Slow kinetics of inositol 1,4,5-trisphosphate-induced Ca^{2+} release: is the release ‘quantal’ or ‘non-quantal’?

Ludwig MISSIAEN, Humbert DE SMEDT, Jan B. PARYS, Ilse SIENAERT, Henk SIPMA, Sara VANLINGEN and Rik CASTEELS

Laboratorium voor Fysiologie, K. U. Leuven Campus Gasthuisberg, Herestraat 49, B-3000 Leuven, Belgium

Inositol 1,4,5-trisphosphate (InsP_3)-induced Ca^{2+} release from intracellular stores is generally assumed to be a ‘quantal’ process because low InsP_3 concentrations mobilize less Ca^{2+} than high concentrations and a submaximal concentration does not release all the InsP_3 -mobilizable Ca^{2+} . However, some recent reports questioned the generally accepted view that a low dose of InsP_3 is unable to empty the whole store. We have now challenged the stores of permeabilized A7r5 cells in InsP_3 for much longer periods than previously reported, to assess directly whether the slow phase of the release would empty the whole store (a non-quantal response) or only a fraction of it (a quantal response). Addition of a maximal $[\text{InsP}_3]$ at the end of a prolonged (92 min) stimulation with a submaximal $[\text{InsP}_3]$ resulted in additional Ca^{2+} release. Experiments in which the stores were challenged with different submaximal InsP_3 concentrations for long time

periods revealed that a lower $[\text{InsP}_3]$ never released the same amount of Ca^{2+} as a higher $[\text{InsP}_3]$. This quantal pattern of Ca^{2+} release occurred both at 25 °C and at 4 °C. There was a time-dependent increase in the fraction of Ca^{2+} recruited by the lower compared with the higher $[\text{InsP}_3]$. This recruitment of Ca^{2+} persisted if the $[\text{InsP}_3]$ was decreased, but was largely prevented by palmitoyl-CoA, a potent blocker of the luminal Ca^{2+} translocation between individual store units. ATP, in the presence of InsP_3 , released Ca^{2+} under conditions permitting the recruitment of no additional InsP_3 receptors, indicating that an all-or-none emptying of a fraction of the stores cannot be the only mechanism responsible for quantal Ca^{2+} release in A7r5 cells. We conclude that some of the previously published evidence for a non-quantal Ca^{2+} release pattern should be reinterpreted.

INTRODUCTION

Many hormones, neurotransmitters and growth factors use inositol 1,4,5-trisphosphate (InsP_3) as intracellular messenger [1]. Low InsP_3 concentrations mobilize less Ca^{2+} than high concentrations, and a submaximal concentration is not capable of releasing all the InsP_3 -mobilizable Ca^{2+} [2]. This partial release of the Ca^{2+} from the intracellular stores was originally called ‘quantal’ Ca^{2+} release. Throughout this paper, ‘quantal’ Ca^{2+} release will therefore be defined as the inability of a submaximal $[\text{InsP}_3]$ to empty the whole store, regardless of the mechanism that is responsible for such behaviour. This quantal InsP_3 -induced Ca^{2+} release contrasts with the non-quantal Ca^{2+} release induced by a Ca^{2+} ionophore, where the rate of Ca^{2+} release, but not the final extent of the Ca^{2+} release, depends on the stimulation intensity [2].

The concept of quantal or partial Ca^{2+} release was based on the absence of an additional Ca^{2+} release after the initial rapid release phase in permeabilized cells [2–6]. Many reports on both intact [7–10] and permeabilized cells [11–15] have since revealed that the InsP_3 receptor (InsP_3R) can set up a long-lasting Ca^{2+} leak. However, this slow release in the presence of submaximal InsP_3 concentrations has not been monitored for a sufficiently long period and with sufficient accuracy to establish whether it will eventually empty the whole InsP_3 -sensitive store. The concept of quantal or partial Ca^{2+} release was recently challenged when imaging techniques made it possible to measure directly the effects of InsP_3 on the luminal $[\text{Ca}^{2+}]$. An almost total (non-quantal) release of the stores by a submaximal $[\text{InsP}_3]$ was reported when the luminal $[\text{Ca}^{2+}]$ was monitored with chlorotetracycline [16], Calcium Green-5N [17] or with Magfura 2 in the presence of thapsigargin [18]. However, other experiments with fura 2 [17,19], furaptra [20] or Magfura 2 [21] resulted in a quantal or partial release pattern. Measurements of the InsP_3 -

induced retrograde entry of Mn^{2+} into stores also yielded conflicting results with respect to the possible quantal or partial nature of the InsP_3R function. These experiments are not easily comparable with the above-mentioned studies because Mn^{2+} -entry experiments are for technical reasons done at free cytosolic Ca^{2+} concentrations (e.g. 2.9 μM in [17]) that are inhibitory for the InsP_3R [22–25]. InsP_3 -induced Mn^{2+} entry in InsP_3 -sensitive Ca^{2+} stores has been reported to be both non-quantal [17,26] and quantal [19]. Differences in free cytosolic $[\text{Mn}^{2+}]$ might contribute to these divergent results, especially because Mn^{2+} , like Ca^{2+} , both activates and, at higher concentrations, inhibits the InsP_3R [27]. Finally, the reported effects of low temperature on quantal or partial Ca^{2+} release are also conflicting because quantal (i.e. partial) responses were lost at 0–11 °C under some experimental conditions [28–30], but not in others [4,31,32].

The aim of the present study was to settle some of these conflicting findings with respect to the detailed kinetics of the InsP_3 -induced Ca^{2+} release and to assess whether the slow phase of the release would eventually empty the whole store (a non-quantal response) or only a part of it (a quantal response). We have for that purpose challenged the stores of attached permeabilized A7r5 cells with various InsP_3 concentrations for extremely long periods (up to 100 min). Addition of a maximal $[\text{InsP}_3]$ at the end of a long (92 min) stimulation with a submaximal $[\text{InsP}_3]$ resulted in an additional Ca^{2+} release. We also observed that the fraction of Ca^{2+} released by a lower $[\text{InsP}_3]$ compared with that by a higher $[\text{InsP}_3]$ increased with time. This ratio finally stabilized, indicating that a prolonged stimulation with a submaximal $[\text{InsP}_3]$ never released the same amount of Ca^{2+} as a long-lasting incubation at a higher concentration. An experimental protocol in which stores were exposed to 0.2 μM InsP_3 after a preceding stimulation with higher InsP_3 concentrations indicates that the slow phase of the release was not due to the presumed intrinsic InsP_3 -dependent inactivation of the InsP_3R .

Although the release remains quantal, as indicated by the observation that lower concentrations of InsP_3 never released the same amount of Ca^{2+} as a higher dose, we also observed a very pronounced slow response component that, at least during a certain period, mobilized an increasing fraction of the Ca^{2+} released by higher InsP_3 concentrations. Luminal transfer of Ca^{2+} between individual store units seems to contribute to the slow release. The relevance of these findings for interpreting the conflicting data with respect to the quantal or partial nature of the release will be discussed.

MATERIALS AND METHODS

A7r5 cells, an established cell line derived from embryonic rat aorta, were used between the 10th and the 20th passage after receipt from the American Type Culture Collection (Bethesda, MD, U.S.A.) and subcultured weekly after trypsinization. The cells were cultured at 37 °C in a 9% CO_2 incubator in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal-calf serum, 3.8 mM L-glutamine, 0.9% (v/v) non-essential amino acids, 85 i.u./ml penicillin and 85 $\mu\text{g}/\text{ml}$ streptomycin. The cells were seeded in 12-well dishes (4 cm^2 ; Costar Europe, Badhoevedorp, The Netherlands) at a density of approx. 10^4 cells/ cm^2 .

$^{45}\text{Ca}^{2+}$ fluxes on permeabilized cells were done on a thermostatically controlled plate. The culture medium was aspirated and replaced by 1 ml of permeabilization medium containing 120 mM KCl, 30 mM imidazole/HCl (pH 6.8), 2 mM MgCl_2 , 1 mM ATP, 1 mM EGTA, 20 $\mu\text{g}/\text{ml}$ saponin at 25 °C. The saponin-containing solution was removed after 10 min and the cells were washed once with an identical but saponin-free solution. $^{45}\text{Ca}^{2+}$ uptake into the non-mitochondrial Ca^{2+} stores was accomplished by incubation in 2 ml of loading medium containing 120 mM KCl, 30 mM imidazole/HCl, pH 6.8, 5 mM MgCl_2 , 5 mM ATP, 0.44 mM EGTA, 10 mM NaN_3 , 10 mM creatine phosphate, 50 $\mu\text{g}/\text{ml}$ creatine kinase and 100 nM free $^{45}\text{Ca}^{2+}$ at 25 °C. The loading time was 60 min except in the experiment shown in Figure 8, where it was 120 min. After this phase of $^{45}\text{Ca}^{2+}$ accumulation, the monolayers were incubated in 1 ml of efflux medium containing 120 mM KCl, 30 mM imidazole/HCl, pH 6.8, 2 mM MgCl_2 , 1 mM ATP, 1 mM EGTA, 100 nM free $^{45}\text{Ca}^{2+}$ and 2 μM thapsigargin. The temperature of the efflux medium (25 or 4 °C) is indicated in the Figure legends. At the end of the experiment the $^{45}\text{Ca}^{2+}$ remaining in the stores was released by incubation with 1 ml of a 2% (w/v) SDS solution for 30 min at 25 °C. All efflux curves were corrected for passive $^{45}\text{Ca}^{2+}$ binding, which was taken as the efflux from stores loaded with $^{45}\text{Ca}^{2+}$ in loading medium containing 2 μM thapsigargin and 10 μM A23187.

RESULTS AND DISCUSSION

Kinetics of InsP_3 -induced Ca^{2+} release

Ca^{2+} pumps in the intracellular stores can compensate for the leak through the Ca^{2+} -release channels [33,34]. The detailed kinetics of the release can therefore be assessed only if the internal Ca^{2+} pumps are completely blocked. In the early studies on quantal Ca^{2+} release, this inhibition was often achieved by removing ATP from the medium. However, some Ca^{2+} pumping can still occur under these conditions [34], probably because of compartmentalized ATP production. Our approach of blocking the pumps with thapsigargin [7,13,14,35,36] is free of this artifact [34] and results in a unidirectional Ca^{2+} efflux.

Prolonged stimulation of permeabilized A7r5 cells with a low $[\text{InsP}_3]$ in unidirectional Ca^{2+} -efflux conditions results in a slow

Figure 1 InsP_3 -induced Ca^{2+} release from permeabilized A7r5 cells

The non-mitochondrial stores were loaded for 60 min at 100 nM free $^{45}\text{Ca}^{2+}$ and then incubated in an efflux medium containing 2 μM thapsigargin at 25 °C (line a). The stores in line b were incubated in the presence of 1 μM InsP_3 for 92 min and then challenged with 200 μM InsP_3 for 2 min. The stores in line c were incubated in the presence of 200 μM InsP_3 during the first 2 min of efflux. Panel (A) shows on a logarithmic scale and panel (B) on a linear scale the decrease in store Ca^{2+} content as a function of time. As is apparent from the Figure, the small decrease in Ca^{2+} content induced by 200 μM InsP_3 can only be accurately observed when a logarithmic scale is used. The bold lines on top of the Figure represent the period of stimulation with the indicated $[\text{InsP}_3]$ (in μM). The results are typical of four experiments.

release after the initial more rapid release [13,14,20,36]. This behaviour could still represent quantal (i.e. partial) Ca^{2+} release if this long-lasting leak induced by the low $[\text{InsP}_3]$ did not empty the whole InsP_3 -sensitive store. We have therefore added InsP_3 for up to 100 min to assess directly the final extent of Ca^{2+} release. Such extremely long exposure to InsP_3 using unidirectional Ca^{2+} efflux conditions has, to the best of our knowledge, not been performed previously. Replacing the efflux medium every 2 min prevented a progressive accumulation of InsP_3 metabolites during the efflux.

Line b in Figure 1(A) illustrates the decline in the Ca^{2+} content of the stores during a 92 min incubation in 1 μM InsP_3 . This concentration was submaximal, because the addition of 200 μM InsP_3 at time 0 (line c) resulted in a more rapid decrease in this Ca^{2+} content. The basic question in assessing quantal release is whether the 92 min incubation at 1 μM InsP_3 completely released the mobilizable Ca^{2+} from the InsP_3 -sensitive store (i.e. a non-quantal Ca^{2+} release pattern) or only partly (i.e. a quantal response). A classical approach for addressing this question is to add a maximal $[\text{InsP}_3]$ at the end of the long-lasting incubation in the lower $[\text{InsP}_3]$. No further Ca^{2+} release would be indicative

Figure 2 Quantal InsP_3 -induced Ca^{2+} release from permeabilized A7r5 cells

The efflux medium at 25 °C contained no InsP_3 (line a), 0.2 μM InsP_3 (line b) or 1 μM InsP_3 (line c). Panel (A) shows on a logarithmic scale and panel (B) on a linear scale the decrease in store Ca^{2+} content as a function of time. The percentage of the 1 μM InsP_3 -releasable Ca^{2+} that was mobilized by 0.2 μM InsP_3 as a function of time is shown in the inset to (B). The results are means for five experiments, each done in triplicate.

of a non-quantal release pattern. Figure 1(A) illustrates that 200 μM InsP_3 added after the 92 min incubation at 1 μM InsP_3 released additional Ca^{2+} . This long-lasting stimulation with the submaximal $[\text{InsP}_3]$ therefore did not completely empty the InsP_3 -sensitive store.

Effects of InsP_3 are often presented as linear plots of the total store Ca^{2+} content, the luminal $[\text{Ca}^{2+}]$ or the medium free $[\text{Ca}^{2+}]$ against time [2,17,19,31,37–39]. In addition the signals coming from luminally trapped Ca^{2+} indicators are usually presented as linear plots of fluorescence intensities [16,17] or ratios [18,21] against time. We have therefore replotted the content of the Ca^{2+} stores on a linear scale (Figure 1B). The additional Ca^{2+} release induced by 200 μM InsP_3 at the end of the 92 min incubation in 1 μM InsP_3 was hardly detectable in the linear plot. As a consequence, quantal Ca^{2+} release could easily remain unnoticed in a linear representation of the data.

An immediate Ca^{2+} release also occurred when a maximal $[\text{InsP}_3]$ was given after a 2 min challenge with a submaximal $[\text{InsP}_3]$ in RBL-2H3 cells (Figure 5 in [29]) and after a 3.7 min

challenge with a submaximal $[\text{InsP}_3]$ in hepatocytes (Figure 7 in [17]). However, in both these cases this additional Ca^{2+} release was considered insignificant and therefore it was concluded that the low $[\text{InsP}_3]$ mobilized the whole store, implying that the release was non-quantal (i.e. complete). However, InsP_3 becomes less effective in emptying largely depleted stores in both RBL-2H3 cells [40] and hepatocytes [41–44]. It is therefore unclear whether the relatively small Ca^{2+} release on adding a maximal $[\text{InsP}_3]$ at the end of a submaximal challenge in RBL-2H3 cells and hepatocytes really reflects non-quantal Ca^{2+} release or merely represents the reduced effectiveness of InsP_3 in releasing Ca^{2+} if stores are largely depleted. No conclusions can therefore be drawn from the relatively small Ca^{2+} release induced by 200 μM InsP_3 after the long-lasting challenge with 1 μM $[\text{InsP}_3]$ in A7r5 cells (Figure 1).

A 92 min incubation in 1 μM InsP_3 failed to mobilize the whole InsP_3 -sensitive store (Figure 1). Although these are the longest incubations in InsP_3 in unidirectional Ca^{2+} -efflux conditions ever reported, it is still possible that even longer incubations would be needed to mobilize the whole InsP_3 -sensitive store. However, much longer challenges with InsP_3 could not be done for technical reasons, because the non-specific binding then amounted to more than 50 % of the total Ca^{2+} uptake, whereas it was less than 3 % after 10 min. We therefore developed another experimental protocol to test the possible quantal nature of the release. Two different concentrations of InsP_3 (0.2 and 1 μM) were added for 100 min to establish whether the low dose would eventually release the same amount of Ca^{2+} as the high dose (Figure 2). The Ca^{2+} released by 0.2 μM InsP_3 relative to that mobilized by 1 μM InsP_3 is shown in the inset as a function of time. After 2 min, 0.2 μM InsP_3 released 44.0 ± 1.3 % (mean $\pm$ S.E.M.; $n = 5$) of the Ca^{2+} released by 1 μM InsP_3 . This percentage increased as the time of incubation in both concentrations of InsP_3 was prolonged and reached a plateau of 78.2 ± 1.2 % (mean $\pm$ S.E.M.; $n = 5$) after 80 min. After this time point, the fraction of Ca^{2+} released by the lower $[\text{InsP}_3]$ relative to the fraction released by the higher $[\text{InsP}_3]$ no longer increased. The 0.2 μM InsP_3 was therefore unable to release the same amount of Ca^{2+} as the 1 μM InsP_3 , indicating that the release was indeed a quantal process. This quantal release occurred despite the existence of a pronounced slow phase, which, at least for a limited period, mobilized an increasing fraction of the Ca^{2+} released by the higher $[\text{InsP}_3]$. Figure 2(B) represents a linear representation of the Ca^{2+} content of the stores as a function of time. The quantal or partial release behaviour was again less obvious in the linear plot.

Effects of low temperature

Figure 3 shows the effect of a 60 min stimulation with 0.1 and 1 μM InsP_3 at 4 °C, with the inset showing the Ca^{2+} mobilized by the 0.1 μM InsP_3 relative to that released by 1 μM InsP_3 as a function of time. The lower $[\text{InsP}_3]$ released 32.9 ± 3.4 % (mean $\pm$ S.E.M.; $n = 5$) of the Ca^{2+} mobilized by the higher $[\text{InsP}_3]$ after 2 min. This percentage increased to a plateau at 80.3 ± 1.6 % (mean $\pm$ S.E.M.; $n = 5$) after 40 min, which was maintained for the last 20 min. The release at 4 °C was therefore also quantal or partial because the lower $[\text{InsP}_3]$ never released the same amount of Ca^{2+} as the higher dose.

The kinetics of the release at 4 °C (Figure 3) differed from that at 25 °C (Figure 2). Although the lower $[\text{InsP}_3]$ at both temperatures eventually released approx. 80 % of the Ca^{2+} released by the higher $[\text{InsP}_3]$, the release after 2 min was less pronounced at 4 °C than at 25 °C. As a consequence, the additional Ca^{2+} release after the first 2 min was more pronounced at 4 °C than at 25 °C.

Figure 3 InsP_3 -induced Ca^{2+} release from permeabilized A7r5 cells is also quantal at 4 °C

The stores were loaded with $^{45}\text{Ca}^{2+}$ at 25 °C. The efflux medium at 4 °C contained no InsP_3 (line a), 0.1 μM InsP_3 (line b) or 1 μM InsP_3 (line c). Panel (A) shows on a logarithmic scale and panel (B) on a linear scale the decrease in store Ca^{2+} content as a function of time. The inset to (B) represents the percentage of the 1 μM InsP_3 -releasable Ca^{2+} that was mobilized by 0.1 μM InsP_3 as a function of time at 4 °C. The results are means for five experiments, each done in triplicate.

Our present finding of a quantal Ca^{2+} release at 4 °C contrasts with several reports claiming that, at lower temperatures, sub-maximal InsP_3 concentrations empty the whole store and thereby present a non-quantal response [28–30]. The experimental approach for assessing the final extent of Ca^{2+} release differed, however, in some of these studies. For example, Patel and Taylor [30] added 10 nM InsP_3 until 30% of the InsP_3 -sensitive store had been released (7 min) and concluded that the whole InsP_3 -sensitive store would be mobilized if InsP_3 were to be added for long enough. In contrast, we have added InsP_3 for 60 min at 4 °C and concluded from the absence of additional Ca^{2+} release between 40 and 60 min (Figure 3, inset) that the release was quantal (i.e. partial). Kindman and Meyer [29] observed that 28 nM InsP_3 released a greater percentage of the total mobilizable Ca^{2+} at 11 °C than at 37 °C and concluded that quantal release was lost at 11 °C. However, the higher sensitivity to InsP_3 at lower temperatures was not taken into consideration [30,32].

Figure 4 Effect of decreasing the $[\text{InsP}_3]$ after an initial stimulation with a higher $[\text{InsP}_3]$

The stores were first challenged at 25 °C with 0.5 μM (line b) or 1 μM InsP_3 (line c) for 2 min and then incubated in the absence of InsP_3 for 12 min. At 14 min they were incubated in the presence of 0.2 μM InsP_3 for the rest of the experiment. Line a is the control in the absence of InsP_3 stimulation. Panel (A) shows on a logarithmic scale and panel (B) on a linear scale the decrease in store Ca^{2+} content as a function of time. The inset to (B) shows the Ca^{2+} release in line b as a percentage of that in line c as a function of time; the arrow indicates the addition of 0.2 μM InsP_3 . The results are the means for seven experiments, each done in triplicate.

Nature of the slow release

The kinetics of the InsP_3 -induced Ca^{2+} release cannot be described by a single exponential because the release consists of a fast component and a slow component [13,14,20,36,39,45,46]. Figure 2 indicates that a low $[\text{InsP}_3]$, added for a long time, recruits a progressively increasing fraction of the Ca^{2+} that was released by a long exposure to a higher $[\text{InsP}_3]$. This finding indicates that low InsP_3 concentrations elicit a relatively more pronounced slow phase of the Ca^{2+} release than high InsP_3 concentrations. Intrinsic InsP_3 -dependent inactivation of the InsP_3R [47] would provide an obvious explanation for the more rapid decline of the slow release phase at higher InsP_3 concentrations because the rate of inactivation would depend on the $[\text{InsP}_3]$. This possibility was further investigated, as shown in Figure 4. The stores were first challenged for 2 min with 0.5 or 1 μM InsP_3 , then incubated for 12 min in the absence of InsP_3 , and finally stimulated with a

Figure 5 Kinetics of InsP_3 -induced Ca^{2+} release in the presence of $2 \mu\text{M}$ palmitoyl-CoA

The efflux medium at 25°C contained no InsP_3 (line a), $0.2 \mu\text{M}$ InsP_3 (line b) or $1 \mu\text{M}$ InsP_3 (line c). Panel (A) shows on a logarithmic scale and (B) on a linear scale the decrease in store Ca^{2+} content as a function of time. The percentage of the $1 \mu\text{M}$ InsP_3 -releasable Ca^{2+} that was mobilized by $0.2 \mu\text{M}$ InsP_3 as a function of time of incubation in $2 \mu\text{M}$ palmitoyl-CoA is shown in the inset to (B). The inset in (A) shows the percentage of the $10 \mu\text{M}$ A23187 releasable Ca^{2+} that was released by a 2 min incubation at the indicated $[\text{InsP}_3]$ in the absence (open bars) and presence (filled bars) of $2 \mu\text{M}$ palmitoyl-CoA. The results are the means for three experiments, each done in triplicate.

lower $[\text{InsP}_3]$ ($0.2 \mu\text{M}$) for the rest of the experiment. The initial application of $0.5 \mu\text{M}$ InsP_3 released 74.4% of the Ca^{2+} mobilized by $1 \mu\text{M}$ InsP_3 . This percentage remained constant during the following 12 min of incubation in the absence of InsP_3 (Figure 4, inset), indicating that the progressive mobilization of an increasing amount of Ca^{2+} by the low $[\text{InsP}_3]$ relative to the high $[\text{InsP}_3]$ in Figures 2 and 3 depended on the presence of InsP_3 . The subsequent addition of $0.2 \mu\text{M}$ InsP_3 induced a pronounced slow release in the trace originally stimulated by $0.5 \mu\text{M}$ InsP_3 but not in the trace originally stimulated by $1 \mu\text{M}$ InsP_3 . As a consequence, the final $[\text{Ca}^{2+}]$ of the stores after the earlier 0.5 and $1.0 \mu\text{M}$ InsP_3 additions became almost identical after 60 min. The slow recruitment of additional Ca^{2+} can therefore also be achieved by a lower $[\text{InsP}_3]$, which was identical for both conditions, after washing out the initial higher $[\text{InsP}_3]$. This

finding excludes the intrinsic inactivation of the InsP_3R as underlying the progressive recruitment of additional Ca^{2+} because this recruitment can occur during incubation in conditions that were identical with respect to $[\text{InsP}_3]$ and hence InsP_3 -induced InsP_3R inactivation.

Explanations of non-quantal Ca^{2+} responses in attached cells rely on the existence of luminal communications between stores, through which Ca^{2+} might move from store units with InsP_3Rs that are not sensitive to a low $[\text{InsP}_3]$ to regions of the endoplasmic reticulum that are sufficiently sensitive [16,17,26]. The disruption of these connections probably could explain why non-quantal responses no longer occur in suspensions of cells [16,17,26]. The appearance of a large InsP_3 -insensitive store is a possible criterion for such damage [6,16,26]. A maximal $[\text{InsP}_3]$ mobilizes more than 94% of the releasable Ca^{2+} in permeabilized A7r5 cells [14], indicating that no significant damage has occurred and therefore that the proposed connections are still present. Ca^{2+} transfer through these communication pathways is completely and reversibly blocked by palmitoyl-CoA [48,49]. Figure 5 represents the effect of two different concentrations of InsP_3 (0.2 and $1 \mu\text{M}$) in an efflux medium containing $2 \mu\text{M}$ palmitoyl-CoA. The inset to Figure 5(B) shows the percentage of the $1 \mu\text{M}$ InsP_3 -releasable Ca^{2+} that was mobilized by $0.2 \mu\text{M}$ InsP_3 as a function of time. A plateau was reached after 10 min in the presence of palmitoyl-CoA, whereas it took 80 min to reach a plateau in its absence (Figure 2B, inset). The long-lasting slow component of the release was therefore largely prevented by palmitoyl-CoA. To test the effect of palmitoyl-CoA on the fast phase of the release, we challenged the store with 2 or $100 \mu\text{M}$ InsP_3 for only 2 min in the absence or presence of $2 \mu\text{M}$ palmitoyl-CoA (Figure 5A, inset). The release, expressed as a percentage of the A23187 ($10 \mu\text{M}$)-releasable Ca^{2+} , was not affected by palmitoyl-CoA. This finding excludes non-specific effects of palmitoyl-CoA on the InsP_3R . The palmitoyl-CoA-induced inhibition of the slow release phase therefore implies that the transfer of Ca^{2+} through luminal connections contributes to the slow InsP_3 -induced Ca^{2+} release in A7r5 cells. This finding also implies that this component of the slow release phase, which depends on this luminal Ca^{2+} transfer, is rate-limited by the transfer of Ca^{2+} into pools that have their InsP_3Rs activated, from other pools that do not.

Mechanism for quantal Ca^{2+} release

One possibility to explain the kinetics of the Ca^{2+} release is an all-or-none Ca^{2+} -release response of individual store units expressing receptors with different sensitivities to InsP_3 [2–5,16,17,26,46]. A low $[\text{InsP}_3]$ will completely empty the more sensitive store units; the subsequent addition of a higher $[\text{InsP}_3]$ will then recruit the less sensitive units. Low doses of InsP_3 will in addition drain some Ca^{2+} from store units that are not sensitive to this low $[\text{InsP}_3]$ if luminal communications between the stores are preserved [16,17,26]. A7r5 cells express both $\text{InsP}_3\text{R-I}$ and $\text{InsP}_3\text{R-III}$ [31,50], which differ in their affinities for InsP_3 [51], and our findings represented in Figure 5 are compatible with functionally active luminal connections. The fundamental question to be addressed is whether both InsP_3R heterogeneity and luminal connections are sufficient to explain the observed kinetics of the release in A7r5 cells. All published experiments on the quantal or partial nature of the Ca^{2+} release rely on challenging the stores with at least two different concentrations of InsP_3 or related compounds (InsP_3 analogues or adenophostins). However, applying a higher $[\text{InsP}_3]$ after an initial challenge with a lower $[\text{InsP}_3]$ will induce two effects in an all-or-none model for Ca^{2+} release: (1) an increase in the channel activity of those InsP_3Rs that have already responded to the lower $[\text{InsP}_3]$, so that these

Figure 6 Effect of ATP on the InsP_3 -induced Ca^{2+} release

The stores were challenged at 25 °C with 1 μM InsP_3 in a medium with (○) or without (●) 1 mM ATP, as indicated by the bars above the traces. The efflux medium contained no Mg^{2+} and no added Ca^{2+} . The results are the means for two experiments, each done in triplicate.

store units release their Ca^{2+} more rapidly, and (2) an opening of those channels that failed to open at the lower $[\text{InsP}_3]$, thereby recruiting additional stores. Although a discrimination between these two effects would be very instructive for assessing the validity of the all-or-none model for quantal or partial Ca^{2+} release, this cannot be achieved by varying the $[\text{InsP}_3]$.

ATP is a unique tool in this respect because in the presence of InsP_3 it increases the frequency and average duration of channel openings [52] in those InsP_3Rs that have already responded to the lower $[\text{InsP}_3]$. ATP does not, however, open channels that failed to open at the lower $[\text{InsP}_3]$ because low ATP concentrations stimulate the release without affecting the K_d or the B_{max} of InsP_3 binding [53]. The possibility that ATP might induce channel opening in InsP_3Rs that bound InsP_3 but did not release Ca^{2+} can be excluded because ATP does not lower the threshold for InsP_3 -induced Ca^{2+} release in functional assays [36,54]. The addition of ATP in the presence of a low $[\text{InsP}_3]$ will therefore only increase the frequency and duration of channel openings in store units that have already responded to InsP_3 , without recruiting store units that were refractory to this low $[\text{InsP}_3]$.

Figure 6 illustrates the effect of a stimulatory [ATP] (1 mM) in the presence of 1 μM InsP_3 . The medium also contains 2 μM palmitoyl-CoA to block the luminal continuity. If the quantal or partial Ca^{2+} release represented the complete emptying of only those store units that are sensitive to 1 μM InsP_3 , then stimulatory concentrations of ATP, which do not recruit additional stores, would only increase the initial rate but not the final percentage of Ca^{2+} release. However, Figure 6 illustrates that ATP also increased the final degree of Ca^{2+} release. This stimulatory effect of ATP was also observed if ATP was added at the end of a 20 min incubation at 1 μM InsP_3 . This additional Ca^{2+} release under conditions permitting the recruitment of neither additional InsP_3Rs nor store units indicates that an all-or-none emptying of a fraction of the stores cannot be the only mechanism responsible for quantal (i.e. partial) Ca^{2+} release in A7r5 cells.

The proposed regulation of the InsP_3R by luminal Ca^{2+} [13,17,21,31,32,36,40–44,55–58] implies that the release in the presence of a low $[\text{InsP}_3]$ will slow down and eventually stop while Ca^{2+} is still present in each store unit [59]. This steady-state mechanism for quantal Ca^{2+} release, in contrast with the all-or-none hypothesis, can easily explain why ATP triggers further Ca^{2+} release: ATP allows each store unit to release more Ca^{2+} in

Figure 7 Effect of preincubation with a higher $[\text{InsP}_3]$ on the Ca^{2+} release induced by a lower $[\text{InsP}_3]$

The stores in lines b and c were first challenged at 25 °C with 1 μM InsP_3 for 2 min and then incubated in the absence of InsP_3 for 12 min. Control stores (line a) were not challenged with InsP_3 for the first 14 min. At 14 min, they were incubated in the presence of 20 μM InsP_3 for 2 min (line c) or with 0.2 μM InsP_3 (lines a and b) for the rest of the experiment. The lines show on a logarithmic scale the decrease in store Ca^{2+} content as a function of time. The results are the means for three experiments, each done in triplicate.

the presence of a submaximal $[\text{InsP}_3]$. Regulation by luminal Ca^{2+} can also explain why the addition of 0.2 μM InsP_3 after an initial challenge with 1 μM InsP_3 failed to release Ca^{2+} (Figure 7). The InsP_3 -sensitive stores were not empty, because 20 μM InsP_3 triggered a pronounced Ca^{2+} release. We explain this inability of 0.2 μM InsP_3 to set up the slow leak when added after an initial challenge with 1 μM InsP_3 by there being too low a luminal $[\text{Ca}^{2+}]$ for InsP_3 to open the channel. Regulation by luminal Ca^{2+} can finally explain why a low $[\text{InsP}_3]$ did not eventually release the same amount of Ca^{2+} as a higher concentration (Figure 2). There seems to be an inverse relation between the luminal Ca^{2+} content and the $[\text{InsP}_3]$ required to activate the channel.

The finding that ATP increased the efflux rate in the continuous presence of InsP_3 and in the presence of palmitoyl-CoA, i.e. with inactive connections between the stores, again confirms that luminal transfer of Ca^{2+} cannot be the only mechanism underlying the slow phase of the release. This finding suggests that the InsP_3R must have been in some kind of suboptimal activated state, whereas the addition of ATP pushed the equilibrium more to the open state.

The recruitment of additional Ca^{2+} in the absence of palmitoyl-CoA was never complete (i.e. the release remained quantal). Although we cannot exclude the possibility that the linking of the stores was only partial, an alternative explanation is based on the regulation of the InsP_3R by luminal Ca^{2+} [13,17,21,31,32,36,40–44,55–58]. When, in the presence of a low $[\text{InsP}_3]$, this Ca^{2+} content becomes too low, the InsP_3R is no longer opened and the release stops, despite the fact that less Ca^{2+} is released than with a higher $[\text{InsP}_3]$.

Bi-directional Ca^{2+} fluxes

The slow phase of the InsP_3 -induced Ca^{2+} release in pancreatic acinar cells only occurred in the absence of Ca^{2+} pumping and was prevented when the Ca^{2+} pumps were functional [18,33]. A similar phenomenon was observed for the Ca^{2+} release via the ryanodine receptor in smooth muscle [34]. We therefore investi-

Figure 8 InsP_3 -induced Ca^{2+} release in a bi-directional $^{45}\text{Ca}^{2+}$ flux

The non-mitochondrial Ca^{2+} stores were incubated for 120 min in loading medium containing 100 nM free $^{45}\text{Ca}^{2+}$ at 25 °C. The stores reached a steady-state loading after 40 min, which was maintained up to 120 min. InsP_3 [0.3 μM (line a) or 100 μM (line b)] was added for a period ranging from 1 to 60 min before the end of the loading period. The Ca^{2+} contents of the stores after this incubation in the indicated $[\text{InsP}_3]$ were plotted on the vertical axis ($n = 3$).

gated the contribution of Ca^{2+} pumping to the release kinetics in A7r5 cells (Figure 8). Stores were loaded with $^{45}\text{Ca}^{2+}$ to steady state and then incubated under loading conditions with 0.3 or 100 μM InsP_3 for 1–60 min. The low $[\text{InsP}_3]$ released $43.7 \pm 2.4\%$ (mean $\pm$ S.E.M.; $n = 3$) of the ionophore-releasable Ca^{2+} within 1 min and $83.3 \pm 1.3\%$ (mean $\pm$ S.E.M.; $n = 3$) after 60 min. The pronounced slow release was therefore also present under conditions of active Ca^{2+} pumping. The 60 min incubation at 0.3 μM InsP_3 did not lower the store Ca^{2+} content to the same low level as that induced by the continuous presence of 100 μM InsP_3 , indicating that the release remained quantal or partial.

We previously presented evidence that stores loaded with $^{45}\text{Ca}^{2+}$ subsequently responded to a low $[\text{InsP}_3]$ with a fast and subsequent slow release phase [13]. Reloading the stores with $^{40}\text{Ca}^{2+}$ resulted in an additional release of $^{45}\text{Ca}^{2+}$; this was taken as important evidence in favour of a mechanism for quantal Ca^{2+} release depending on a stimulatory effect exerted by luminal Ca^{2+} . Reloading of the stores probably occurred via the Ca^{2+} pumps present in each of the store units. Refilling of the stores via the luminal connections will probably be less important, taking into consideration the relatively slow time course of such transfer in A7r5 cells.

Conclusions

We have added InsP_3 for up to 100 min to assess directly whether or not the release is quantal (defined here as partial). A maximal $[\text{InsP}_3]$ at the end of a prolonged incubation in a sub-maximal $[\text{InsP}_3]$ was able to release a further fraction of the accumulated Ca^{2+} . By challenging the stores with two sub-maximal InsP_3 concentrations for very long time periods, we observed that the lower $[\text{InsP}_3]$ never mobilized the same amount of Ca^{2+} as the higher $[\text{InsP}_3]$, indicating that the release in A7r5 cells remained a quantal or partial process over the extended time period. We conclude that some of the published arguments in favour of a non-quantal release pattern should be reinterpreted.

Although the nature of the slow phase of the release is too complicated for a straightforward explanation of its underlying mechanism, we have tried to find out which components contribute to it, although their relative contributions are difficult to assess. The decreasing luminal $[\text{Ca}^{2+}]$ seems to be the main reason for the inability of the low $[\text{InsP}_3]$ to mobilize the whole InsP_3 -

sensitive Ca^{2+} store. The lower $[\text{InsP}_3]$ was, however, at least for a limited period, able to recruit an increasing fraction of the Ca^{2+} mobilized by the higher $[\text{InsP}_3]$. Part of this recruitment most probably represented luminal transfer of Ca^{2+} between individual store subunits.

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REFERENCES

- Berridge, M. J. (1993) *Nature* (London) **361**, 315–325
- Muallem, S., Pandol, S. J. and Beeker, T. G. (1989) *J. Biol. Chem.* **264**, 205–212
- Taylor, C. W. and Potter, B. V. L. (1990) *Biochem. J.* **266**, 189–194
- Oldershaw, K. A., Nunn, D. L. and Taylor, C. W. (1991) *Biochem. J.* **278**, 705–708
- Bootman, M. D., Berridge, M. J. and Taylor, C. W. (1992) *J. Physiol. (London)* **450**, 163–178
- Bird, G. St. J., Obie, J. F. and Putney, Jr., J. W. (1992) *J. Biol. Chem.* **267**, 18382–18386
- Missiaen, L., Parys, J. B., De Smedt, H. and Casteels, R. (1993) *Biochem. Biophys. Res. Commun.* **193**, 6–12
- Favre, C. J., Lew, D. P. and Krause, K.-H. (1994) *Biochem. J.* **302**, 155–162
- Smith, P. M. and Gallacher, D. V. (1994) *Biochem. J.* **299**, 37–40
- Toescu, E. C. and Petersen, O. H. (1994) *Pflügers Arch.* **427**, 325–331
- Champeil, P., Combettes, L., Berthon, B., Doucet, E., Orlowski, S. and Claret, M. (1989) *J. Biol. Chem.* **264**, 17665–17673
- Meyer, T. and Stryer, L. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 3841–3845
- Missiaen, L., De Smedt, H., Droogmans, G. and Casteels, R. (1992) *Nature* (London) **357**, 599–602
- Missiaen, L., Parys, J. B., De Smedt, H., Lemaire, F. X., Sienart, I., Bootman, M. D. and Casteels, R. (1995) *Cell Calcium* **18**, 100–110
- Loomis-Husselbee, J. W. and Dawson, A. P. (1993) *Biochem. J.* **289**, 861–866
- Renard-Rooney, D. C., Hajnóczky, G., Seitz, M. B., Schneider, T. G. and Thomas, A. P. (1993) *J. Biol. Chem.* **268**, 23601–23610
- Combettes, L., Cheek, T. R. and Taylor, C. W. (1996) *EMBO J.* **15**, 2086–2093
- van de Put, F. H. M. M. and Elliott, A. C. (1996) *J. Biol. Chem.* **271**, 4999–5006
- Short, A. D., Klein, M. G., Schneider, M. F. and Gill, D. L. (1993) *J. Biol. Chem.* **268**, 25887–25893
- Sugiyama, T. and Goldman, W. F. (1995) *Am. J. Physiol.* **269**, C813–C818
- Tanimura, A. and Turner, R. J. (1996) *J. Cell Biol.* **132**, 607–616
- Iino, M. (1990) *J. Gen. Physiol.* **95**, 1103–1122
- Bezprozvanny, I., Watras, J. and Ehrlich, B. E. (1991) *Nature* (London) **351**, 751–754
- Finch, E. A., Turner, T. J. and Goldin, S. M. (1991) *Science* **252**, 443–446
- Parys, J. B., Sernett, S. W., DeLisle, S., Snyder, P. M., Welsh, M. J. and Campbell, K. P. (1992) *J. Biol. Chem.* **267**, 18776–18782
- Hajnóczky, G., Lin, C. and Thomas, A. P. (1994) *J. Biol. Chem.* **269**, 10280–10287
- Striggow, F. and Ehrlich, B. E. (1996) *J. Gen. Physiol.* **108**, 115–124
- Meyer, T., Wensel, T. and Stryer, L. (1990) *Biochemistry* **29**, 32–37
- Kindman, L. A. and Meyer, T. (1993) *Biochemistry* **32**, 1270–1277
- Patel, S. and Taylor, C. W. (1995) *Biochem. J.* **312**, 789–794
- Parys, J. B., Missiaen, L., De Smedt, H. and Casteels, R. (1993) *J. Biol. Chem.* **268**, 25206–25212
- Xu, X., Zeng, W. and Muallem, S. (1996) *J. Biol. Chem.* **271**, 11737–11744
- van de Put, F. H. M. M., De Pont, J. J. H. M. and Willems, P. H. G. M. (1994) *J. Biol. Chem.* **269**, 12438–12443
- Steenbergen, J. M. and Fay, F. S. (1996) *J. Biol. Chem.* **271**, 1821–1824
- Missiaen, L., De Smedt, H., Droogmans, G. and Casteels, R. (1992) *J. Biol. Chem.* **267**, 22961–22966
- Missiaen, L., De Smedt, H., Parys, J. B., Sienart, I., Vanlingen, S. and Casteels, R. (1996) *J. Biol. Chem.* **271**, 12287–12293
- Somlyo, A. V., Horiuti, K., Trentham, D. R., Kitazawa, T. and Somlyo, A. P. (1992) *J. Biol. Chem.* **267**, 22316–22322
- Mezna, M. and Michelangeli, F. (1995) *J. Biol. Chem.* **270**, 28097–28102
- Hirota, J., Michikawa, T., Miyawaki, A., Furuichi, T., Okura, I. and Mikoshiba, K. (1995) *J. Biol. Chem.* **270**, 19046–19051
- Horne, J. H. and Meyer, T. (1995) *Biochemistry* **34**, 12738–12746
- Missiaen, L., Taylor, C. W. and Berridge, M. J. (1991) *Nature* (London) **352**, 241–244
- Nunn, D. L. and Taylor, C. W. (1992) *Mol. Pharmacol.* **41**, 115–119
- Marshall, I. C. B. and Taylor, C. W. (1993) *J. Biol. Chem.* **268**, 13214–13220
- Oldershaw, K. A. and Taylor, C. W. (1993) *Biochem. J.* **292**, 631–633
- Hirose, K. and Iino, M. (1994) *Nature* (London) **372**, 791–794
- Tortorici, G., Zhang, B.-X., Xu, X. and Muallem, S. (1994) *J. Biol. Chem.* **269**, 29621–29628
- Hajnóczky, G. and Thomas, A. P. (1994) *Nature* (London) **370**, 474–477

- 48 Comerford, J. G. and Dawson, A. P. (1993) *Biochem. J.* **289**, 561–567
- 49 Rys-Sikora, K. E., Ghosh, T. K. and Gill, D. L. (1994) *J. Biol. Chem.* **269**, 31607–31613
- 50 De Smedt, H., Missiaen, L., Parys, J. B., Bootman, M. D., Mertens, L., Van Den Bosch, L. and Casteels, R. (1994) *J. Biol. Chem.* **269**, 21691–21698
- 51 Newton, C. L., Mignery, G. A. and Südhof, T. C. (1994) *J. Biol. Chem.* **269**, 28613–28619
- 52 Bezprozvanny, I. and Ehrlich, B. E. (1993) *Neuron* **10**, 1175–1184
- 53 Kaplin, A. I., Snyder, S. H. and Linden, D. J. (1996) *J. Neurosci.* **16**, 2002–2011
- 54 Ferris, C. D., Huganir, R. L. and Snyder, S. H. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2147–2151
- 55 Bootman, M. D., Missiaen, L., Parys, J. B., De Smedt, H. and Casteels, R. (1995) *Biochem. J.* **306**, 445–451
- 56 Sugiyama, T. and Goldman, W. F. (1995) *Am. J. Physiol.* **269**, C813–C818
- 57 Camacho, P. and Lechleiter, J. D. (1995) *Cell* **82**, 765–771
- 58 Gamberucci, A., Fulceri, R., Tarroni, P., Giunti, R., Marcolongo, P., Sorrentino, V. and Benedetti, A. (1995) *Cell Calcium* **17**, 431–441
- 59 Irvine, R. F. (1990) *FEBS Lett.* **263**, 5–9

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