

# D-*myo*-Inositol 1,4,5-trisphosphate phosphatase in skeletal muscle

Daria MILANI\*

Centro di Studio delle Biomembrane del CNR, Istituto di Patologia Generale dell'Università di Padova, via Loredan 16, 35131 Padova, Italy,

Pompeo VOLPE†

Centro di Studio per la Biologia e la Fisiopatologia Muscolare del CNR, Istituto di Patologia Generale dell'Università di Padova, via Loredan 16, 35131 Padova, Italy, and Tullio POZZAN

Istituto di Patologia Generale dell'Università di Ferrara, via Borsari, 44100 Ferrara, Italy

The presence and subcellular distribution of D-*myo*-inositol 1,4,5-trisphosphate phosphatase (InsP<sub>3</sub>ase) in rabbit fast-twitch skeletal muscle were investigated. A specific InsP<sub>3</sub>ase was found in both sarcotubular-membrane and soluble fractions. Membrane-bound InsP<sub>3</sub>ase accounted for 60–65% of total activity. The InsP<sub>3</sub>ase was detected both on the surface membranes and on the InsP<sub>3</sub>-sensitive intracellular Ca<sup>2+</sup> store, i.e. the sarcoplasmic reticulum. The *K<sub>m</sub>* for inositol 1,4,5-trisphosphate (InsP<sub>3</sub>) ranged between 15 and 18 μM, and the highest *V<sub>max</sub>* (19.6 nmol of InsP<sub>3</sub> hydrolysed/min per mg of protein) was measured in a membrane fraction enriched in transverse tubules. Several known inhibitors of InsP<sub>3</sub>ase, e.g. 2,3-bisphosphoglycerate, CdCl<sub>2</sub> and EDTA, were active on skeletal-muscle InsP<sub>3</sub>ase. Total InsP<sub>3</sub>ase activity of both rabbit and frog skeletal muscle was comparable with that of rabbit brain, liver and main pulmonary artery (smooth muscle). The present results are consistent with the hypothesis that InsP<sub>3</sub> plays a role in excitation–contraction coupling in skeletal muscle [Volpe, Salviati, Di Virgilio & Pozzan (1985) *Nature* (London) **316**, 347–349].

## INTRODUCTION

Ca<sup>2+</sup> release from the TC of skeletal-muscle SR is triggered by the depolarization of sarcolemma and T-tubules. It is well established that signal transduction for Ca<sup>2+</sup> release occurs at the triad where TC and T-tubules are junctionally associated, yet the nature of the excitation–contraction coupling mechanism is still largely unknown (Somlyo, 1985).

Among the hypotheses proposed to explain excitation–contraction coupling, one states that, as a consequence of T-tubule depolarization, a chemical messenger, e.g. InsP<sub>3</sub>, produced at the level of the T-tubule membrane and released within the triad junction, opens Ca<sup>2+</sup> channels localized in the junctional SR membrane (Volpe *et al.*, 1986). This model reproduces with some major kinetics and anatomical modifications what has been previously proposed for non-muscle cells (Berridge & Irvine, 1984): receptor activation at the plasma-membrane level evokes phospholipase C-dependent breakdown of PtdInsP<sub>2</sub> to InsP<sub>3</sub> and diacylglycerol. InsP<sub>3</sub> acts as a second messenger and releases Ca<sup>2+</sup> from intracellular non-mitochondrial stores. InsP<sub>3</sub> was shown to induce Ca<sup>2+</sup> release from the SR of both isolated fractions (Volpe *et al.*, 1985) and skinned fibres (Volpe *et al.*, 1985; Vergara *et al.*, 1985) of skeletal muscle. Other laboratories have subsequently confirmed our findings (Donaldson *et al.*, 1987; Walker *et al.*, 1987;

Volpe *et al.*, 1987a; but see Palade, 1987). The model involving InsP<sub>3</sub> in excitation–contraction coupling requires that the complex enzymic machinery responsible for the synthesis and hydrolysis of PtdInsP<sub>2</sub>, the membrane-bound precursor of InsP<sub>3</sub> (Berridge & Irvine, 1984), and for the catabolism of InsP<sub>3</sub> should be present in skeletal muscle. In this respect, Hidalgo *et al.* (1986) and Varsanyi *et al.* (1986) have shown that PtdInsP<sub>2</sub> is localized in the T-tubule along with the relevant PtdIns and PtdInsP kinases (Hidalgo *et al.*, 1986).

A major argument against the physiological role of the InsP<sub>3</sub> in excitation–contraction coupling has been the relatively high concentrations of InsP<sub>3</sub> required to elicit contractions in skinned fibres. The *ad hoc* explanation has been that high concentrations of InsP<sub>3</sub> were needed to overcome a potent InsP<sub>3</sub>ase (Vergara *et al.*, 1985; Donaldson *et al.*, 1987). However, the skeletal-muscle InsP<sub>3</sub>ase activity was directly measured in single skinned fibres, and the specific activity appeared to be extremely low (Walker *et al.*, 1987). This finding was considered a blow to the proposed role of InsP<sub>3</sub> in excitation–contraction coupling (Walker *et al.*, 1987).

In the present paper we show, instead, that a specific InsP<sub>3</sub>ase is present in skeletal muscle. The total activity of skeletal-muscle InsP<sub>3</sub>ase was comparable with that of other tissues, e.g. brain, liver and smooth muscle, where a role for InsP<sub>3</sub> in stimulus–activation coupling is undisputed. Here we also demonstrate that skeletal-

Abbreviations used: SR, sarcoplasmic reticulum; LSR, longitudinal SR; TC, terminal cisternae; JFM, junctional SR; T-tubule, transverse tubule; InsP<sub>3</sub>, D-*myo*-inositol 1,4,5-trisphosphate; InsP<sub>2</sub>, D-*myo*-inositol 1,4-bisphosphate; InsP<sub>1</sub>, D-*myo*-inositol 1-phosphate; InsP<sub>3</sub>ase, D-*myo*-inositol 1,4,5-trisphosphate phosphatase; PtdInsP<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; PtdInsP, phosphatidylinositol 4-phosphate; PtdIns, phosphatidylinositol.

\* Present address: Fidia Research Laboratories, Abano Terme, Padova, Italy.

† To whom correspondence should be addressed. Present address: Department of Physiology and Biophysics, University of Texas Medical Branch, Galveston, TX 77550, U.S.A.

muscle  $\text{InsP}_3$ ase is present not only at the plasma-membrane level and in the myoplasm, as in other tissues, but also at the level of the SR, the intracellular  $\text{InsP}_3$ -sensitive target (Volpe *et al.*, 1985). Thus the skeletal-muscle  $\text{InsP}_3$ ase seems to have a strategic localization at the triad level, on both T-tubules and TC membranes, where signal transduction for muscle activation occurs.

## EXPERIMENTAL

### Isolation of SR fractions

SR was isolated from rabbit fast-twitch skeletal muscles and fractionated by isopycnic sucrose-density-gradient centrifugation into R1 (10%/27%-sucrose interface), R2 (27%/32%-sucrose interface), R3 (32%/38%-sucrose interface) and R4 (38%/45%-sucrose interface) as previously described (Saito *et al.*, 1984; Zorzato *et al.*, 1985). SR fractions were resuspended in 0.3 M-sucrose/5 mM-imidazole, pH 7.4 (buffered sucrose), and stored at  $-80^\circ\text{C}$  until used. JFM was obtained from the TC fraction by the procedure developed by Costello *et al.* (1986), in the presence of either 0.7% Triton X-100 or 1% octaethylene glycol mono-n-dodecyl ether ( $\text{C}_{12}\text{E}_8$ ). Protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

### KCl wash of SR fractions

Samples of fractions R1, R2 and R4 were incubated in buffered sucrose supplemented with 0.6 M-KCl for 10 min at  $0^\circ\text{C}$  and then centrifuged at 150000 g for 60 min. Pellets were resuspended in buffered sucrose, and the supernatants were dialysed at  $0^\circ\text{C}$  for 12 h against buffered sucrose. Both pellets and supernatants were stored at  $-80^\circ\text{C}$  until used.

### Isolation of muscle soluble fractions

Soluble fractions were obtained during purification of SR (Saito *et al.*, 1984). The supernatant obtained from the centrifugation of the first homogenate at 7700 g for 10 min was further centrifuged at 120000 g for 90 min, and the resulting supernatant (soluble fraction A) was collected and stored at  $-80^\circ\text{C}$ . A second soluble fraction (fraction B) was obtained by re-homogenizing the 7700 g pellet and treating the supernatant as described above.

### Homogenates of rabbit liver, brain, main pulmonary artery and skeletal muscle and of frog skeletal muscle

The tissues were homogenized with 10 vol. of 0.3 M-sucrose, 5 mM-imidazole, pH 7.4, containing 200  $\mu\text{g}$  of phenylmethanesulphonyl fluoride/l in a motor-driven Potter-Elvehjem homogenizer. Homogenates were centrifuged at 2500 g for 10 min to remove intact cells, debris and nuclei, and supernatants were frozen in liquid  $\text{N}_2$  and stored at  $-80^\circ\text{C}$  until used. Several main pulmonary arteries were pooled for a single experiment, and their adventitia were removed as indicated by Somlyo *et al.* (1985).

### Assay for degradation of [ $^3\text{H}$ ]InsP<sub>3</sub>

For analysis of  $\text{InsP}_3$ ase activity,  $\text{InsP}_3$  (final concn. 5–400  $\mu\text{M}$ ) and [ $^3\text{H}$ ]InsP<sub>3</sub> (20000 d.p.m.) were diluted into medium (final vol. 0.1 ml) containing 110 mM-KCl, 10 mM-NaCl, 1 mM- $\text{KH}_2\text{PO}_4$ , 20 mM-Hepes, pH 7.2,

**Table 1. [ $^3\text{H}$ ]InsP<sub>2</sub> production by homogenates of frog skeletal muscle and of rabbit skeletal muscle, brain, main pulmonary artery (MPA) and liver**

Preparation of homogenates and  $\text{InsP}_3$ ase assay (standard medium) were carried out as described in the Experimental section. The  $\text{InsP}_3$  concentration was 50  $\mu\text{M}$ . Data are from triplicate determinations carried out on two different preparations. The amount of  $\text{InsP}_1$  formed was relatively high in brain and main pulmonary artery (2.55 and 1.01 nmol produced/mg of protein in 1 min) and low in liver and rabbit skeletal muscle (0.38 and 0.24 nmol produced/mg of protein in 1 min).

Tissue	Specific activity (nmol of $\text{InsP}_2$ produced/min per mg of protein)	Total activity (nmol of $\text{InsP}_2$ produced/min per g wet wt.)
Frog muscle	0.63	77
Rabbit muscle	1.36	111
Rabbit liver	1.43	190
Rabbit brain	4.70	305
Rabbit MPA	2.95	203

3 mM- $\text{MgSO}_4$  (standard medium at  $30^\circ\text{C}$ ) (Joseph & Williams, 1985). The reaction was started by adding an equal volume of the assay medium containing the protein (final concn. 1–0.5 mg/ml). The reaction was stopped by adding 0.2 ml of ice-cold 15% (v/v) trichloroacetic acid. After centrifugation at 12000 rev./min for 5 min, the supernatants were extracted with  $4 \times 0.4$  ml of diethyl ether, neutralized with 0.1 ml of 0.1 M-sodium tetraborate and loaded on columns containing 0.6 ml of Dowex-1 (formate form). Elution was performed as previously described (Downes & Michell, 1981); 0.6 ml fractions were collected, and the radioactivity was measured by liquid-scintillation spectrometry.

### Materials

D-*myo*-[ $^3\text{H}$ ]InsP<sub>3</sub> and D-*myo*-InsP<sub>3</sub> were obtained from Amersham. One batch of especially purified  $\text{InsP}_3$  was kindly given by Dr R. F. Irvine. 2,3-Bisphosphoglycerate was obtained from Boehringer, and D-( $\alpha$ )-fructose 1,6-bisphosphate, D-( $\alpha$ )-fructose 2,6-disphosphate and D-( $\alpha$ )-glucose 1,6-bisphosphate were from Sigma. Dowex AG1-X8 (formate form) was purchased from Bio-Rad. All other reagents were of analytical or higher grade.

## RESULTS

An enzyme that specifically hydrolyses  $\text{InsP}_3$  to  $\text{InsP}_2$  has been demonstrated in many cell types (Downes & Michell, 1981; Downes *et al.*, 1982; Storey *et al.*, 1984; Joseph & Williams, 1985; Connolly *et al.*, 1986; Sasaguri *et al.*, 1985; Kukita *et al.*, 1986; Rana *et al.*, 1986; Erneux *et al.*, 1986; Guillemette *et al.*, 1987; Walker *et al.*, 1987). From the data presented in Table 1, it appears that a similar  $\text{InsP}_3$ ase activity is also present in homogenates of both rabbit and frog skeletal muscle. The total  $\text{InsP}_3$ ase activity of skeletal muscle, expressed as nmol of  $\text{InsP}_2$  produced/g wet wt. of tissue, is slightly lower than that of brain, liver and main pulmonary artery (smooth muscle). If allowance is made for the myofibrillar protein content (more than 80% of total

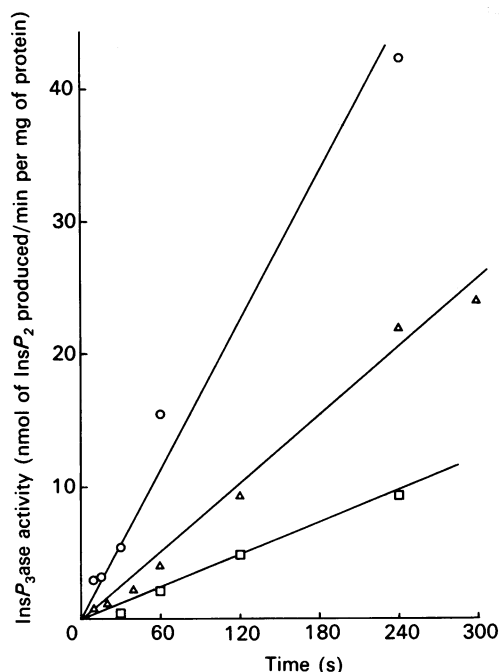


Fig. 1. Time course of  $\text{InsP}_3$  hydrolysis by sarcotubular membrane fractions of rabbit skeletal muscle

The  $\text{InsP}_3$ ase assay was carried out at 30 °C in the standard medium with 50  $\mu\text{M}$ - $\text{InsP}_3$  and 1 mg of protein for fractions R1 (○), R2 (△) and R4 (□). Each curve (each point performed in duplicate) is representative of at least two different experiments and was drawn by linear-regression analysis (correlation coefficient  $\geq 0.98$ ).

protein), the total  $\text{InsP}_3$ ase activity in skeletal muscle is comparable with that of other tissues.

After 5 min incubation at 30 °C, a small amount of radioactivity was found in the fractions attributable to inositol and  $\text{InsP}_1$  for homogenates of skeletal muscles and rabbit liver. On the other hand, a relatively high percentage of radioactivity co-eluted with  $[^3\text{H}]\text{InsP}_1$  in the case of homogenates of brain and main pulmonary artery (Table 1).

#### Membrane-bound $\text{InsP}_3$ ase

By following the fractionation procedure developed by Saito *et al.* (1984), we have isolated membrane fractions derived from different portions of the sarcotubular system of rabbit skeletal muscle. As reported by Saito *et al.* (1984), fraction R1 contains mostly longitudinal cisternae (LSR), with some T-tubules and, probably, sarcolemma; fraction R2 is enriched in LSR; fraction R3 is a mixture of LSR and TC; and fraction R4 is enriched in TC. The JFM corresponds to the junctional SR (Costello *et al.*, 1986). All membrane fractions were tested for their ability to hydrolyse  $[^3\text{H}]\text{InsP}_3$ . Unless otherwise specified, experiments were carried out in the presence of 3 mM- $\text{MgCl}_2$  and 50  $\mu\text{M}$ - $\text{InsP}_3$ , a concentration at least double the  $K_m$  (see below).

The time course of  $[^3\text{H}]\text{InsP}_2$  production by R1, R2 and R4 fractions is shown in Fig. 1. The kinetics of formation of the product is linear over at least 2 min for R1, and over 4 min for R2 and R4. Similar results are obtained for R3 and JFM (not shown). The enzyme activity seems to be specific for  $\text{InsP}_3$ , since  $[^3\text{H}]\text{InsP}_1$

Table 2.  $K_m$ ,  $V_{\max}$  and specific activity of  $\text{InsP}_3$ ase in sarcotubular membrane fractions

$\text{InsP}_3$ ase assay was carried out in the standard medium with  $\text{InsP}_3$  concentrations in the range 5–400  $\mu\text{M}$  and incubation times of 1–2 min.  $K_m$  and  $V_{\max}$  were determined by Lineweaver–Burk-plot regression analysis. Data are averages of several experiments carried out in duplicate on different preparations. Specific activity was measured in the presence of 50  $\mu\text{M}$ - $\text{InsP}_3$ ; data are means  $\pm$  S.D. when at least six experiments were performed. Abbreviation: ND, not determined.

Fraction activity	$V_{\max}$ (nmol of $\text{InsP}_2$ produced/ min per mg)	$K_m$ ( $\mu\text{M}$ )	Specific activity (nmol of $\text{InsP}_2$ produced/ min per mg)
R1	19.6	15.5	$13.5 \pm 2.2$ (8)
R2	7.7	16.6	$5.9 \pm 0.2$ (6)
R3	ND	ND	$6.6 \pm 1.2$ (6)
R4	$4.2 \pm 0.3$	18.7	$2.7 \pm 0.7$ (7)
JFM	ND	ND	$3.4 \pm 0.9$ (6)

production is almost negligible (2–2.5 % of  $\text{InsP}_2$  formed) even after a 5 min incubation.

$[^3\text{H}]\text{InsP}_3$  hydrolysis by muscle membranes displays normal Michaelis–Menten kinetics: values of  $V_{\max}$ ,  $K_m$  and specific activity at 50  $\mu\text{M}$ - $\text{InsP}_3$  are summarized in Table 2. The  $K_m$  for  $\text{InsP}_3$  ranges between 15.5 and 18.7  $\mu\text{M}$ . The enzyme appears to be distributed in LSR as well as in TC and JFM fractions, whereas fraction R1 shows the highest  $V_{\max}$ . Given the membrane composition of R1 (Saito *et al.*, 1984), these results imply that the  $\text{InsP}_3$ ase is heavily concentrated in T-tubules and, probably, sarcolemma. It might be argued that the  $\text{InsP}_3$ ase activity of R2, R4 and JFM fractions is due to contamination by T-tubules. Although we cannot rule out this possibility for the R2 fraction we think it is unlikely for R4 and JFM, which are devoid of T-tubules as judged by enzyme markers and electron microscopy (Saito *et al.*, 1984; Costello *et al.*, 1986; Volpe *et al.*, 1987b).

In order to establish whether the  $\text{InsP}_3$ ase is membrane-bound or only loosely bound to muscle membranes, fractions R1, R2 and R4 were treated with high ionic strength (0.6 M-KCl), and the distribution of the enzyme activity between supernatants and pellets was investigated. The results of this experiment are shown in Table 3. Since only 2–8 % of the  $\text{InsP}_3$ ase is found in the supernatants, and more than 90 % is recovered in the particulate fractions, we infer that the enzyme is in a tightly membrane-bound form.

#### Soluble $\text{InsP}_3$ ase

We also looked for the presence of a soluble  $\text{InsP}_3$ ase in rabbit skeletal muscle. Two soluble fractions (A and B; see the Experimental section for details) were obtained and  $\text{InsP}_3$ ase activity was measured. The soluble  $\text{InsP}_3$ ase accounts for 35–40 % of the total  $\text{InsP}_3$ ase activity (cf. Table 1). The  $K_m$  of the soluble enzyme (fraction A) is about 24  $\mu\text{M}$ , i.e. slightly higher than that of the membrane-bound enzyme. The  $V_{\max}$  is 1.5 nmol of  $\text{InsP}_2$  produced/min per mg of protein, and the specific activity at 50  $\mu\text{M}$ - $\text{InsP}_3$  is about 0.6 nmol of  $\text{InsP}_2$  produced/min per mg of protein.

**Table 3. Distribution of  $\text{InsP}_3$ ase activity after KCl wash of sarcotubular membrane fractions**

$\text{InsP}_3$ ase activity was measured in the standard medium by using R1, R2 and R4 fractions before and after treatment with 0.6 M-KCl. The  $\text{InsP}_3$  concentration was 50  $\mu\text{M}$ . Determinations were done in duplicate on two different preparations. Abbreviations: sp, supernatant; and pt, pellet after KCl wash (see the Experimental section for details).

Fraction	Specific activity (nmol of $\text{InsP}_3$ produced/min per mg)	Activity recovered (%)
R1	10.40	
sp R1	1.30	1.9
pt R1	9.50	98.1
R2	3.10	
sp R2	2.50	1.7
pt R2	5.25	98.3
R4	2.76	
sp R4	2.45	8.0
pt R4	2.53	92.0

**Table 4. Effect of various substances on  $\text{InsP}_3$ ase activity**

Various compounds at indicated concentrations were added to the standard incubation medium containing 50  $\mu\text{M}$ - $\text{InsP}_3$ . The reaction time was 2 and 5 min for membrane-bound (R1) and soluble (fraction A)  $\text{InsP}_3$ ase respectively. In preliminary studies on R4 fraction at 7  $\mu\text{M}$ - $\text{InsP}_3$ , addition of 100  $\mu\text{M}$ - $\text{Ca}^{2+}$  to the standard medium did not change  $\text{InsP}_3$ ase activity. Results are expressed as percentage inhibition, and are means  $\pm$  S.D. for triplicate determinations carried out on two different preparations; ND, not determined.

	$\text{InsP}_3$ ase activity (% inhibition)	
	Membrane- bound	Soluble
3 mM-2,3-Bisphosphoglycerate	68	65 $\pm$ 2
5 mM-Fructose 2,6-bisphosphate	44	29 $\pm$ 3
5 mM-Fructose 1,6-bisphosphate	60	50
5 mM-Glucose 1,6-bisphosphate	48	51
0.1 mM- $\text{CdCl}_2$	94 $\pm$ 4	84
0.5 mM-EGTA (free $[\text{Ca}^{2+}] < 1 \text{ nM}$ )	ND	10
10 mM-EDTA	98	96
3 mM-ATP*	54	38

\* In these experiments the total  $\text{Mg}^{2+}$  concentration was 6 mM.

### $\text{InsP}_3$ ase inhibitors

Various agents have been reported to inhibit  $\text{InsP}_3$ ase activity, and the effects of some of them are presented in Table 4. The results are very similar for the membrane-bound (R1) and the soluble (fraction A) enzyme.  $\text{Mg}^{2+}$  is essential for enzymic activity (Downes *et al.*, 1982), as demonstrated by 96–98 % inhibition obtained with 10 mM-EDTA.  $\text{CdCl}_2$  (0.1 mM) (Downes *et al.*, 1982) is also a potent inhibitor (84–94 % inhibition). 2,3-Bis-

phosphoglycerate (3 mM), which seems to act as a competitive inhibitor (Downes *et al.*, 1982), decreases the  $\text{InsP}_3$ ase activity by 60 %. Three bisphosphorylated glucose metabolites, at 5 mM (Rana *et al.*, 1986), give an inhibition in the range 30–70 %. Negligible effects are detected with 0.5 mM-EGTA, i.e. at a nominal free  $[\text{Ca}^{2+}] < 1 \text{ nM}$ . Interestingly, ATP (3 mM) inhibits by about 50 % both membrane-bound and soluble  $\text{InsP}_3$ ase.

### DISCUSSION

In this study we show for the first time the occurrence of a specific powerful  $\text{InsP}_3$ ase in skeletal muscle, which shares several biochemical properties with the homologous enzyme from many different tissues (Downes & Michell, 1981; Downes *et al.*, 1982; Storey *et al.*, 1984; Joseph & Williams, 1985; Connolly *et al.*, 1986; Sasaguri *et al.*, 1985; Kukita *et al.*, 1986; Rana *et al.*, 1986; Erneux *et al.*, 1986; Guillemette *et al.*, 1987; Walker *et al.*, 1987). It is present in membrane-bound (60–65 %) and soluble form (35–40 %), has an absolute requirement for  $\text{Mg}^{2+}$ , has a  $K_m$  for  $\text{InsP}_3$  around 20  $\mu\text{M}$ , and is inhibited by compounds such as 2,3-bisphosphoglycerate and  $\text{Cd}^{2+}$ . The  $\text{InsP}_3$ ase of the R1 fraction displays the highest  $V_{\text{max}}$  so far reported for the membrane-bound form from any other tissue.

The existence of a powerful  $\text{InsP}_3$ ase in skeletal muscle has been previously postulated on the basis of experiments where the  $\text{Ca}^{2+}$ -releasing action of  $\text{InsP}_3$  was enhanced by manipulations capable of inhibiting  $\text{InsP}_3$ ase. Vergara *et al.* (1985) showed that low  $\text{Mg}^{2+}$  or  $\text{Cd}^{2+}$ ,  $\text{Ni}^{2+}$  and 2,3-bisphosphoglycerate all potentiate the action of  $\text{InsP}_3$  on frog skinned fibres. A similar observation was made by Rojas *et al.* (1987), though Lea *et al.* (1986) did not. Donaldson *et al.* (1987) observed that microinjected  $\text{InsP}_3$  (1  $\mu\text{M}$ ) in rabbit skinned fibres was as effective as 100–300  $\mu\text{M}$ - $\text{InsP}_3$  added to the bathing solution. The explanation for this result was that 'endogenous phosphatases create a radially decreasing concentration gradient' of exogenously added  $\text{InsP}_3$  (Donaldson *et al.*, 1987).  $\text{InsP}_3$ ase was also implicated in the transient nature of  $\text{InsP}_3$ -induced  $\text{Ca}^{2+}$  release, since 'microinjection of more  $\text{InsP}_3$  during the decline of an  $\text{InsP}_3$ -induced tension transient elicits an abrupt increase in fibre tension' (Donaldson *et al.*, 1987).

Our present data and the previous suggestions (Vergara *et al.*, 1985; Donaldson *et al.*, 1987; Rojas *et al.*, 1987) are in conflict with the results of Walker *et al.* (1987). The latter authors have reported that: (a)  $\text{InsP}_3$ ase activity of frog skinned fibres is 35 times slower than that of skinned stripes of rabbit main pulmonary artery (smooth muscle); (b) there is no diffusible ('soluble')  $\text{InsP}_3$ ase in either skeletal or smooth muscle; (c)  $\text{InsP}_3$ ase activity was negligible in both types of muscle. They concluded that rapid  $\text{InsP}_3$  degradation cannot be accounted for by an active  $\text{InsP}_3$ ase in skeletal muscle. On the other hand, we have shown that: (a) total  $\text{InsP}_3$ -ase activity of main pulmonary artery is comparable with that of skeletal muscles (Table 1); we also note that the occurrence of rapid  $\text{InsP}_3$  degradation has been indirectly shown in intact frog skeletal muscles (Vergara *et al.*, 1987); (b)  $V_{\text{max}}$  of  $\text{InsP}_3$ ase of several different skeletal-muscle fractions (see Table 2 and the Results section) is similar to (or even higher than) that reported for soluble and particulate fractions of pig coronary artery

(2–2.4 nmol of  $\text{InsP}_2$  produced/min per mg of protein in Sasaguri *et al.*, 1985); (c) the existence of  $\text{InsP}_2$ ase activity is indicated by the recovery of a significant amount of  $\text{InsP}_1$  in homogenates of main pulmonary artery after incubation with [ $^3\text{H}$ ] $\text{InsP}_3$  (Table 1; see also Sasaguri *et al.*, 1985); (d) a soluble  $\text{InsP}_3$ ase exists in skeletal muscle as well as in smooth muscle (Sasaguri *et al.*, 1985). Although the experimental models and protocols of our study are different from those of Walker *et al.* (1987), the reasons for the discrepancy remain unknown.

In other cell types (e.g. liver, endocrine pancreas)  $\text{InsP}_3$ ase has been preferentially, but not exclusively, localized in the plasma membrane (Seyfred *et al.*, 1984; Joseph & Williams, 1985; Rana *et al.*, 1986). Likewise, our data also indicate that membrane-bound  $\text{InsP}_3$ ase activity is higher in a fraction enriched in T-tubules. Moreover, the  $\text{InsP}_3$ ase is localized on the junctional SR too. This anatomical organization would be ideally suited to dispose rapidly of the  $\text{InsP}_3$  being formed at the T-tubule level, and to terminate  $\text{Ca}^{2+}$  release (Volpe *et al.*, 1988). A rough calculation based on the content of  $\text{PtdInsP}_2$  (8 pmol/g of muscle; Vergara *et al.*, 1987) and on the total  $\text{InsP}_3$ ase activity (1.8 pmol of  $\text{InsP}_3$  hydrolysed/ms per g of muscle; Table 1) indicates that skeletal muscle  $\text{InsP}_3$ ase would be capable of hydrolysing all  $\text{InsP}_3$  formed within a few milliseconds, i.e. on the same time scale as a single twitch (provided that the turnover number of the enzyme, which is at present unknown, was compatible).

In conclusion, our data are consistent with the hypothesis that  $\text{InsP}_3$  plays a role in excitation–contraction coupling. In fact, not only do all the enzymic steps involved in the generation of the lipid precursors (Hidalgo *et al.*, 1986; Varsanyi *et al.*, 1986) and the  $\text{InsP}_3$ -sensitivity (Volpe *et al.*, 1985) appear to be strategically localized in the triad, but also the  $\text{InsP}_3$ ase which hydrolyses the second messenger seems to be present in the T-tubule membrane. However, other key aspects of the  $\text{InsP}_3$  hypothesis (Volpe *et al.*, 1986) have to be addressed before giving a definitive appraisal on the role of  $\text{InsP}_3$  in excitation–contraction coupling. For instance, it remains to be ascertained whether and how T-tubule depolarization is linked to phospholipase C-dependent  $\text{PtdInsP}_2$  hydrolysis (see Di Virgilio *et al.*, 1986), whether  $\text{PtdInsP}_2$  turnover is compatible with the time scale of excitation–contraction coupling ( $\sim 3$  ms; Vergara *et al.*, 1987; Maylie *et al.*, 1987), and how fast are rates of  $\text{InsP}_3$ -induced  $\text{Ca}^{2+}$  release from SR.

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## REFERENCES

- Berridge, M. J. & Irvine, R. F. (1984) *Nature* (London) **321**, 315–321.
- Connolly, T. M., Lawing, W. J. & Majerus, P. W. (1986) *Cell* **46**, 951–958.
- Costello, B., Chadwick, C., Saito, A., Chu, A., Maurer, A. & Fleischer, S. (1986) *J. Cell Biol.* **103**, 741–753.
- Di Virgilio, F., Salviati, G., Pozzan, T. & Volpe, P. (1986) *EMBO J.* **5**, 259–262.
- Donaldson, S. K., Goldberg, N. D., Walseth, T. F. & Heuttemann, D. A. (1987) *Biochim. Biophys. Acta* **927**, 92–99.
- Downes, C. P. & Michell, R. H. (1981) *Biochem. J.* **198**, 133–140.
- Downes, C. P., Mussat, M. C. & Michell, R. H. (1982) *Biochem. J.* **203**, 169–177.
- Erneux, C., Delvaux, A., Moreau, C. & Dumont, J. E. (1986) *Biochem. Biophys. Res. Commun.* **134**, 351–358.
- Guillemette, G., Balla, T., Baukal, A. J., Spat, A. & Catt, K. J. (1987) *J. Biol. Chem.* **262**, 1010–1015.
- Hidalgo, C., Carrasco, M. A., Magendzo, K. & Jaimovich, E. (1986) *FEBS Lett.* **202**, 69–73.
- Joseph, S. K. & Williams, R. J. (1985) *FEBS Lett.* **180**, 150–154.
- Kukita, M., Hirata, M. & Koga, T. (1986) *Biochim. Biophys. Acta* **885**, 121–128.
- Lea, T. J., Griffiths, P. J., Tregear, R. T. & Ashley, C. C. (1986) *FEBS Lett.* **207**, 153–161.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
- Maylie, J., Irving, M., Leung Sizto, N. & Chandler, W. K. (1987) *J. Gen. Physiol.* **89**, 83–143.
- Palade, P. (1987) *J. Biol. Chem.* **262**, 6149–6154.
- Rana, R. S., Sekar, M. C., Hokin, L. E. & MacDonald, M. J. (1986) *J. Biol. Chem.* **261**, 5237–5240.
- Rojas, E., Nassar-Gentina, V., Luxoro, M., Pollard, M. E. & Carrasco, M. A. (1987) *Can. J. Physiol. Pharmacol.* **65**, 672–680.
- Saito, A., Seiler, S., Chu, A. & Fleischer, S. (1984) *J. Cell Biol.* **99**, 875–885.
- Sasaguri, T., Hirata, M. & Kuriyama, H. (1985) *Biochem. J.* **231**, 497–503.
- Seyfred, M. A., Farrel, L. E. & Wells, W. W. (1984) *J. Biol. Chem.* **259**, 13204–13208.
- Somlyo, A. P. (1985) *Nature* (London) **316**, 298–299.
- Somlyo, A. V., Bond, M., Somlyo, A. P. & Scarpa, A. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 5231–5235.
- Storey, D. J., Shears, S. B., Kirk, C. J. & Michell, R. H. (1984) *Nature* (London) **312**, 374–376.
- Varsanyi, M., Messer, M., Brandt, N. R. & Heilmeyer, L. M. G., Jr. (1986) *Biochem. Biophys. Res. Commun.* **138**, 1395–1401.
- Vergara, J., Tsien, R. Y. & Delay, M. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 6352–6356.
- Vergara, J., Asotra, K. & Delay, M. (1987) in *Cell Calcium and the Control of Membrane Transport* (Mandel, L. J. & Eaton, D. C., eds.), pp. 133–151, Rockefeller University Press, New York.
- Volpe, P., Salviati, G., Di Virgilio, F. & Pozzan, T. (1985) *Nature* (London) **316**, 347–349.
- Volpe, P., Di Virgilio, F., Pozzan, T. & Salviati, G. (1986) *FEBS Lett.* **197**, 1–4.
- Volpe, P., Di Virgilio, F. & Pozzan, T. (1987a) *Trends Biochem. Sci.* **12**, 139–140.
- Volpe, P., Gutweniger, H. E. & Montecucco, C. (1987b) *Arch. Biochem. Biophys.* **253**, 138–145.
- Volpe, P., Di Virgilio, F., Bruschi, G., Regolisti, G. & Pozzan, T. (1988) in *Inositol Lipids and Cell Signalling* (Michell, R. H., Drummond, A. R. & Downes, C. P., eds.), Academic Press, New York, in the press.
- Walker, J. W., Somlyo, A. V., Goldman, Y. E., Somlyo, A. P. & Trentham, D. R. (1987) *Nature* (London) **327**, 249–252.
- Zorzato, F., Salviati, G., Facchinetti, T. & Volpe, P. (1985) *J. Biol. Chem.* **260**, 7349–7355.