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Ca^{2+} regulation of 1-(3-*sn*-phosphatidyl)-1D-*myo*-inositol 4-phosphate formation and hydrolysis on sarcoplasmic-reticular Ca^{2+}-transport ATPase

A new principle of phospholipid turnover regulation

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Lipid phosphorylation was shown to occur on the isolated sarcoplasmic-reticulum (SR) Ca^{2+}-transport ATPase. More than 95% of the radioactivity incorporated on incubation of the SR ATPase with [γ-^{32}P]ATPMg can be extracted with acidic organic solvents and was identified as 1-(3-*sn*-phosphatidyl)-1D-*myo*-inositol 4-phosphate (PtdIns4*P*) [Varsányi, Toelle, Heilmeyer, Dawson & Irvine (1983) EMBO J. **2**, 1543–1548]. This lipid phosphorylation is only observed at nanomolar concentrations of free Ca^{2+}; in the presence of micromolar free Ca^{2+} PtdIns4*P* disintegrates rapidly. Also, upon blockade of the kinase reaction PtdIns4*P* decomposes, indicating a PtdIns/PtdIns4*P* turnover. The PtdIns4*P* concentration is dependent on the free Ca^{2+} concentration, being half-maximal at 35 nM-Ca^{2+}. PtdIns4*P* hydrolysis is catalysed by a PtdIns4*P* phosphomonoesterase; accordingly no diacylglycerol is formed, which would be a product of a phosphodiesteratic cleavage. Fluoride inhibits this phosphomonoesterase. Ca^{2+} does not influence directly either the PtdIns kinase or the PtdIns4*P* phosphomonoesterase. PtdIns4*P* forms a tight complex with the transport ATPase, from which it can be removed only by chromatography on heparin–agarose in the presence of Triton X-100. It is concluded that Ca^{2+} regulates the PtdIns/PtdIns4*P* turnover by availability of substrate, depending on the Ca^{2+}-transport-ATPase conformation, which traps or exposes the respective lipid head groups.

INTRODUCTION

It is generally accepted that PtdIns4*P* and PtdIns(4,5)P_2 are intermediates in a reaction sequence leading to two second messengers, InsP_3 and DG (for review see Sekar & Hokin, 1986; Berridge, 1986). Furthermore, PtdIns and its phosphorylated derivatives interact strongly with membrane proteins, which raises the question of whether they could exert a regulatory function (Choquette *et al.*, 1984; Lin & Fain, 1984; cf. Lin *et al.*, 1983). It is conceivable that polyphosphoinositides not only may serve as metabolites but may also play a functional role, e.g. as lipophilic membrane-localized effectors. Alternatively, complexing of PtdIns or its derivatives with membrane proteins might influence the metabolic flux of these phospholipids through the signalling cascade.

Previously we have described for the first time that mainly PtdIns4*P* can be formed on the isolated skeletal-muscle SR Ca^{2+}-transport ATPase (Varsányi *et al.*, 1983). Only trace amounts of phosphate are incorporated into protein with either the isolated Ca^{2+}-transport ATPase (Varsányi *et al.*, 1983) or isolated SR vesicles (Georgoussi & Heilmeyer, 1986*b*). PtdIns4*P* formation is catalysed either by an endogenously present PtdIns kinase or exogenously added PtdIns kinase. As a source of exogenous PtdIns kinase phosphorylase kinase was employed; we have shown recently that this protein kinase expresses the above lipid kinase activity, too (Georgoussi & Heilmeyer, 1986*a*). Neither on the isolated Ca^{2+}-transport ATPase nor in longitudinal SR does a further phosphorylation to PtdIns(4,5)P_2 take place (Varsányi *et al.*, 1986*a*). In other membranes, e.g. rat liver lysosomal membranes (Collins & Wells, 1983) or Golgi membranes (Jergil & Sundler, 1983), PtdIns4*P* and only traces of PtdIns(4,5)P_2 (not more than 5%) were also formed. The apparent lack of PtdIns4*P* kinase in all these organelles excludes the possibility that there PtdIns4*P* serves as a second-messenger precursor.

PtdIns4*P* formation on the isolated SR Ca^{2+}-transport ATPase is only observed at nanomolar free Ca^{2+}; in presence of micromolar free Ca^{2+} a fast disintegration occurs (Heilmeyer *et al.*, 1985). It is not known which reaction, a phosphodiesteratic cleavage or hydrolysis by a phosphomonoesterase, is responsible for this disintegration, or if any of the enzymes involved are themselves regulated by micromolar Ca^{2+}.

The present paper shows that a PtdIns4*P* phosphomonoesterase catalyses PtdIns4*P* hydrolysis on the Ca^{2+}-transport ATPase and that micromolar Ca^{2+} regulates the PtdIns/PtdIns4*P* turnover by availability of substrate on the lipid–membrane-protein complex.

Abbreviations used: PtdIns, 1-(3-*sn*-phosphatidyl)-1D-*myo*-inositol; PtdIns4*P*, 1-(3-*sn*-phosphatidyl)-1D-*myo*-inositol 4-phosphate; PtdIns(4,5)P_2, 1-(3-*sn*-phosphatidyl)-1D-*myo*-inositol 4,5-bisphosphate; InsP_3, D-*myo*-inositol 1,4,5-trisphosphate; DG, diacylglycerol; PA, 3-phosphatidic acid; SR, sarcoplasmic reticulum.

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MATERIALS AND METHODS

Materials

[^{32}P]P_i (carrier-free) was obtained from New England Nuclear, and the reagents for [γ-^{32}P]ATP synthesis (Walseth & Johnson, 1979) were purchased from Boehringer-Mannheim. [^{3}H]PtdIns was from Amersham. Inositol phospholipids used as standards were from Sigma. Silica-gel plates (20 cm × 20 cm; 0.25 mm thickness) were obtained from Merck. Hexokinase from yeast as an $(NH_4)_2SO_4$ suspension (140 units/mg) was obtained from Boehringer-Mannheim.

Preparations and assays

SR membranes were isolated as described by De Meis & Hasselbach (1971) and the Ca^{2+}-transport ATPase was prepared as described by MacLennan (1970). Protein concentration was measured by the methods of Lowry *et al.* (1951) and Bradford (1976). Lipid-bound radioactivity associated with the Ca^{2+}-transport ATPase was assayed as described by Varsányi *et al.* (1983). Phospholipid phosphate was determined as described by Bartlett (1959). Phosphorylase kinase was prepared by the method of Cohen (1973), as modified by Jennissen & Heilmeyer (1975) and Hessová *et al.* (1985). This protein kinase exhibits PtdIns kinase activity of approx. 1 nmol/min per mg (Varsányi *et al.*, 1986*a*; Georgoussi & Heilmeyer, 1986*a*). DG kinase from rabbit skeletal muscle was purified as described by Georgoussi & Heilmeyer (1986*a*). T.l.c. was carried out as described by Shaigk & Palmer (1977). Lipid-bound radioactivity was determined as described by Schacht (1981). Free Ca^{2+} concentrations in Ca/EGTA buffers were calculated by using the binding constants reported by Sillén & Martel (1970) given for 25 °C.

Preparation of [^{32}P]PtdIns4*P* as substrate for the PtdIns4*P* monoesterase

PtdIns associated with the heat-denatured Ca^{2+}-transport ATPase (5 mg/ml) was phosphorylated with exogenous PtdIns kinase (625 μg of phosphorylase kinase/ml) as described by Varsányi *et al.* (1983). After 4–6 h at 26 °C a preparation of approx. 5 mol of PtdIns4*P*/100000 g of protein was obtained. The phosphorylation was stopped by heat treatment of the reaction mixture at 85 °C for 5 min. Additionally, free Mg^{2+} was complexed by excess of EDTA to protect [^{32}P]PtdIns4*P*. The denatured membranes were sedimented by centrifugation (100000 *g* for 70 min at 23 °C).

After addition of 30 mM-Hepes, pH 7.5, to the collected membranes, the suspension obtained was extensively dialysed for 2.5 h against 1% $Na_4P_2O_7$/20 mM-Hepes, and for a further 2.5 h against 20 mM-Hepes, pH 7.5. The non-covalently bound ^{32}P radioactivity (i.e. P_i) yields 1–2% of the total radioactivity incorporated into PtdIns4*P*.

Assay for PtdIns4*P* monoesterase activity

As substrate, [^{32}P]PtdIns4*P* associated with the heat-treated Ca^{2+}-transport ATPase was used (for preparation of the substrate see above); 49 nmol of PtdIns4*P*/ml of reaction mixture was incubated in a total volume of 150 μl (at pH 7.5, 26 °C) containing 50 mM-Hepes/KOH, 1% Triton X-100, 2 mM-EGTA and final free Ca^{2+} concentrations of 1 nM up to 0.1 mM with PtdIns4*P* monoesterase containing probes of 0.2–2.5 mg/ml. The reaction was started by the addition of [4-^{32}P]PtdIns4*P*. During the dephosphorylation, samples were withdrawn and mixed with 7% trichloroacetic acid containing 2.8 mg of bovine serum albumin/ml. After storage of the samples for 10 min on ice and centrifugation at 1100 *g* for 15 min at room temperature, ^{32}P radioactivity of the supernatant was determined by scintillation counting. The initial phosphate liberation rate was used for calculation of PtdIns4*P* monoesterase activity. The effect of 1% Triton X-100 on the solubilization of the [^{32}P]PtdIns4*P*-containing heat-treated ATPase was determined and taken into consideration.

Preparation of phosphatidyl[2-^{3}H]inositol [4-^{32}P]phosphate ([^{3}H/^{32}P]PtdIns4*P*)

[2-^{3}H]PtdIns (10 μCi) obtained from Amersham in toluene/ethanol (1:1, v/v) was dried under N_2 stream. To this dried lipid 2.5 mg of PtdIns (Sigma) in 500 μl of 100 mM-KCl/100 mM-Tris/HCl (pH 7.5)/2.4% Triton X-100 was added, and the suspension was sonicated for 1 h at 40–50 °C. Phosphorylation of [^{3}H]PtdIns was carried out at a final PtdIns concentration of 1 mM in 100 mM-KCl/100 mM-Tris/HCl (pH 7.5)/10 mM-EDTA/1 mM-EGTA/1 mM-dithioerythritol/0.4% Triton X-100 with 750 μg of PtdIns kinase/ml. After preincubation at 4 °C for 15 min, the temperature was raised to 36 °C and 10 mM-[γ-^{32}P]ATP was added to the

Table 1. Triton X-114 extraction of PtdIns kinase and PtdIns4*P* phosphomonoesterase from SR membranes

Extraction was carried out as described in the Materials and methods section. PtdIns kinase activity was determined by assaying lipid-bound radioactivity as described by Georgoussi & Heilmeyer (1986*a*). PtdIns4*P* monoesterase activity was determined as described in the Materials and methods section.

		Protein		PtdIns kinase activity		PtdIns4*P* monesterase activity	
Fraction	Volume	Concn. (mg/ml)	Total (mg)	Specific (pmol/min per mg)	Total (pmol/min)	Specific (pmol/min per mg)	Total (pmol/min)
SR suspension in 0.1 M-KCl	1.00	0.83	0.83	352	292		
Aqueous phase	0.85	0.41	0.35	331	116		
SR suspension in 0.1 M-KCl	1.00	2.50	2.50			630	1575
Aqueous phase	0.85	1.23	1.05			1014	1065

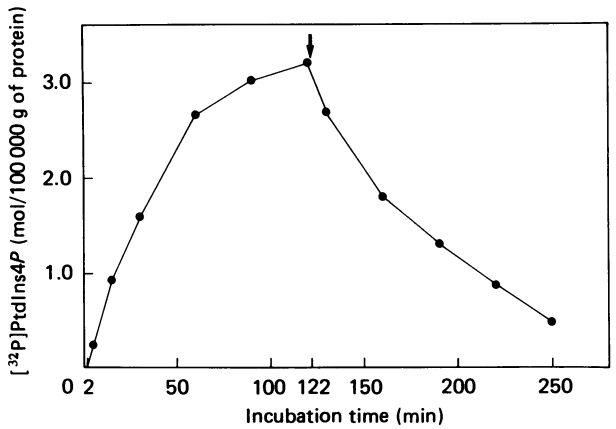

Fig. 1. Ca^{2+} effect on PtdIns4*P* content on the isolated SR Ca^{2+}-transport ATPase

PtdIns bound to the isolated Ca^{2+}-transport ATPase (5 mg/ml of reaction mixture) was preincubated for 1.5 min in a total volume of 300 μl with added PtdIns kinase (0.45 mg of phosphorylase kinase/ml) at pH 7.5 and 26 °C in 100 mM-KCl/100 mM-Tris/HCl/10 mM-EDTA/1 mM-EGTA/10 mM-[γ-^{32}P]ATP. Samples (15 μl) were withdrawn after 0.5, 1 and 1.5 min to determine the radioactivity bound non-specifically. After 2 min the phosphorylation reaction was started by addition of 14 μl of 400 mM-Mg^{2+}. The resulting free Ca^{2+} and Mg^{2+} concentrations were 1.6 nM and 1.2 mM respectively. During the incubation, samples (15 μl) were withdrawn and applied to Whatman GF/C filter-paper discs to determine the amount of PtdIns4*P* bound to the Ca^{2+}-transport ATPase. At 122 min (arrow) the free Ca^{2+} concentration was raised to 200 nM, and samples were withdrawn as described above. For determination of the amount of PtdIns4*P* on the ATPase, see Varsányi *et al.* (1983) and Georgoussi & Heilmeyer (1986*a*).

phosphorylation mixture. To determine the non-lipid-bound radioactivity extractable into the organic phase, two 15 μl samples were removed and each was added to 300 μl of chloroform/methanol (2:1, v/v). Phosphorylation was started by addition of 30 mM-Mg^{2+}; during the phosphorylation reaction 15 μl samples were removed. After phase separation as described by Schacht (1981), the lipid-bound ^{32}P and ^{3}H radioactivities were determined in the chloroform phase. Approx. 60% of the PtdIns had been converted into PtdIns4*P*. After 9 h, 4 ml of chloroform/methanol (1:2, v/v), 1 ml of 2.4 M-HCl and 2 ml of chloroform were added to 3 ml of phosphorylation mixture. After phase separation, the organic phase was washed with 2 m of methanol/1 M-HCl (1:1, v/v) and finally concentrated to approx. 400 μl in an N_2 stream. The [^{3}H/^{32}P]PtdIns4*P*-containing organic phase was applied (in eight spots) to a silica-gel plate. After autoradiography for identification of [^{3}H/^{32}P]-PtdIns4*P*, the radioactive material was recovered from the thin-layer plate by extracting the silica gel with 0.7 ml of chloroform/methanol (1:2, v/v), 0.4 ml of 1 M-HCl and 0.4 ml of chloroform. After phase separation, the chloroform phase containing [^{3}H/^{32}P]PtdIns4*P* was removed and washed once with 1% NaCl. The yield of the [^{3}H/^{32}P]PtdIns4*P* recovered from the plate was approx. 5–10%. After evaporation of the chloroform phase to dryness under a stream of N_2, the dried [^{3}H/^{32}P]PtdIns4*P* was taken up in 100 mM-KCl/100 mM-Tris/HCl (pH 7.5)/2.4% Triton X-100 and was ultrasonicated for 60 min at approx. 40 °C. The suspension was stored at −70 °C. The resulting [^{3}H/^{32}P]PtdIns4*P* had a specific ^{3}H radioactivity of 15 Ci/mmol and a specific ^{32}P radioactivity of 1.1 Ci/mmol.

Solubilization of PtdIns kinase and PtdIns4*P* monoesterase from the SR membranes

The solubilization was carried out with Triton X-114 essentially as described by Bordier (1981). SR was suspended in the solubilizing buffer, consisting of 1.5% precondensed Triton X-114, 150 mM-NaCl, 1 mM-dithioerythritol, 10 mM-Tris/HCl, pH 7.5, at a final protein concentration of 1 mg of SR protein/ml (for PtdIns kinase) or 2.5 mg of SR protein/ml (for PtdIns4*P* monoesterase) respectively. Both enzymes were obtained in the water phase after phase separation (Table 1). PtdIns kinase was solubilized to an extent of approx. 40% and PtdIns4*P* monoesterase to approx. 70%.

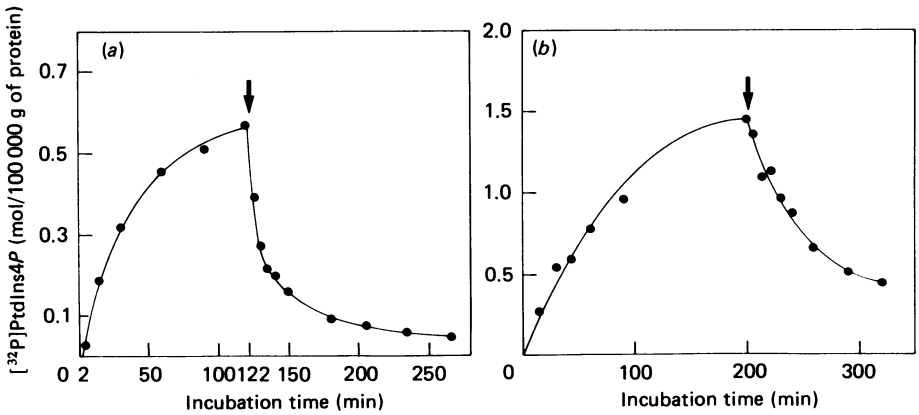

Fig. 2. Initiation of PtdIns4*P* disintegration on blockade of PtdIns kinase by substrate removal

PtdIns on the ATPase was phosphorylated as described in the legend of Fig. 1. (*a*) At 122 min (arrow) 50 mM-glucose and 17.3 units of hexokinase/ml were added to the incubation mixture. (*b*) At 200 min, free Mg^{2+} was decreased to 1.5 μM by addition of 25.7 mM-EDTA to the reaction mixture. Samples (15 μl) were withdrawn, and the amount of PtdIns4*P* was determined as described in the legend of Fig. 1.

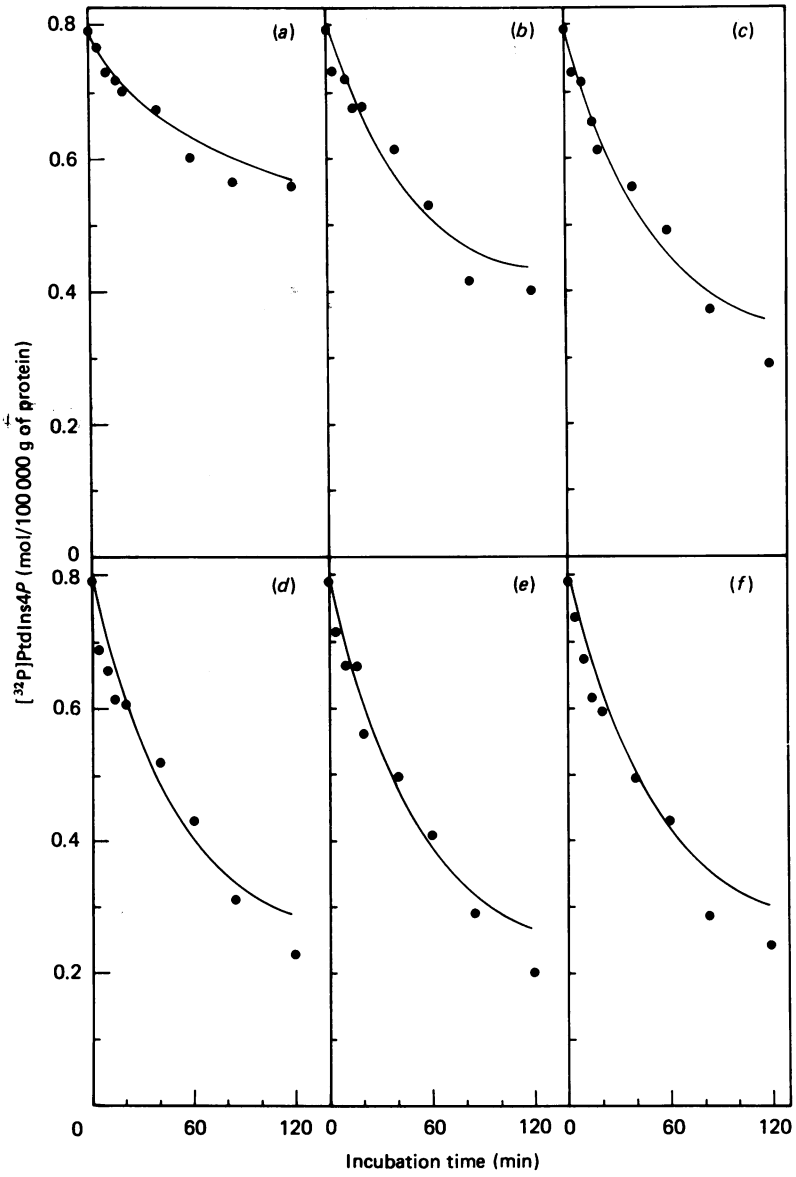

Fig. 3. Ca^{2+}-dependence of the PtdIns4*P* disintegration

PtdIns on the Ca^{2+}-transport ATPase was phosphorylated as described in the legend of Fig. 1. After 120 min, in portions the free [Ca^{2+}] was enhanced to 20 (*a*), 50 (*b*), 100 (*c*), 200 (*d*), 500 (*e*) and 850 (*f*) nM respectively. PtdIns4*P* decomposition was measured as described in the legend of Fig. 1. The experimental data are given by the filled symbols. The continuous line represents the theoretical curve calculated according to the equation:

$$a = x + (a_0 - x) \cdot e^{-t/\tau}$$

where a is the amount of PtdIns4*P* determined at time t, x represents the amount at $t = \infty$, a_0 is the amount at $t = 0$, and τ is the time constant of the relaxation, i.e. PtdIns4*P* disintegration process.

RESULTS

PtdIns, which is associated with the isolated SR Ca^{2+}-transport ATPase, can be phosphorylated by either endogenous or exogenously added PtdIns kinase, resulting in the formation of ATPase-bound PtdIns4*P*. Fig. 1 shows that at 1.6 nM free Ca^{2+} radioactively labelled PtdIns4*P* is formed on incubation of the isolated ATPase with [γ-^{32}P]ATPMg. The amount of PtdIns4*P* formed is a function of the ATPase preparation and ranges between 0.5 and 3 mol of PtdIns4*P*/mol of ATPase (for explanation see below). Increasing the free Ca^{2+} concentration to 0.2 μM (arrow in Fig. 1) results in an immediate decrease in radioactively labelled PtdIns4*P*. This decrease indicates a turnover of PtdIns and PtdIns4*P*. Indeed, if the kinase reaction is stopped, either by removing ATP or by chelation of Mg^{2+} with EDTA, the PtdIns4*P* formed disappears with a half-time of 24 and 22 min respectively (Fig. 2). This turnover is influenced by the free Ca^{2+} concentration. Increasing the free Ca^{2+} concentration in the incubation mixture to 20 nM (Fig. 3*a*) already initiates disintegration of PtdIns4*P*. The time course can be calculated by assuming a relaxation time of approx. 56 min (continuous line in

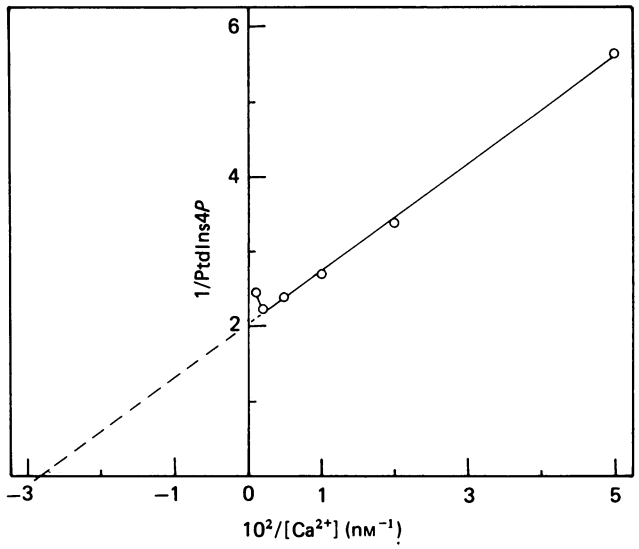

Fig. 4. Dependence of the steady-state amount of PtdIns4*P* on the free Ca^{2+} concentration

Reciprocals of the steady-state amount of PtdIns4*P* (mol/100000 g of protein) on the ATPase (equivalent to the values at $t = \infty$ in Fig. 3) were plotted against the reciprocals of the free Ca^{2+} concentrations. The intercept on the abscissa yields the $K_{0.5}$ value.

Fig. 3; for calculation see the legend). Increasing the free Ca^{2+} concentration further does not change the relaxation time, but decreases the steady-state amount of PtdIns4*P* (Figs. 3*a*–3*f*). The half-maximal effect of Ca^{2+} is obtained at 35 nM, which can be calculated from a plot of the reciprocals of these steady-state values versus the free Ca^{2+} concentrations (Fig. 4).

There are several pathways by which PtdIns4*P* could be disintegrated. For example, after phosphodiesteratic cleavage of PtdIns4*P*, the products DG and D-*myo*-inositol 1,4-bisphosphate would be expected after completion of a phosphorylation/disintegration cycle. A small amount of PA is formed on the ATPase if DG kinase is added after the phosphorylation/disintegration cycle. However, PA formation occurs with the same velocity and to the same degree as in a parallel reaction without a previous phosphorylation/disintegration cycle (results not shown). It demonstrates that DG has not been formed during the phosphorylation/disintegration cycle, which excludes a phosphodiesteratic cleavage. It rather indicates that PtdIns4*P* has been cleaved by a phosphomonoesterase. In this case, the products P_i and PtdIns are expected and the re-formed PtdIns should be once more phosphorylatable. Indeed, after completion of a phosporylation/disintegration cycle, rephosphorylation of PtdIns can be initiated and the amount of PtdIns4*P* formed reaches the same value as that obtained in the first PtdIns phosphorylation phase (results not shown).

Finally, to identify the enzyme catalysing this PtdIns4*P* degradation, doubly labelled phosphatidyl[2-^{3}H]inositol [4-^{32}P]phosphate ([^{3}H/^{32}P]PtdIns4*P*) has been synthesized and used as substrate (see the Materials and methods section). This doubly labelled material has been incubated with the isolated ATPase, which contains the PtdIns4*P*-degradative activity. After various time periods samples were removed and the cleavage products analysed by enriching the lipids in an organic solvent and the water-soluble components in the water phase. From the doubly labelled [^{3}H/^{32}P]PtdIns4*P* only ^{32}P radioactivity is released, which appears in the water phase; ^{3}H radioactivity remains quantitatively in the organic phase (Figs. 5*a* and 5*b*). ^{32}P radioactivity has been identified as P_i by high-voltage electrophoresis (results not shown). This observtion and the previous experiments show that the hydrolysis of PtdIns4*P* is catalysed by a phosphomonoesterase.

This PtdIns4*P* phosphomonoesterase is inhibited by 20 mM-fluoride. In the presence of this anion, PtdIns4*P* formation is accelerated, whereas the dephosphorylation is completely inhibited when the kinase reaction is stopped either by removal of Mg^{2+} with EDTA or by removal of ATP with glucose/hexokinase (Figs. 6*a* and 6*b*).

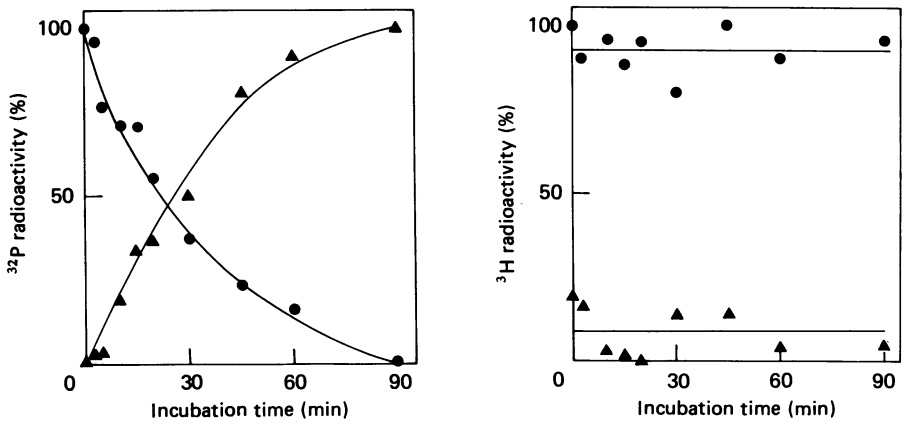

Fig. 5. Fate of ^{3}H and ^{32}P radioactivity after degradation of doubly labelled [^{3}H/^{32}P]PtdIns4*P*

To 100 μl of [^{3}H/^{32}P]PtdIns4*P* after ultrasonication for 30 min at 4 °C in 100 mM-Tris/HCl/100 mM-KCl/10 mM-EDTA/1 mM-EGTA, pH 7.5, and increase of the temperature to 30 °C, isolated Ca^{2+}-transport ATPase (4.5 mg/ml), which carries the PtdIns4*P*-degradative activity, was added. During the incubation, samples (15 μl) were withdrawn and added to 300 μl of chloroform/methanol (1:2, v/v). The ^{3}H and ^{32}P radioactivities of the chloroform (●) and water phase (▲) were determined after phase separation as described by Schacht (1981).

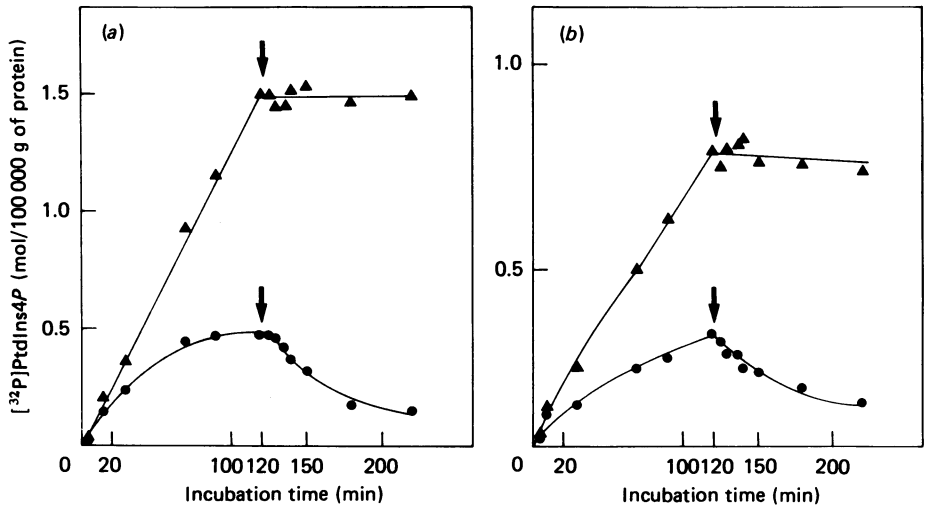

Fig. 6. Effect of fluoride on the PtdIns4*P* content of the isolated ATPase

PtdIns bound to Ca^{2+}-transport ATPase was phosphorylated in the absence (●) and the presence (▲) of 20 mM-NaF as described in the legend of Fig. 1. Arrows show the time intervals at which the dephosphorylation of PtdIns4*P* was started by (*a*) removing ATP by the hexokinase reaction, or (*b*) chelating Mg^{2+} with excess of EDTA (for details see Figs. 2*a* and 2*b*). Bound PtdIns4*P* was determined as described in the legend of Fig. 1.

The amount of PtdIns4*P* formed on the isolated ATPase is a function of the relative activities of the PtdIns kinase and PtdIns4*P* monoesterase. This ratio differs in various ATPase preparations. Furthermore, the amount of PtdIns4*P* on the ATPase has been shown to be dependent on the free Ca^{2+} concentration (Fig. 3). The decrease in PtdIns4*P* could be due either to Ca^{2+} inhibition of PtdIns kinase or to Ca^{2+} activation of PtdIns4*P* monesterase. Alternatively, Ca^{2+} could act indirectly, e.g. by changing the complexing of the substrate on the Ca^{2+}-transport ATPase, which would result in an exposed, i.e. phosphorylatable, or trapped, i.e. non-phosphorylatable, form of the PtdIns/PtdIns4*P* headgroup. Fig. 7 shows that heat denaturation of the Ca^{2+}-transport ATPase and use of PtdIns and PtdIns4*P* associated with this denatured protein as substrates abolishes the Ca^{2+} effect; neither the PtdIns kinase nor the PtdIns4*P* monoesterase activity shows any change when the free Ca^{2+} concentration is varied between 1 nM and 0.1 mM. The same result is obtained when a mixture of PtdIns solubilized in Triton X-100 is employed as substrate for the PtdIns kinase (not shown). Therefore it is evident that the interaction of the native Ca^{2+}-transport ATPase protein with the phospholipids, PtdIns and PtdIns4*P*, plays a decisive role in this Ca^{2+}-regulated phenomenon.

Indeed, PtdIns4*P* is tightly associated with the Ca^{2+}-transport ATPase protein. Fig. 8 shows that, during chromatography on DEAE-Sepharose in the presence of Triton X-100, phospholipids can be removed from the transport-ATPase protein. Only minimal amounts of PtdIns4*P* and approx. 90% of lipids displaced from the ATPase appear in fractions 10–37 (Fig. 8). In the presence of 250 mM-NaCl the main radioactively labelled PtdIns4*P* is eluted with one part of the ATPase protein (fractions 67–78). A later-eluted ATPase fraction (78–85) contains no PtdIns4*P* but a residual amount of approx. 10% of the total lipids. PtdIns4*P* can be displaced from the ATPase protein (pool of fractions 78–85) by competition with the acidic carbohydrate heparin.

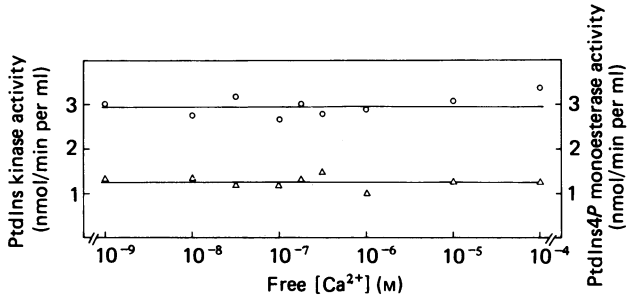

Fig. 7. Lack of Ca^{2+} effect on PtdIns kinase and PtdIns4*P* monoesterase

PtdIns associated with heat-denatured (20 min, 87 °C) Ca^{2+}-transport ATPase (2 mg/ml) was phosphorylated with 250 μg of PtdIns kinase (phosphorylase kinase)/ml as described in the legend of Fig. 1. Free Ca^{2+} concentrations were established with Ca/EGTA buffer as described in the Materials and methods section. PtdIns kinase (○) activity was determined by measuring the initial PtdIns4*P* formation rate on the heat-treated Ca^{2+}-transport ATPase, and PtdIns kinase activity was calculated as described by Georgoussi & Heilmeyer (1986*a*). PtdIns4*P* monoesterase activity (△) was assayed as described in the Materials and methods section. PtdIns4*P* monoesterase was in the water phase after Triton X-114 solubilization of SR membranes (see the Materials and methods section).

Without salt the radioactively labelled PtdIns4*P* is eluted from heparin–agarose; when salt is included, the PtdIns4*P*-free ATPase protein is obtained (Fig. 9).

DISCUSSION

Evidence is accumulating that PtdIns present in fast-skeletal-muscle SR can be phosphorylated to form PtdIns*P*, but no further phosphorylation to PtdIns(4,5)P_2 seems to occur in these membranes (Varsányi *et al.*, 1983). In agreement, PtdIns kinase is present in longitu-

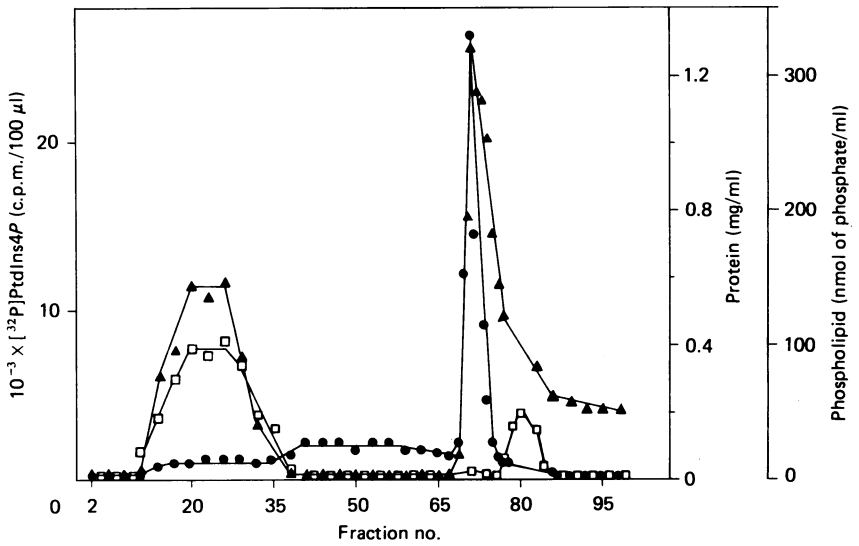

Fig. 8. DEAE-Sepharose chromatography of solubilized SR Ca^{2+}-transport ATPase

PtdIns bound to the ATPase (5 mg/ml) was phosphorylated by the endogenous PtdIns kinase as described in the legend of Fig. 1: 0.5 mol of PtdIns4*P*/mol of ATPase was obtained. After extensive dialysis against 10 mM-Hepes/20 mM-NaF/250 mM-sucrose, pH 7.0, the membranes were solubilized with 1% Triton X-100, and the mixture was centrifuged at 40000 *g* for 30 min. The clear supernatant was applied on a column of DEAE-Sepharose CL6B (2.5 cm × 9 cm) in 1% Triton X-100 containing 20 mM-Hepes/20 mM-NaF, pH 7.5. PtdIns4*P*-containing ATPase was eluted by addition of 0.25 M-NaCl to the above buffer. Determinations of PtdIns4*P*-bound radioactivity (●), protein (■) and phospholipid (□) of the fractions were carried out as described in the Materials and methods section.

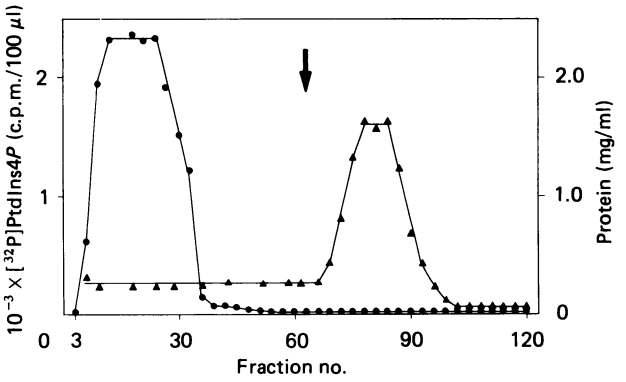

Fig. 9. Chromatography of PtdIns4*P*-containing ATPase on heparin–agarose

A pool of PtdIns4*P*-containing ATPase from the DEAE-Sepharose (see Fig. 8) was diluted 4-fold in 20 mM-Hepes/20 mM-NaF/1% Triton X-100, pH 7.5, and was applied to heparin–agarose (1 cm × 8 cm; flow rate 5 ml/h) which was pre-equilibrated with the dilution buffer. Bound protein (▲) was eluted by addition of 0.5 M-NaCl to the buffer (arrow). ●, $[^{32}P]$PtdIns4*P*.

dinal SR, and PtdIns4*P* kinase is only present in T-tubules (Varsányi *et al.*, 1986*b*; Hidalgo *et al.*, 1986). These observations do not point towards a role of PtdIns4*P* as an intermediate in these membranes for production of the second messengers, InsP_3 and DG. PtdIns4*P* could, however, be a membrane-localized effector; it activates the Ca^{2+}-transport ATPase of SR (Varsányi *et al.*, 1983). Similarly, the nuclear-envelope ATPase is stimulated by PtdIns4*P* (Smith & Wells, 1983). Furthermore, PtdIns4*P* and PtdIns(4,5)P_2 enhance the plasma-membrane Ca^{2+}-transport ATPase activity of hepatocytes (Lin & Fain, 1984) and erythrocytes (Choquette *et al.*, 1984). These observations indicate that PtdIns4*P* serves as a membrane-localized effector more widely than in SR only.

PtdIns4*P* can be used as a signalling molecule only if it is degraded. Here we demonstrate, for the first time, that a hydrolytic pathway for PtdIns4*P* exists in SR, involving a phosphomonoesterase.

This conclusion is based on the following three observations: (*a*) the disintegration of PtdIns4*P* on the ATPase yields PtdIns, which can be rephosphorylated to PtdIns4*P*; (*b*) during decomposition of PtdIns4*P*, no DG is produced, which would be expected as the product of a phosphodiesteratic cleavage; (*c*) exogenously added doubly labelled PtdIns4*P* is split into PtdIns and P_i (Fig. 5). Therefore, the prerequisite for PtdIns4*P* being a signalling molecule in SR is fulfilled.

The combination of an active PtdIns kinase with an active PtdIns4*P* phosphomonesterase results in a turnover of PtdIns and PtdIns4*P*. Turnover in the system employed here has been demonstrated in several ways: blocking the kinase reaction by removing ATP or Mg^{2+} results in an immediate dephosphorylation of PtdIns4*P* (Fig. 2); blocking the phosphomonoesterase with fluoride increases the amount of PtdIns4*P* formed on the ATPase and prevents its dephosphorylation when the kinase reaction is stopped (Figs. 6*a* and 6*b*).

Turnover is characterized by the turnover rate as well as by the amount and the time at which steady state is reached. The turnover rate and the time in which this steady state can be reached are dependent on the relative activities of the PtdIns kinase and PtdIns4*P* monoesterase. The observed time course of PtdIns4*P* formation on the isolated ATPase is slow. The extrapolation to the situation in the intact cell is not yet possible, since the

respective enzymes, the PtdIns kinase and the PtdIns4*P* phosphomonesterase, have not yet been purified. However, it can be stated with certainty that this time must be faster in the cell, since during purification of the ATPase both enzymes, PtdIns kinase and PtdIns4*P* phosphomonoesterase, have been lost to an as yet undetermined degree.

In order for PtdIns4*P* to serve as a regulator of the Ca^{2+}-transport ATPase activity, there must exist a possibility to influence its steady-state amount. It might be even more important to what degree such a control system is sensitive to changes in its environment. In a reversible covalent modification system like that described here for PtdIns and PtdIns4*P*, under certain conditions 'zero order ultrasensitivity' can be obtained, i.e. an abrupt change in the PtdIns4*P* steady-state value in response to an external stimulus. This phenomenon occurs when the interconverting enzymes are saturated, i.e. they work in the zero-order range (La Porte & Koshland, 1983). The occurrence of ultrasensitivity in a futile-cycling system depends critically on the total amount of substrate available for interconversion. The availability of substrate provided as a complex with the ATPase seems to be regulated by micromolar Ca^{2+}. As demonstrated here, a dramatic change in the steady-state amount of PtdIns4*P* on the ATPase is observed as function of the free Ca^{2+} concentration (Figs. 1 and 3). This change in PtdIns4*P* amount is produced by substrate availability, and not by changes in the activities of the interconverting enzymes (Fig. 7). No change in the enzyme activities with Ca^{2+} is obtained when the PtdIns or PtdIns4*P*, respectively, was presented in form of a Triton X-100 micelle or bound to heat-denatured ATPase. The same conclusion, namely that PtdIns complexing on the ATPase limits the substrate availability for the PtdIns kinase, was drawn from experiments with vanadate (Varsányi *et al.*, 1986*a*). A conformational change in the ATPase induced by vanadate leads to exposure of PtdIns, which then can be phosphorylated. Furthermore, a direct complexing of PtdIns4*P* with the ATPase has been demonstrated here: except for PtdIns4*P*, other phospholipids can be separated from the ATPase by chromatography on DEAE-Sepharose in Triton X-100 (see Fig. 8). To remove PtdIns4*P* from the ATPase, competition with the highly negatively charged molecule heparin is required, which indicates a strong interaction of PtdIns4*P* with the ATPase (see Fig. 9). Therefore we conclude that Ca^{2+} regulates indirectly the complex-formation of PtdIns or PtdIns4*P* with the ATPase by inducing a conformational change, which in turn modifies the substrate availability.

Such a regulation of PtdIns/PtdIns4*P* turnover by a Ca^{2+}-sensitive membrane protein seems to circumvent the difficulty of allosteric regulation of the respective enzyme activities, PtdIns kinase or PtdIns4*P* phosphomonoesterase. Phospholipids are integrated into the membranes. Therefore, diffusion of low-M_r lipophilic substrances to these enzymes is hindered. Thus simple feedback loops are not possible, and regulation by substrate availability could be an equivalent mechanism for lipophilic components. It would be interesting to see if substrate availability could also regulate the whole signal cascade in which PtdIns4*P* is involved.

The observed Ca^{2+} dependence of the PtdIns4*P* formation and degradation in SR is compatible, in principle, with the activity cycle of the living muscle cell: simultaneously to the increase in sarcoplasmic free Ca^{2+} concentration during Ca^{2+} release from SR, the PtdIns4*P*-regulated Ca^{2+}-transport ATPase of SR would decrease and thereby support the increase in sarcoplasmic Ca^{2+}. After termination of Ca^{2+} release, the free Ca^{2+} concentration of the sarcoplasm is believed to decay quickly towards its resting value, owing to Ca^{2+} binding to cellular proteins and Ca^{2+} uptake into the SR. In this state the Ca^{2+}-dependent Ca^{2+}-uptake activity of the SR, which is proportional to the free sarcoplasmic concentration, would no longer contribute effectively to the reaccumulation of Ca^{2+} still present in the sarcoplasm. In contrast, the PtdIns4*P*-activated Ca^{2+}-transport ATPase of the SR could contribute to the restoration of the resting state, and therefore PtdIns4*P* on the ATPase might determine the steady-state concentration of free Ca^{2+} in resting muscle.

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