca^{2+}. This inability to explain the descending phase of the bell-shaped curve as a Ca^{2+}-dependent inhibition of Ins(1,4,5)P_3-binding has led to the development of a multistate model which can explain the Ca^{2+}-dependent changes both in terms of binding and channel activity. The model assumes that there are three ligand-binding sites on the receptor: an Ins(1,4,5)P_3-binding site, an activating Ca^{2+}-binding site and an inhibitory Ca^{2+}-binding site. In view of the fact that the experiments discussed in our study are carried out at saturating Ins(1,4,5)P_3 concentrations, the results strongly indicate that the effects Ca^{2+} on channel conductance are independent of Ins(1,4,5)P_3 binding, and that they are directly on the receptor. However, none of the present multi-state models explicitly include a receptor state with a decreased conductance, rather a decreased open probability.

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