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Do submaximal $InsP_3$ concentrations only induce the partial discharge of permeabilized hepatocyte calcium pools because of the concomitant reduction of intraluminal Ca^{2+} concentration?

Laurent Combettes[a], Michel Claret[a] and Philippe Champeil[b]

[a]*Unité de Recherche U274, Institut National de la Santé et de la Recherche Médicale, Université Paris Sud, bat. 443, 91405 Orsay, France,* and [b]*Département de Biologie Cellulaire et Moléculaire, Centre d'Etudes Nucléaires de Saclay, 91191 Gif sur Yvette Cedex, France*

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In several types of cells whose cytoplasmic Ca^{2+} is regulated by inositol phosphate derivatives, low concentrations of $InsP_3$ added to permeabilized cell suspensions induce the rapid discharge of part of the $InsP_3$-sensitive Ca^{2+} pool instead of slow monophasic release of Ca^{2+} from the entire pool. As a tentative explanation for this puzzling observation, sometimes called 'quantal release', it was suggested that the reduced intraluminal Ca^{2+} concentration remaining in the Ca^{2+} pool after a certain amount of Ca^{2+} had been released might allosterically reduce the channels' affinity for $InsP_3$ and the corresponding $InsP_3$-dependent Ca^{2+} efflux, and thus result in partial pool discharge (Irvine, R.F. (1990) FEBS Lett. 263, 5–9). We have tested this hypothesis by manipulating the Ca^{2+} pool contents with ionophore, and found that the rate of $InsP_3$-dependent Ca^{2+} efflux after ionophore-induced partial discharge of the Ca^{2+} pools was much faster than what was predicted on the basis of this hypothesis. Heterogeneity of the Ca^{2+} pools appears to be a more likely reason for the 'quantal release' behavior.

Ca^{2+} release; Submaximal [$InsP_3$]; Permeabilized rat hepatocyte

1. INTRODUCTION

In many hormone-regulated cellular processes, $InsP_3$, produced as a result of phospholipase C stimulation, activates the efflux of Ca^{2+} from intracellular storage compartments [1]. An $InsP_3$-binding protein purified from cerebellum or smooth muscle was observed to act as both the functional $InsP_3$ receptor and the Ca^{2+} channel, as after its reconstitution into lipid vesicles these vesicles were able to release Ca^{2+} in response to stimulation by $InsP_3$ [2]. Subsequently, however, it was suspected that the process of Ca^{2+} release by $InsP_3$ presented an additional complexity, since the addition of low concentrations of $InsP_3$ to permeabilized cells was found to induce the fast release of a part of the $InsP_3$-sensitive calcium pool instead of the slow discharge of the pool [3]. Meyer and Stryer [4] and Taylor and Potter [5] confirmed that neither $InsP_3$ degradation nor active re-filling of the Ca^{2+} stores was responsible for this behavior.

Various ideas have been put forward to account for this fast partial discharge. On the one hand, Muallem and co-workers [3] initially proposed that each cell contained several Ca^{2+} stores with different sensitivities to $InsP_3$, each of which could be emptied in an 'all-or-nothing' manner, leading to what was called 'quantal' release. On the other hand, the possibility that this partial Ca^{2+} release might reflect the spontaneous attenuation with time of the channel's response to $InsP_3$ in a homogeneous population of Ca^{2+} stores was also explored. A very attractive hypothesis was proposed recently by Irvine [6], who suggested that the lowering of the Ca^{2+} concentration on the intralumenal side of the Ca^{2+} store might reduce the affinity of the receptor for $InsP_3$, thus allowing only partial Ca^{2+} release in the presence of submaximal doses of $InsP_3$. This suggestion was further discussed recently [7–9].

In the present work, the effect of partial store depletion on the relative potency of low concentrations of $InsP_3$ in inducing Ca^{2+} release was examined in a very simple way, using saponin-permeabilized rat hepatocytes and a fluorometric assay with the Ca^{2+}-sensitive dye, quin2. The experiment consisted of first adding a small amount of ionomycin to loaded stores, thus allowing the stores to come to a new and lower steady state Ca^{2+} load, and then adding a submaximal concentration of $InsP_3$, previously found in control experiments to induce the same reduction of the $InsP_3$-sensitive Ca^{2+} load. We found that the kinetics of the release induced by this concentration of $InsP_3$ were too fast to be consis-

Abbreviations: $InsP_3$, inositol 1,4,5-trisphosphate; quin2, 2-[(2-amino-5-methylphenoxy)methyl]-6-methoxy-8-amino-quinoline-*N,N,N',N'*-tetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Correspondence address: L. Combettes, Unité de Recherche U274, Institut National de la Santé et de la Recherche Médicale, Université Paris Sud, bat. 443, 91405 Orsay, France. Fax: (33) (1) 69410574.

tent with a steady-state model of partial release from identical stores, i.e. the simplest form of Irvine's model [7]. Our results support the view that the fractional release of Ca^{2+} observed in the presence of low concentrations of $InsP_3$ is more probably due, at least partly, to the heterogeneous nature of the Ca^{2+} stores.

2. MATERIALS AND METHODS

Rat hepatocytes were isolated as previously described [10] and permeabilized directly in the fluorometer cuvette by resuspension ($3 \cdot 10^6$ cells/ml) in a cytosol-like medium (37°C) containing 50 μg/ml saponin, 20 μM of the fluorescent Ca^{2+} buffer, quin2, 100 mM KCl, 20 mM NaCl, 5 mM $MgCl_2$, 0.96 mM NaH_2PO_4, 25 mM HEPES buffer, 1.5 mM Na_2ATP, 5 mM creatine phosphate and 5 U/ml creatine kinase (pH 7.1 at room temperature). Ca^{2+} movements were deduced from the observed changes in the fluorescence of quin2 (excitation wavelength, 336 nm; emission wavelength, 495 nm). The free Ca^{2+} in the medium was calculated from the dye fluorescence level using the apparent dissociation constant of 115 nM for the Ca^{2+}–quin2 complex.

3. RESULTS

Panel A of Fig. 1 shows a typical control experiment performed with saponin-treated rat hepatocytes: addition of a submaximal concentration of $InsP_3$ (0.17 μM, single arrow) rapidly released only part of the total $InsP_3$-sensitive Ca^{2+} pool, since a subsequent addition of a maximally efficient concentration of $InsP_3$ (2.5 μM $InsP_3$, double arrow) released more Ca^{2+}. This partial rapid release was also observed in the additional presence of glucose and hexokinase or in the presence of a non-metabolizable $InsP_3$ analog (data not shown; see [4,5]).

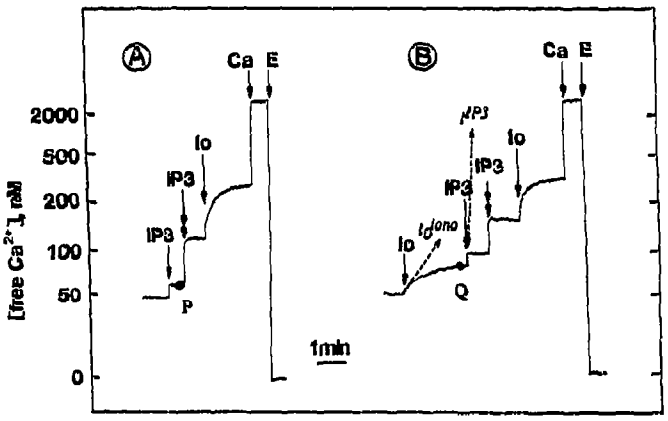

Fig. 1. Effect of ionophore-induced store depletion on $InsP_3$-induced partial Ca^{2+} release. Hepatocytes were permeabilized at 37°C in a fluorometer cuvette in the presence of quin2 and ATP (see Materials and Methods), and the Ca^{2+} pools were allowed to load until steady state was reached. In the experiment illustrated in panel A, 0.17 μM (single arrow) and 2.5 μM (double arrow) $InsP_3$ (IP3) were added sequentially, and then 4 μM ionomycin (Io), 40 μM Ca^{2+} (Ca) and 2 mM EGTA (E). In the experiment illustrated in panel B, 1 μM ionomycin (Io) was first added; a new steady state was reached, and then the same sequence of additions was performed as in the experiment illustrated in panel A.

According to the steady-state single compartment model put forward by Irvine [6], the reason why net Ca^{2+} efflux is only transient after addition of a low concentration of $InsP_3$ is that, as the Ca^{2+} concentration in the lumen falls, so does the apparent affinity of $InsP_3$ for its binding site on the channel, until a new set point in intraluminal Ca^{2+} is eventually established. Panel B of Fig. 1 illustrates the experiment designed to test this model. Here, we first added a low concentration of ionomycin, so that the calcium concentration in the $InsP_3$-sensitive pool was slowly reduced to a concentration equivalent to or even lower than that resulting from the addition of the submaximal concentration of $InsP_3$ used for the previous experiment; since ionomycin partially depleted both the $InsP_3$-sensitive- and the $InsP_3$-insensitive Ca^{2+} stores, the amount of Ca^{2+} released by ionomycin and detected by quin2 in panel B was of course larger than that released by 0.17 μM $InsP_3$ in panel A (see Table I below). Under these new steady-state conditions, we then added the same submaximal concentration of $InsP_3$ to the partially depleted pools.

In a steady-state model for the $InsP_3$-sensitive Ca^{2+} pool, if an identical Ca^{2+} load or intraluminal Ca^{2+} concentration is established in the presence of either the submaximal concentration of $InsP_3$ (point P in panel A) or the low concentration of ionomycin (point Q in panel B), the leaks under both conditions, l^{IP3} and l^{iono}, *must be similar* (given no change in pumping rate). An overestimation for the rate of the ionophore-induced leak from the partially depleted pool, l^{iono}, is provided by the magnitude of the initial leak observed when ionophore was added to the fully saturated pools, l_O^{iono} (panel B). As for the rate of the $InsP_3$-dependent leak after the new Ca^{2+} load was established at point P in the experiment illustrated in panel A (l^{IP3}), in a model with a homogeneous population of identical $InsP_3$-sensitive Ca^{2+} stores, it must be identical to the rate of the leak observed when the same concentration of $InsP_3$ was added to the already depleted Ca^{2+} pools in the experiment illustrated in panel B. Obviously, in contrast with the prediction of the steady-state model, panel B shows that the small $InsP_3$-induced Ca^{2+} efflux observed (l^{IP3}) was *much faster* than the initial leak created by ionophore (l_O^{iono}), and therefore also much faster than the leak created by ionophore under the conditions of reduced Ca^{2+} load (l^{iono}). This is experimental evidence against the steady-state model. Allowing, because of the slightly higher free Ca^{2+}, for a pumping rate in the presence of ionophore slightly higher than in its absence (point Q in panel B vs. point P in panel A) makes the experimental result even more contradictory to the behaviour predicted by the steady-state model. In connection with this, it was previously noted, in a quantitative simulation of the model, that it was not easy to simulate a release of part of the stored Ca^{2+} which was initially *very* rapid relative to the one way fluxes [7].

Table I shows the average results corresponding to

five separate experiments, which all displayed the same quantitative features as the one illustrated in Fig. 1. The results make sure that in these measurements, 1 μM ionomycin reduced the Ca^{2+} load in the $InsP_3$-sensitive compartment to a new load (0.77 nmol/mg at point Q, compared to 1.10 before addition of ionomycin) comparable to and in fact even smaller than that resulting from the addition of 0.17 μM $InsP_3$ (0.81 nmol/mg at point P). As a result of these measurements, the relative efficiency of 0.17 μM $InsP_3$ in emptying the $InsP_3$-sensitive Ca^{2+} stores was found not to differ greatly in control and partially depleted stores (26 ± 3 and 28.5 ± 2% of the effect of maximal $InsP_3$ concentrations, respectively).

4. DISCUSSION

The hypothesis that the partial release of Ca^{2+} induced by submaximal concentrations of $InsP_3$ might be due to rapid attenuation of the Ca^{2+} channels' permeability was explored by various groups. For instance, in synaptosome-derived microsomal vesicles, Finch et al. recently described a rapid decay in the rate of $InsP_3$-induced Ca^{2+} release which was accounted for by conductance inactivation rather than store depletion [11], and suggested that the rise in cytosolic Ca^{2+} accompanying the $InsP_3$-induced Ca^{2+} release might modulate the properties of the Ca^{2+} channel. In our permeabilized hepatocytes, however, the possibility that the partial release was dependent on local increase of the Ca^{2+} concentration around the storage compartments was excluded by $^{45}Ca^{2+}$ experiments (not shown) in which we found that partial discharge by submaximal concentrations of $InsP_3$ was also observed both when the external free Ca^{2+} concentration was strictly buffered to pCa 7 (by the addition in the efflux medium of large amounts of EGTA and $^{45}Ca^{2+}$) and when it was dramatically reduced (by the addition of EGTA alone). Note that Meyer and Stryer [4] also found that increasing the free Ca^{2+} concentration from 150 to 800 nM did not significantly modify the kinetics of Ca^{2+} release in permeabilized rat basophilic leukemia cells.

Our initial suggestion that the putative partial channel inactivation might be associated with the conversion of the $InsP_3$ receptor into a form with a higher affinity for its ligand [12] soon appeared to be in contradiction with the fact that further addition of $InsP_3$ to the partially emptied pools resulted in further releases of Ca^{2+}. However, Meyer and Stryer [4] pointed out that if this putative inactivation (occurring as a result of a change in the binding of a ligand) were associated with the conversion of the $InsP_3$ receptor into a form with a lower affinity for $InsP_3$, the responsiveness of the half-unloaded pools to further addition of $InsP_3$ would be preserved.

Irvine then made the very attractive suggestion that partial channel closure and the putative conversion of the $InsP_3$ receptor into a form with a lower affinity for $InsP_3$ might occur as a result of the drop in the Ca^{2+} concentration *inside* the stores [6]. In other words, dissociation of Ca^{2+} from a lumenal site on the $InsP_3$ receptor or on an associated protein might reduce the affinity of the receptor for cytosolic $InsP_3$, and this would provide an explanation for quantal release, even if the Ca^{2+} stores formed a homogeneous population. The purpose of the present work was to test this model. To our disappointment, the kinetic predictions derived from the model, at least in its initial form, were not verified in suspensions of permeabilized liver cells (Fig. 1 of this report). Moreover, judging from the results of experiments performed under different Ca^{2+} loading conditions (results of $^{45}Ca^{2+}$ experiments are not shown, but see Table 1), the relative efficiency of $InsP_3$ in allowing Ca^{2+} release did not appear to be greatly dependent on the intraluminal Ca^{2+} content.

Consequently, the above various models, based on spontaneous or Ca^{2+}-mediated putative attenuation of the sensitivity to $InsP_3$ of identical release channels in a homogeneous population of Ca^{2+} stores, do not fully explain why submaximal concentrations of $InsP_3$ release less Ca^{2+} than higher concentrations of this ligand. This brings us back to the original suggestion of Muallem et al. that submaximal $InsP_3$ concentrations released all the Ca^{2+} from a fraction of the $InsP_3$-sensitive stores of permeabilized pancreatic acini, whereas none of the Ca^{2+} was released from the remaining fraction, a process which they called quantal release [3]. Using intact *Xenopus* oocytes and laser confocal microscopy, Parker and co-workers [13,14] provided evidence for all-or-

Table 1

$InsP_3$-induced and/or ionophore-induced partial store depletion*

	Ca^{2+} released (nmol/mg)	$InsP_3$-sensitive fraction	
		Total pool (nmol/mg)	Released by 0.17 μM $InsP_3$ (%)
Panel A			
released by 0.17 μM $InsP_3$	0.29±0.04[a]		26.0±3[d]
further released by 2.5 μM $InsP_3$	0.81 0.13[b]	1.10±0.14[c]	
further released by 4 μM ionophore	0.68±0.10		
Panel B			
released by 1 μM ionophore	0.55±0.05		
released by 0.17 μM $InsP_3$	0.22±0.01[f]		28.5±2[i]
further released by 2.5 μM $InsP_3$	0.55±0.03[g]	0.77±0.03[h]	
further released by 4 μM ionophore	0.58±0.03		

*Mean ±S.D. of five experiments.
Superscripts: c=a+b; d=a/c; h=f+g; i=f/h.

nothing localized release of Ca^{2+} in these large cells. As regards the possible reasons for pool heterogeneity in permeabilized smaller cells, one obvious reason is that such experiments are performed with large populations of cells, which may have different individual patterns of Ca^{2+} response [15,16]. Inside a given cell, various compartments with different behaviour might also coexist, for instance with different receptor surface densities, or different surface-to-volume ratios, or different ratios of $InsP_3$-sensitive channels to re-pumping ATPase units [17]. The fact that a very large number of small and independent Ca^{2+} storage compartments exist within a single cell (instead of a large single compartment) seems to be implied by the fact that Ca^{2+} oscillations spread through the cell in the form of a Ca^{2+} wave [14,18], although the exact identity of the relevant stores is still open to debate (e.g. [19]). With regards to individual $InsP_3$ receptors, plasticity has been observed in response to acute [20] or chronic [21] hormone stimulation; receptor heterogeneity might be mediated by different levels of phosphorylation [22,23], different sensitivities to Ca^{2+} [24], variable posttranscriptional modifications [7], or the presence of several types of transcripts [25,26].

In this work, we tested whether a reduction of intralumenal Ca^{2+} was responsible for the partial release of Ca^{2+} induced by submaximal concentrations of $InsP_3$ in permeabilized hepatocytes, and obtained a negative answer. Obviously, this does not deny that the intralumenal Ca^{2+} load might be critical in other respects, as appears to be the case for caffeine-sensitive Ca^{2+} stores in chromaffin granules [27] and, under stringent conditions, for overloaded $InsP_3$-sensitive Ca^{2+} stores in hepatocytes [19]. Irvine [6] also previously described how a direct effect of luminal Ca^{2+} on the $InsP_3$ receptor would contribute to the understanding of the Ca^{2+} entry into stimulated cells.

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