Interaction of Luminal Calcium and Cytosolic ATP in the Control of Type 1 Inositol (1,4,5)-Triphosphate Receptor Channels*

Edwin C. Thrower‡, Hamid Mobasheri, Sheila Dargan, Phedra Marius, Edward J. A. Lea, and Alan P. Dawson$ 

From the School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, United Kingdom

Ca$^{2+}$ within intracellular stores (luminal Ca$^{2+}$) is believed to play a role in regulating Ca$^{2+}$ release into the cytosol via the inositol (1,4,5)-trisphosphate (Ins(1,4,5)-P$_3$)-gated Ca$^{2+}$ channel (or Ins(1,4,5)-P$_3$ receptor). To investigate this, we incorporated purified Type 1 Ins(1,4,5)-P$_3$ receptor from rat cerebellum into planar lipid bilayers and monitored effects at altered luminal [Ca$^{2+}$] using K$^+$ as the current carrier. At a high luminal [Ca$^{2+}$] and in the presence of optimal [Ins(1,4,5)-P$_3$] and cytosolic [Ca$^{2+}$], a short burst of Ins(1,4,5)-P$_3$ receptor channel activity was followed by complete inactivation. Lowering the luminal [Ca$^{2+}$] caused the channel to reactivate indefinitely. At luminal [Ca$^{2+}$], reflecting a partially empty store, channel activity did not inactivate. The addition of cytosolic ATP to a channel inactivated by high luminal [Ca$^{2+}$] caused reactivation. We provide evidence that luminal Ca$^{2+}$ is exerting its effects via a direct interaction with the luminal face of the receptor. Activation of the receptor by ATP may act as a device by which cytosolic Ca$^{2+}$ overload is prevented when the energy state of the cell is compromised.

The second messenger inositol (1,4,5)-trisphosphate (Ins(1,4,5)-P$_3$) binds to Ins(1,4,5)-P$_3$ receptors in the endoplasmic reticulum, causing release of stored Ca$^{2+}$ into the cell. The subsequent changes in localized levels of cytosolic-free Ca$^{2+}$ have been shown to exert both stimulatory and inhibitory effects on the Ins(1,4,5)-P$_3$ receptor (1–15). This regulation of Ins(1,4,5)-P$_3$ receptors by cytosolic Ca$^{2+}$ is highly concentration-dependent. Small elevations in cytosolic [Ca$^{2+}$] (<300 nM) potentiate Ca$^{2+}$ release and channel opening, whereas higher concentrations are inhibitory (4–6). Whereas the role of cytosolic Ca$^{2+}$ is well documented, the potential function of intraluminal Ca$^{2+}$ within the stores remains controversial. A role for intraluminal Ca$^{2+}$ has been proposed (16) in which Ca$^{2+}$ content of the stores modulates the sensitivity of the Ins(1,4,5)-P$_3$ receptor to Ins(1,4,5)-P$_3$, and there is some evidence supporting this (9, 17–25), although there is also evidence against it (8, 26–31). A luminal high affinity Ca$^{2+}$ binding site has been identified in mouse type I receptor (32) mapped to the non-conserved acidic sub-region of the luminal loop between amino acids 2463 and 2528, although whether it participates in the proposed regulation is unclear. A further possible role for luminal Ca$^{2+}$ interaction with the Ins(1,4,5)-P$_3$ receptor is in the control of capacitative Ca$^{2+}$ entry. The idea of conformational coupling between Ins(1,4,5)-P$_3$ receptor and a Ca$^{2+}$ entry channel was proposed by Irvine (16) and elaborated by Berridge (33). Recently, direct evidence for interaction between Ins(1,4,5)-P$_3$ receptors and Ca$^{2+}$ entry channels in controlling capacitative Ca$^{2+}$ entry in a variety of cells has been presented (34–36). A key question now is whether or not Ins(1,4,5)-P$_3$ receptors are capable of direct transduction of the signal concerning the Ca$^{2+}$ filling status of the endoplasmic reticulum to plasma membrane Ca$^{2+}$ entry channels or whether accessory proteins are involved. Planar lipid bilayer experiments allow manipulation of the free [Ca$^{2+}$] on either the cytosolic (cis) or luminal (trans) face of the Ins(1,4,5)-P$_3$ receptor, thereby providing a means for studying intraluminal Ca$^{2+}$ effects under conditions where the free Ca$^{2+}$ concentrations on either side are clamped at a set value throughout the experiment. Bezprozvanny and Ehrlich (37) investigated the effects of trans (or intraluminal) Ca$^{2+}$ using cerebellar microsomes fused to planar lipid bilayers, with Sr$^{2+}$, Ba$^{2+}$, or Mg$^{2+}$ as current carrier (the use of K$^+$ was precluded by the presence of K$^+$ channels in the microsomal membranes). They found that, in contrast to the effects predicted by Irvine (16), intraluminal Ca$^{2+}$ slightly inhibited channel activity at concentrations greater than 1 mM. However, interpretation of the data is complicated by the possibility of interactions of the divalent cations used as current carriers with potential Ca$^{2+}$ binding sites on the luminal face of the receptor. To resolve this complication, we have used purified cerebellar Type 1 receptor inlaid into bilayers prepared by the method of Schindler (38). This has enabled us to use K$^+$ as current carrier, and since the luminal face of the Ins(1,4,5)-P$_3$ receptor is likely to be exposed physiologically to around 100 μM K$^+$, K$^+$ is unlikely to interfere with any physiologically relevant properties of the receptor, unlike Sr$^{2+}$ and other divalent cations (39, 40). Published estimates of luminal free Ca$^{2+}$ are quite varied depending on the method of measurement (41). However, most recent estimates seem to put the value for a full Ca$^{2+}$ store at around 0.5 mM (42). Accordingly, we have looked at the effects of luminal Ca$^{2+}$ at 1 mM (the upper end of the estimate for a full store), 0.6 mM (around the consensus estimate for a full store), 100 μM (the lower end of the estimate for a full store and the consensus value for a partially empty store), and 300 mM (a totally depleted store).

In essence, we find that at 1 and 0.6 mM luminal Ca$^{2+}$, there is a very rapid run-down of channel activity. This channel activity can be restored by decreasing the Ca$^{2+}$ on the trans side of the bilayer. Importantly, channel run-down is not ob-
FIG. 1. Western blot analysis of InsP$_3$R preparations. i, purified Type 1 InsP$_3$ receptor (InsP$_3$R-1) was probed with anti-Type 1 InsP$_3$ receptor, and only one band was observed. Purified Type 1 InsP$_3$ receptor (P) and brain homogenate (B) (20 mg of total protein/lane) were probed with antibodies against Type 1 InsP$_3$ receptor (ii), calmodulin (iii), and FKBP12 (iv). The arrows indicate the positions of the 205-kDa marker, purified calmodulin, and recombinant FKBP12, respectively. The purified protein lane was deliberately overloaded with InsP$_3$R receptor to try to detect associated FKBP12 and calmodulin. The blots are typical results from six independent preparations. c shows the intensity of staining of 50 ng of recombinant FKBP12 run on the same blot as (ii).

RESULTS

Channel Activity at 1 mM Intraluminal (trans) Ca$^{2+}$—Initially, experiments were carried out with 1 mM Ca$^{2+}$ on the trans side and 300 mM on the cis side. The cis side is defined as the cytosolic face of the receptor by being the side to which Ins$_{1,4,5}$P$_3$ is added. A typical recording is shown in Fig. 2. No channel activity was observed until after addition of Ins$_{1,4,5}$P$_3$ (5 mM) to the cis compartment. After Ins$_{1,4,5}$P$_3$ addition, a short burst of channel activity was seen. The time that elapsed between Ins$_{1,4,5}$P$_3$ addition and commencement of channel activity varied between experiments, probably because it was not possible to mix the contents of the compartment rapidly and maintain the bilayer. However, activity normally began within 60 s and ceased completely after a further few seconds. Typical channel activity is shown in Fig. 2A, where the applied p.d. was -100 mV. The traces have been expanded, and details of the individual channels can be seen in Fig. 2B. The record shows short-lived events. The lifetime of some of the events is close to the resolution time (0.5 ms) for data acquisition, and the channel events are thus not as clean as they might otherwise be. Nevertheless, the open state is fitted by a single Gaussian distribution (Fig. 2C), with a peak current of 2.3 ± 0.7 pA (S.D.), equivalent to a conductance of 23 pS. For the section of the record shown in Fig. 2B, the areas under the Gaussian curves describing the closed state (marked I in Fig. 2C) and open state (marked II in Fig. 2C) show that the channel spent 50% of the time in each state. After the initial burst of channel events, activity did not resume, even up to 2 h.
indicating inhibition by 1 mM luminal Ca$^{2+}$. However, the channel was still present and intact in the bilayer because it could be re-activated by potential changes (see below and Fig. 3). The data shown in Fig. 2, A and B, is representative of four similar experiments. Similar data was obtained at 0.6 mM Ca$^{2+}$.

In some experiments, reversing the applied p.d. from −100 to +100 mV resulted in brief re-activation of the channel before it inactivated again. A typical example of this behavior is shown in Fig. 3.

**Effects of 300 nM Intraluminal (trans) Ca$^{2+}$**—After adjusting the trans [Ca$^{2+}$] to 300 nM, channel activity recommenced (Fig. 4A). After the initial channel activation and inactivation at −100 mV, bursts continued for the duration of the experiment or until the addition of heparin (15 μg/ml) to the cis compartment. A selection of typical traces from this record was expanded and can be seen in Fig. 4B. The characteristics of the channels observed at 300 nM trans Ca$^{2+}$ are very different from those seen at 1 and 0.6 mM trans Ca$^{2+}$. An all-points amplitude histogram of the complete record (not shown) gave a value of 29.6 ± 8.8 pS (S.D.), and analysis of mean open time revealed two populations of open times with values of 0.7 and 6.2 ms using a maximum likelihood fit. Extremely long open times were also observed (in the region of tens of milliseconds, e.g. Fig. 4B, i and ii), but they were few in number, making conventional lifetime analysis difficult. They were therefore excluded from the analysis. Long open times have been observed for the Ins(1,4,5)P$_3$ receptor in previous studies (15). In one particular experiment at 300 nM trans [Ca$^{2+}$], the channel was seen to be open for up to 8 s continuously in a higher conductance state (approximately 55 pS) before flickering between two lower conductance levels (approximately 37 and 24 pS) and finally closing (data not shown). The channel remained closed for approximately 1 min and then suddenly opened again to the highest conductance state (again 55 pS) before exhibiting the same behavior (transition to two lower levels, then closure).

Thus, in contrast to the data at 1 mM trans Ca$^{2+}$, the channel at 300 nM trans Ca$^{2+}$ shows much less spontaneous inactivation, longer open times, and a higher conductance as well as multiple conductance states.
Fig. 4. Single channel activity in the presence of 300 nM intraluminal (trans) Ca$^{2+}$ and 5 μM cytosolic (cis) Ins(1,4,5)P$_3$. A, setting the trans [Ca$^{2+}$] to 300 nM resulted in ongoing channel activity at −100 mV in the presence of 5 μM Ins(1,4,5)P$_3$. Again, openings were defined as upward deflections from the base line (dashed line). Channel activity can be seen over a period of between 10 and 15 s at the beginning of the record, followed by intermittent bursts of activity. Note the difference in time scale compared with Fig. 2A. See “Materials and Methods” for experimental conditions. B, sections i through to iv were expanded from A, representing a range of channel behavior seen throughout this recording (openings are upward deflections from the base line). Exceptionally long open times are seen in trace ii, although events of this nature are few, and their inclusion in the lifetime analysis would obscure the data.

Effects of 100 μM Intraluminal (trans) Ca$^{2+}$ — The previous sets of experiments provided a way to observe effects of a high luminal [Ca$^{2+}$] (0.6–1 mM) and an effectively empty store (300 nM). As an intermediate value we chose 100 μM trans Ca$^{2+}$, which represents the sort of values for [Ca$^{2+}$]$_{luc}$ likely to be found in partly depleted stores. Ins(1,4,5)P$_3$ receptor was incorporated into the bilayer as described previously via proteoliposomes, and no channel activity was observed in the absence of Ins(1,4,5)P$_3$ at −100 mV applied p.d. Almost immediately after the addition of 5 μM Ins(1,4,5)P$_3$, channel activity started (see Fig. 5A). Four sub-conductance levels were quite clearly defined as upward deflections (commencing at region i). This behavior is typical of three similar separate experiments. Regions i, ii, and iii have been expanded and are shown in more detail in B, openings are seen as upward deflections from the base line (dashed line). Trace i shows the transition to a 12-pS sub-state, in which the channel remains for a high percentage of recording time (see Table I). Trace ii shows three transitions. The full transition from the base line (seen at the beginning of trace A, ii) corresponds to a value of 48 pS. The channel then closes to a lower state seen halfway through the trace, with a value of 24 pS. There is a momentary transition to the 12-pS level before opening to the 24-pS level for the duration of this trace. Trace iii illustrates a 36-pS level (seen as full openings in this trace) with closures to the 12-pS level. C, all-points amplitude histogram. The base line is set at 0 pA, and four additional peaks can be seen: 1.5 ± 0.2 pA (S.D.), 2.6 ± 0.2 pA (S.D.), 3.3 ± 0.2 pA (S.D.), and 4.5 ± 0.2 pA (S.D.). These values are slightly higher than those seen from the representative traces due to the fact that there is a slight base line drift.

The lifetime histogram would have obscured the data to such an extent that statistical analysis would not have been possible. Hence, these long lifetimes were calculated as a percentage of the total open time to give an indication of their relevance (see Table I). As can be seen from Fig. 5B, iii, the channel opens quite early in the recording to a 12-pS sub-state and remains in this state for approximately 64% of the total recording time (Table I). There are transitions from this 12-pS state to three other conductance levels of approximately equal value, although the channel never fully closed throughout the remainder of the recording. To summarize, ongoing channel activity is observed at 100 μM trans Ca$^{2+}$ with no inactivation and four clear conductance levels, the predominant level being the smallest at 12 pS and, for 300 nM trans Ca$^{2+}$, a range of open times from short (1.50 ± 0.10 ms) to long.

Observations at Lower Ins(1,4,5)P$_3$ Concentrations — A previously proposed model (16) suggests that full Ca$^{2+}$ stores sen-
sitize Ins(1,4,5)P$_3$ receptors to Ins(1,4,5)P$_5$; hence, high trans [Ca$^{2+}$] should potentiate channel activity at lower Ins(1,4,5)P$_3$ concentrations. However, in the current investigation, we found no systematic differences between the threshold Ins(1,4,5)P$_3$ concentrations needed to see channel activity (~500 nM) at 1 mM or 300 nM trans Ca$^{2+}$.

**Effects of Cytosolic ATP Overcoming the Inhibition by 1 mM trans Ca$^{2+}$**—In an earlier study on regulation by intraluminal Ca$^{2+}$ at the single channel level (37), an elevation in the trans Ca$^{2+}$ level to 1 mM reduced the single channel open probability by 67% of its control level, i.e. there was slight inhibition of Ins(1,4,5)P$_3$-gated channel activity. Although, as described above, these experiments were complicated by the use of divalent cations as current carrier, there is a marked difference between this very limited inhibition and the essentially complete (after the first few seconds) inhibition that we observe. One potential factor that may contribute to this discrepancy is that in the earlier work (37) 500 μM ATP was present in the cis compartment of the bilayer chamber. We therefore repeated the initial experiments at 1 mM trans Ca$^{2+}$ in the presence of 100 μM ATP in the cis compartment (a value already shown to maximize Ins(1,4,5)P$_3$ receptor activity in our hands (47)). No channel activity was seen in the presence of 100 μM ATP until Ins(1,4,5)P$_3$ was added.

However, on addition of 5 μM Ins(1,4,5)P$_3$ channel activity was observed (Fig. 6A) at an applied p.d. of ±100 mV. As can be seen from the entire recording (Fig. 6A) and representative traces of the recording (Fig. 6B), there is continuing channel activity at 1 mM trans Ca$^{2+}$ in the presence of ATP. From the expanded traces of the recording, different sub-states can be seen, and the channel is active at both polarities (±100 mV) for a very extended period. By comparing Fig. 6A with Fig. 2A, it is very clear that ATP on the cytosolic face of the receptor is somehow overcoming the inhibition induced by 1 mM trans Ca$^{2+}$. All-points amplitude histogram analysis (not shown) from traces totaling more than 1000 s of recording time showed opening to one major conductance level (40 ± 2.8 pS (S.D.)), substantially larger than in the absence of ATP, although other sub-conductance levels are evident from individual traces (see Fig. 6B and Fig. 7). The all-points histogram did not define these levels clearly, and for the majority of the recording time, the channel was open to one state. A few very long events were also seen, although the majority are short openings. A histogram for mean open times (not shown) showed two populations: 1.30 ± 0.1 and 6.0 ± 0.8 ms. Under the conditions of Fig. 2, it was found that the addition of ATP to the cis face led to a very rapid reactivation of the channel (Fig. 7), with channel characteristics similar to those seen when ATP was present initially. Thus, cytosolic ATP can reverse as well as prevent the inhibition due to luminal Ca$^{2+}$.

**Effects of Cytosolic ATP at 300 nM trans Ca$^{2+}$**—In a continuation of the previous experiments, the [Ca$^{2+}$] in the trans compartment was chelated to 300 nM using 10 mM HEDTA to see if lowering luminal [Ca$^{2+}$] had any major effects on channel activity in the presence of 100 μM cytosolic (cis) ATP. As can be seen in Fig. 8A (whole recording) and from the selection of traces in Fig. 8B, channel activity continues relatively unchanged at both polarities (±100 mV). All-points amplitude histogram analysis yielded one major peak (33.2 ± 4.7 pS (S.D.)) in addition to the closed one, a slightly smaller conductance than that seen at 300 nM trans Ca$^{2+}$ and 100 μM cis ATP. Again, opening to sub-states is not clearly defined from the all-points histogram, as the channel opens to one major conductance level. From the histogram for mean open times two populations were determined, 1.4 ± 0.1 ms and 8.7 ± 2.9 ms, similar to those seen previously.

By comparing experiments at 300 nM trans Ca$^{2+}$ in the absence and presence of cis ATP, it can be seen that in the presence of ATP the channel shows no signs of any inactivation and continues to gate steadily for very long periods of time (Fig. 8A), unlike the bursting pattern of activity seen in Fig. 4A. That is to say, irrespective of trans [Ca$^{2+}$], ATP still has a stimulatory effect.

**DISCUSSION**

A major finding reported here is that at 1 mM luminal Ca$^{2+}$ the Type 1 Ins(1,4,5)P$_3$ receptor appears to show only very transient channel activity unless ATP is present on the cytosolic side. This is consistent with the observations in perfused, permeabilized cell preparations, where ATP increases the amount of Ca$^{2+}$ release at relatively high Ins(1,4,5)P$_3$ concentrations without changing the threshold Ins(1,4,5)P$_3$ concentration required for Ca$^{2+}$ release (48). However, a key question is whether luminal Ca$^{2+}$ is exerting its effect by binding to the luminal face of the receptor or in some other way. Since we
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FIG. 7. Ins(1,4,5)P₃ receptor channel activity in the presence of 1 mM intraluminal (trans) Ca²⁺ and 10 µM cytosolic (cis) Ins(1,4,5)P₃ before and after addition of 100 µM cytosolic (cis) ATP. A, current traces of 10 s of recording of channel activity in the absence of ATP (first section) and presence of ATP at −100 mV and +100 mV (last two sections, respectively). Sections of the recording have been isolated and expanded in B, C, and D to illustrate typical channel behavior. B, approximately 1 min after addition of 5 µM Ins(1,4,5)P₃, to the cis compartment at an applied p.d. of −100 mV, a brief burst of Ins(1,4,5)P₃ receptor channel activity is seen followed by complete inactivation (as before). Channel size is approximately 12 pS. Openings are defined as upward deflections. C, the channel is reactivated upon addition of 100 µM ATP to the cis compartment. From the expanded traces it can be seen that channel conductance is larger with openings of approximately 45 pS. D, further channel activity at an applied p.d. of +100 mV (downward deflections) is observed, similar to that seen in C.

have used purified Ins(1,4,5)P₃ receptor in these experiments, we can eliminate the influence of accessory proteins, unless they are very tightly associated with the receptor and not removed during the purification.

There remains the question of whether or not luminal Ca²⁺ is passing through the channel and having an effect on the cytosolic face, where it is known to have both activating and inhibitory effects (1–15). We feel that this is very unlikely for several reasons. First, Bezprozvanny and Ehrlich found a value of trans [Ca²⁺] free that we have used in these experiments. This implies that the Ins(1,4,5)P₃ receptor changes its conformational state in response to luminal Ca²⁺ and is thus capable, at least in theory, of transducing changes in Ca²⁺ store loading from the cytosolic face of the endoplasmic reticulum to proteins outside of the endoplasmic reticulum such as Ca²⁺ entry channels. Also, particularly at high luminal [Ca²⁺], the channel shows transient activation followed by inactivation. It is tempting to equate this behavior with the pronounced biphasic kinetics of Ca²⁺ release from intracellular stores (for review, see Ref. 50), where there is an initial fast phase of Ca²⁺ release followed by an ongoing slower phase. It should be noted that the experiments at 300 nM trans Ca²⁺ were continuations of experiments begun at 1 mM trans Ca²⁺, the trans Ca²⁺ being chelated with an appropriate HEDTA concentration. It seems, therefore, that a channel that has inactivated in the presence of high luminal Ca²⁺ is capable of reactivation by decreasing the luminal Ca²⁺. Also, at 100 µM trans Ca²⁺, the channel shows no sign of inactivation. Physiologically, these effects appear to provide a further form of positive feedback; once a store starts emptying it will continue at an accelerating rate. Decreased inhibition due to decreasing luminal Ca²⁺ coupled with positive feedback due to released Ca²⁺ would then act as a "push-pull" mechanism.
leading to a very steep channel activation once Ca^{2+} release is initiated. Particularly at lower trans \([\text{Ca}^{2+}]_{\text{lumen}}\), several subconductance states were routinely observed. Our preparations of the Ins(1,4,5)P_3 receptor do not contain detectable levels of FKBP12. Dissociation of this protein from ryanodine receptors has been shown to promote subconductance states in that channel (51). Since the immunosuppressant FK506 dissociates FKBP12. Dissociation of this protein from ryanodine receptors might lead to changes in the conductance states of the Ins(1,4,5)P_3 receptor also, although recent evidence suggests (53) that the effect on Ca^{2+} fluxes in this system is probably due to inhibition of the Ca^{2+} pumps.

It is clear from our observations and those of others that ATP is an extremely important regulator of the Ins(1,4,5)P_3 receptor. Under the conditions used in Fig. 7, it behaves, along with the Ins(1,4,5)P_3 receptor do not contain detectable levels of Ca^{2+}. Since the immunosuppressant FK506 dissociates FKBP12. Dissociation of this protein from ryanodine receptors initiated. Particularly at lower trans \([\text{Ca}^{2+}]_{\text{lumen}}\), several subconductance states were routinely observed. Our preparations of the Ins(1,4,5)P_3 receptor do not contain detectable levels of FKBP12. Dissociation of this protein from ryanodine receptors has been shown to promote subconductance states in that channel (51). Since the immunosuppressant FK506 dissociates FKBP12. Dissociation of this protein from ryanodine receptors might lead to changes in the conductance states of the Ins(1,4,5)P_3 receptor also, although recent evidence suggests (53) that the effect on Ca^{2+} fluxes in this system is probably due to inhibition of the Ca^{2+} pumps.

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