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The role of inorganic phosphate in regulating the kinetics of inositol 1,4,5-trisphosphate-induced Ca^{2+} release: a putative role for endoplasmic reticulum phosphate transporters

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Abstract

The effects of phosphate and acylphosphonate phosphate transporter inhibitors were investigated on inositol 1,4,5-trisphosphate ($InsP_3$)-induced Ca^{2+} release from cerebellar microsomes. Although neither changing the phosphate concentration nor adding phosphate transporter inhibitors affected the percentage (extent) of $InsP_3$-induced Ca^{2+} release, they did, however, affect the transient kinetics of this process. $InsP_3$-induced Ca^{2+} release is biphasic in nature, arising from two populations of $InsP_3$-sensitive Ca^{2+} stores which either release Ca^{2+} in a fast or slow fashion. Altering phosphate concentration or adding phosphate transporter inhibitors appeared to affect only the fast phase component. We therefore suggest that these observations could be explained by the possibility that phosphate transporters only reside in the fast releasing $InsP_3$-sensitive Ca^{2+} stores.

Keywords: Inorganic phosphate; Phosphate transport; Calcium release; Inositol 1,4,5-trisphosphate; Endoplasmic reticulum

1. Introduction

The binding of hormones and neurotransmitters to cell surface receptors triggers the activation of phospholipase C which forms the second messenger inositol 1,4,5-trisphosphate ($InsP_3$) [1]. $InsP_3$ binds to and opens a specific Ca^{2+} channel usually located on the endoplasmic reticulum (ER) membranes, causing an increase in the cytoplasmic Ca^{2+} concentration. It was previously believed from studies using K^+ channel blockers that K^+ ions acted as counterions in order to prevent the build-up of membrane potential which can be caused by Ca^{2+} efflux [2,3]. We and others have shown that inhibition of $InsP_3$-induced Ca^{2+} release by these K^+ channel blockers was in fact due to direct inhibition of the $InsP_3$ receptor itself [4,5]. More recently, we examined the effects of monovalent cations on $InsP_3$-induced Ca^{2+} release from the cerebellar endoplasmic reticulum vesicles, and showed that K^+ is absolutely necessary for Ca^{2+} release. However, a detailed kinetic analysis demonstrated that it behaved untypical of other co-transporter mechanisms, where normally the concentration of one of the ions increases the rate of the other co-transported ion (as long as it is below the J_{max}) [6]. In our case we showed that in-

Abbreviations: $InsP_3$, Inositol 1,4,5-trisphosphate; PMSF, phenylmethylsulfonyl fluoride; PAA, phosphonoacetic acid; PFA, phosphonoformic acid; PPA, phosphonopropionic acid; PhPA, phenylphosphonic acid; EC_{50}, concentration causing half-maximal response; J_{max}, maximum transport rate

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creasing concentrations of K^+ ions had no effect on the rate constant for $InsP_3$-induced Ca^{2+} release; rather, only the extent of Ca^{2+} release increased with increasing K^+ concentration [6]. These results led us to postulate that K^+ ions may modulate Ca^{2+} release by acting as a 'co-factor' aiding channel gating rather than acting as a counter-ion.

In search of other physiological ions which might modulate Ca^{2+} transport through the $InsP_3$-sensitive channel by being co-transported with Ca^{2+}, here we examined the effect of phosphate ions on this transport process. Previous studies have shown that phosphate transport into the lumen of the sarco(endo)-plasmic reticulum vesicles causes an increase in the rate of Ca^{2+} accumulation [7,8]. In these studies, active accumulation of Ca^{2+} was paralleled by phosphate ion movement in the same direction. This movement of both Ca^{2+} and phosphate ions suggests that phosphate ions may play a role in maintaining a low membrane potential across the ER or sarcoplasmic reticulum (SR), in addition to minimizing passive Ca^{2+} leakage by forming $Ca_3(PO_4)_2$. More recently Fulceri et al. [9] have shown that the phosphate-enhanced accumulation of Ca^{2+} into rat brain, rat solid Morris hepatoma and human platelet microsomes caused an augmentation in the $InsP_3$-sensitive Ca^{2+} pool. This study also showed that $InsP_3$ caused phosphate release in parallel with Ca^{2+}.

In previous studies we and others have demonstrated $InsP_3$-induced Ca^{2+} release to be a biphasic process with fast and slow releasing Ca^{2+} stores [10–13]. In this study we have investigated the effects of phosphate and phosphate transporter inhibitors on the $InsP_3$-mediated Ca^{2+} release from cerebellar microsomes, and have shown an unexpected effect on the transient kinetics of this Ca^{2+} release process.

2. Materials and methods

The phosphate transporter inhibitors phosphonoacetic acid (PAA), phosphonoformic acid (PFA), phosphonopropionic acid (PPA) and phenylphosphonic acid (PhPA) were purchased from Aldrich.

Rat cerebellar microsomes were prepared as described in Ref. [12]. Briefly, 20 fresh rat cerebella were homogenized in approx. 10 volumes of buffer containing 0.32 M sucrose, 5 mM Hepes (pH 7.2) in the presence of a cocktail of protease inhibitors (0.1 mM PMSF, 10 μM leupeptin, 10 μM pepstatin A, 50 μM bezamidine), and then centrifuged for 10 min at $500 \times g$. The supernatant was maintained and the pellet was re-homogenized in 5 volumes of the same buffer and centrifuged as above. The supernatants were then mixed and centrifuged for 20 min at $10\,000 \times g$; the resulting supernatant was finally centrifuged for 1 h at $100\,000 \times g$, yielding the microsomal pellet which was re-suspended in approx. 2–3 ml of the same buffer, aliquoted and snap-frozen in liquid nitrogen, and stored at −70°C until used.

Ca^{2+} uptake and release were carried out by monitoring changes in fluo-3 fluorescence as described in Ref. [14]. Briefly, approx. 200 μg of cerebellar microsomes was incubated in 2 ml of buffer containing 40 mM Tris, phosphate (at the desired concentrations), 100 mM KCl (pH 7.2) at 37°C and to this was added 10 mM phosphocreatine, 10 μg/ml creatine kinase and 125 nM fluo-3. The initial free $[Ca^{2+}]$ was determined to be between 4.7 to 5.8 μM depending upon the phosphate concentration used (see Table 1). Ca^{2+} uptake was then initiated by the addition of 1.5 mM MgATP, and when enough Ca^{2+} had been accumulated, the pumps were inhibited by the addition of approx. 100 μM orthovanadate. $InsP_3$ was then added and the Ca^{2+} released was expressed as a percentage of that released by the Ca^{2+} ionophore A23187.

Rapid kinetic measurements were carried out using stopped-flow spectrofluorimetry as described in Refs. [12,13]. Once Ca^{2+} had been accumulated and after the addition of orthovanadate, the mixture was transferred into syringe A of the stopped-flow instrument (Applied Photophysics, Model SX17-MV). To syringe B, $InsP_3$ at 10-times concentration was added (mixing ratio 10:1) in order to avoid any Ca^{2+} contamination. The contents of the two syringes were then mixed, and the changes in Ca^{2+} concentrations were monitored by exciting at 505 nm and measuring the emission above 515 nm using a cut-off filter. The traces were then analysed using non-linear regression analyses programmes supplied by Biosoft and Applied Photophysics. These traces were found to best fit to a biexponential equation as described in Refs. [11–13].

Over the Ca^{2+} concentrations being measured, the fluorescence change was linearly related to $[Ca^{2+}]$

($r > 0.99$). The effects of any small Ca^{2+} re-uptake on the rates of release were negligible [6,13]. [^{3}H]$InsP_3$ binding to cerebellar microsomes was performed as described in Ref. [6].

3. Results

The addition of ATP to a suspension of cerebellar microsomes in the presence of ATP regenerating system leads to Ca^{2+} uptake which can be monitored using the Ca^{2+} indicator fluo-3 [14]. Optimal Ca^{2+} uptake into the cerebellar microsomes occurred using a buffer that contained 25 mM phosphate. Lowering the phosphate concentrations to 10 mM reduced the rate of Ca^{2+} accumulation by approximately 40%. At even lower phosphate concentration (5 mM) this was even more dramatically affected, causing an approximately 40-fold reduction in the rate of Ca^{2+} accumulation. Higher concentrations of phosphate (40 mM), on the other hand, caused little or no effect on the rate of Ca^{2+} uptake compared to that at 25 mM (Table 1). In all cases there was only a small effect ($\approx 20\%$) on the overall amount of Ca^{2+} that was accumulated into the vesicles between 5 and 40 mM phosphate. A lower concentration of phosphate could not be used since little or no Ca^{2+} accumulation could be detected at these concentrations.

Fig. 1 Shows the effect of varying the concentration of phosphate on the dose response of $InsP_3$-Induced Ca^{2+} release from rat cerebellar microsomes. Maximal release using our standard conditions of 25 mM phosphate occurred at 10–20 μM $InsP_3$ causing approximately 40% release of the total accumulated Ca^{2+} pool (EC_{50} of 0.25 ± 0.05 μM). Varying the phosphate concentrations from 5 to 40 mM had no significant effect on either the extent of Ca^{2+} released by $InsP_3$ or the EC_{50} values.

Table 1
The effect of varying [phosphate ions] on Ca^{2+} uptake by cerebellar microsomes

[Phosphate] (mM)	Initial rate of Ca^{2+} uptake (nmol/min/mg)	Initial $[Ca^{2+}]_{free}$ (μM)
5	0.35	5.8
10	8.5	5.1
25	15.0	4.6
40	14.0	4.7

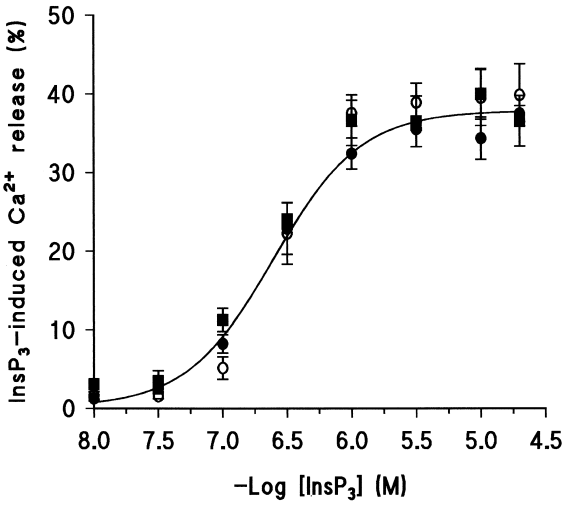

Fig. 1. The effects of varying [phosphate] on the quantal $InsP_3$-induced Ca^{2+} release (IICR) from rat cerebellar microsomes. Ca^{2+} release by different concentrations of $InsP_3$ was measured in a buffer containing 40 mM Tris, 100 mM KCl, in the presence of 5 mM (■), 25 mM (○) and 40 mM (●) phosphate at pH 7.2. Ca^{2+} release was expressed as a percentage of that released by Ca^{2+} ionophore A23187. The data points represent the mean ± S.D. of three or more determinations.

When these experiments are performed on the stopped-flow spectrofluorimeter to gain information on the kinetics of this process, there were clear differences on the time course of Ca^{2+} release. Fig. 2A shows the data of Ca^{2+} release induced by 20 μM $InsP_3$ at 5, 10, 25 and 40 mM phosphate. Each trace was fitted to the biexponential equation given in Ref. [12]. The fits to monoexponential equation were significantly poorer (with χ^2 values of 0.03 and 0.2 for biexponential and monoexponential equations, respectively). In the case of high phosphate concentration (40 mM), there was a slight curvature in the first 0.5 s, which was omitted for the reason of simplification in the fittings. It is clearly shown that varying phosphate concentrations markedly affected the rate constants of Ca^{2+} release. Unexpectedly, phosphate ions appeared to exert their effects on the fast phase where the rate constants increased with increasing phosphate concentrations up to 25 mM, while higher concentrations (40 mM) caused a decrease in the rate constants for this phase. The rate constants for the slow phase, on the other hand, were unaffected. As expected, the amplitudes (extent) of Ca^{2+} for both phases were also insensitive to phosphate (Fig. 2B,C).

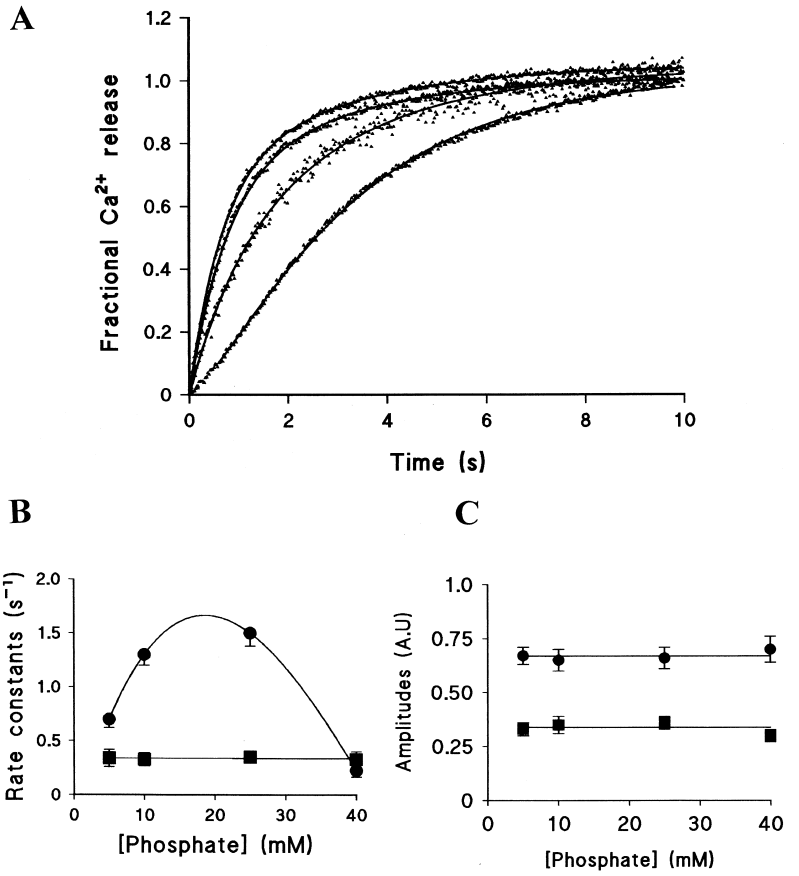

Fig. 2. The effects of phosphate on the transient kinetics of $InsP_3$-induced Ca^{2+} release. (A) Ca^{2+} release by 20 μM $InsP_3$ in the presence of (from top to bottom) 25, 10, 5 and 40 mM phosphate. The traces are the average of 6–10 accumulations. The lines through the data points represent the fits to a biexponential equation as described in Ref. [13]. The rate constants and amplitudes derived from these fits are shown in B and C, where (●) represents the parameters for the fast component and (■) represents the parameters for the slow component.

In order to examine whether the results obtained were due to the concomitant increased phosphate transport via a phosphate transporter across the ER membranes in parallel with Ca^{2+}, acylphosphonate phosphate transporter inhibitors (i.e., PFA, PAA, PPA and PhPA) were used [7,15,18]. The addition of these compounds after Ca^{2+} uptake had taken place, and prior to $InsP_3$ addition again had no apparent effect on the extent of Ca^{2+} release by $InsP_3$ (Fig. 3). Analyses of the traces (Fig. 3), however, showed these compounds to have a strong inhibitory effects on the rate constants of the fast phase, whereas the rate constants for the slow phase were again unaffected (Table 2). The contribution of each phase to the extent of release were also unaffected. The most hydrophobic of these phosphate transporter inhibitors, PhPA, was the most potent in reducing the rate constants for the fast phase by more than 15-fold. The least potent were PFA and PAA, which are the least hydrophobic, and these caused 2–3-fold reduction in the rate constants of the fast phase of Ca^{2+} release.

[^{3}H]$InsP_3$ binding studies showed that these compounds at concentrations used in the present work (1 mM) had no significant effect of $InsP_3$ binding to its receptor (in the absence of phosphate transporter inhibitors binding was set as $100 \pm 7\%$, in the presence of PFA it was $90 \pm 6\%$, with PAA $80 \pm 8\%$, with PPA $93 \pm 5\%$, and PhPA $99 \pm 3\%$).

4. Discussion

Active accumulation of Ca^{2+} in the cell can lead to

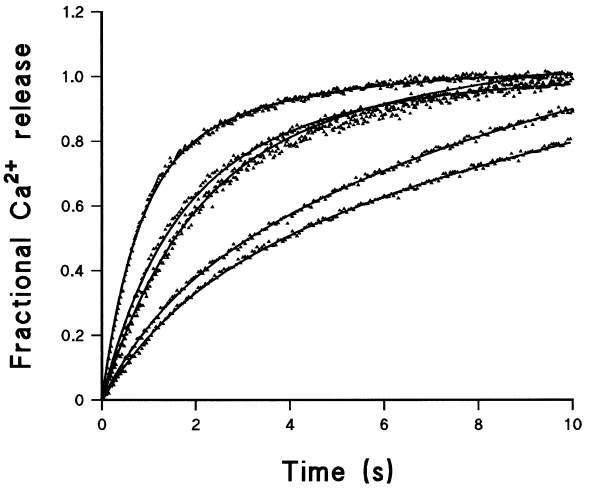

Fig. 3. The effect of phosphate transporter inhibitors on the kinetics of $InsP_3$-induced Ca^{2+} release. The traces (from top to bottom) represent Ca^{2+} release by 20 μM $InsP_3$ in the absence and presence of 1 mM of PAA, PFA, PPA and PhPA, respectively, in a buffer containing 25 mM phosphate. The solid lines through the data points represent the fits to a biexponential profile; the rate constants and amplitudes derived from these fits are summarized in Table 2.

a variety of cytotoxic processes, thus Ca^{2+} concentration in resting cells is kept low in the cytoplasm ($\approx$100 nM) [16]. This low concentration is maintained by either pumping out Ca^{2+} through the plasma membrane via Ca^{2+} pumps and active exchange with Na^+ [17]. Alternatively, Ca^{2+} can be sequestered into different cellular organelles such as the endoplasmic reticulum [1]. The notion that Ca^{2+} movement into or out of the cytoplasm is counterbalanced by the movement of ions such as K^+ in the opposite direction has now been questioned, at least, for Ca^{2+} efflux through the $InsP_3$-sensitive pathway [6]. The fact that Ca^{2+} pumps are electrogenic would require the retention of their electro-neutrality in order to remain active, therefore Ca^{2+} sequestration into various cellular organelles create a large gradient which has to be neutralized by the movement of other ions. Phosphate ions play a significant role in buffering cellular Ca^{2+}, and a recent report by Fulceri et al. [9] highlighted the importance of phosphate in Ca^{2+} sequestration into ER. This study also showed that active accumulation of Ca^{2+} into three different microsomal preparations led to a parallel uptake of phosphate ions and the release of Ca^{2+} by Ca^{2+} ionophore A23187, and $InsP_3$ was also accompanied by phosphate release [9]. The results presented here confirm the importance of phosphate ions in Ca^{2+} transport processes. Although the amount of Ca^{2+} accumulated was not affected by varying phosphate concentrations as observed in Ref. [9], the rate by which it is transported is highly dependent on the presence of phosphate ions, as also demonstrated by Fulceri et al. [9]. It is at present unclear why in our studies we did not observe changes in the extent of $InsP_3$-induced Ca^{2+} release with changes in [phosphate] compared with that observed by Fulceri et al., although differences in the experimental conditions between these two studies may be one possibility. Earlier studies in SR membranes reported the existence of phosphate transporters, which could be inhibited by acylphosphonates [7] and which were previously used has inhibitors of the Na^+/PO_4 transporter [15,18]. As Ca^{2+} release from the cerebellar ER membranes occurs mainly through the $InsP_3$ receptors, it is clear that phosphate and phosphate transporter inhibitors inhibit the fast phase component of this process without affecting the extent of $InsP_3$-induced Ca^{2+} release. Increasing phosphate concentrations caused an increase in the fast rate constants up to an optimal concentration of 25 mM, while higher concentrations resulted in a

Table 2
The effects of phosphate transporter inhibitors on the rate constants and amplitudes of $InsP_3$-induced Ca^{2+} release from rat cerebellar microsomes

	Fast phase rate constant k_{fast} (s^{-1})	Slow phase rate constant k_{slow} (s^{-1})	Amplitude fast phase	Amplitude slow phase
Control	1.50	0.35	0.65	0.35
PFA (1 mM)	0.52	0.31	0.60	0.40
PAA (1 mM)	0.80	0.32	0.61	0.39
PPA (1 mM)	0.13	0.38	0.60	0.40
PhPA (1 mM)	0.09	0.37	0.63	0.37

decrease in the rate constants. The rate constants for the slow phase and for both amplitudes were unaffected.

In the heterogeneous models of $InsP_3$-sensitive Ca^{2+} stores, it is assumed that there are at least two major types of Ca^{2+} stores which release their Ca^{2+} content in response to $InsP_3$ in a fast or slow manner [10–13]. these results may suggest that phosphate only affects the fast phase Ca^{2+} stores but has no effect on the slow phase Ca^{2+} stores. The observed decrease in the fast phase rate constants at high phosphate levels could simply be explained in terms of the formation of less soluble Ca^{2+}/phosphate complexes which dissociate slowly. The use of specific phosphate transporter inhibitors revealed that again only the rate constants of the fast phase were affected, with no apparent effects on either the rate constants of the slow phase or the amplitudes of both phases. The fact that only the rate constants of the fast phase of Ca^{2+} release were affected by changing the phosphate ion concentrations or by the addition of phosphate transporter inhibitors leads to our proposal that only the Ca^{2+} stores which contribute to the fast phase of Ca^{2+} release contain phosphate transporters. From Table 2, it is also clear that some inhibitors decreased the rate constant for the fast phase even lower than that observed for the slow phase, which we have assumed do not contain phosphate transporters. This observation may be explained by the possibility that fast phase Ca^{2+} stores contain a lower density of $InsP_3$ receptors than the slow phase Ca^{2+} stores. Thus it is purely due the fact that these stores contain phosphate transporters which makes them faster. Although it was reported that the purified $InsP_3$ receptor reconstituted into liposomes also released Ca^{2+} in a biphasic manner [19], our re-analysis of this data indicate that it could also be fitted to a monophasic process, when taking into account the high signal-to-noise ratio.

The potency of these inhibitors on the cerebellar ER microsomes was in the order of PAA < PhPA, which would suggest that the most potent inhibitors are the most hydrophobic ones. However, in studies with SR there was a marked difference in the potency of these inhibitors which were in the reverse order [7]. It is clear that for this case the less hydrophobic compounds were the most potent phosphate transport inhibitors. The pattern of inhibition for the Na^+/phosphate transporter, on the other hand, was different again from either of these two [15,18]. This indicates that the cerebellar ER phosphate transporter is different from both the SR and the Na^+/phosphate co-transporter in its inhibitory properties.

Although we suggest in this paper that the effects of phosphate and acylphosphonates on $InsP_3$-induced Ca^{2+} release may be due to the presence of phosphate transporters in the fast releasing Ca^{2+} stores, it must be acknowledged that other possibilities could account for these observations. Once such alternative could be that there are phosphate and acylphosphonate binding sites on the $InsP_3$ receptor itself which can affect its Ca^{2+} releasing properties. However, if such sites do exist they cannot be at the $InsP_3$ binding site displacing the natural ligand, since we have shown that acylphosphonates do not affect [^{3}H]$InsP_3$ binding. In addition, if phosphates act by decreasing the occupancy of the receptor to $InsP_3$, this would have the effect of both decreasing the fast and slow phase rate constants for release [11,12], which is not what is observed.

The fact that phosphate transporter inhibitors appear to preferentially affect only the fast releasing $InsP_3$-sensitive Ca^{2+} stores may prove to be a useful tool in further understanding the role different types of $InsP_3$-sensitive Ca^{2+} stores play in agonist-induced Ca^{2+} mobilization and spatial–temporal Ca^{2+} oscillations [20].

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