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MINI-REVIEW

Inositol Trisphosphate and Excitation–Contraction Coupling in Skeletal Muscle

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Abstract

The role of inositol trisphosphate as a chemical messenger in excitation–contraction coupling is discussed, both in terms of positive and negative results. The evidence presented includes experiments on the effect of inositol trisphosphate in intact and skinned fibers, in calcium release from isolated sarcoplasmic reticulum vesicles, in activation of single calcium release channels incorporated in planar bilayers, and biochemical experiments that have established the presence of all the intermediate steps involved in the metabolism of phosphoinositides, both in intact muscle and in isolated membranes. From these results, it is clear that a role for inositol triphosphate in skeletal muscle function is highly likely; whether this molecule is the physiological messenger in excitation–contraction coupling remains to be established.

Key words: Calcium channels; excitation–contraction coupling; inositol trisphosphate; phosphoinositide metabolism; sarcoplasmic reticulum; skeletal muscle; skinned fibers; transverse tubules.

Introduction

The early studies by Hokin and Hokin (1953) have led to an impressive development in the field of phosphoinositide research, resulting in the current knowledge on the role of inositol 1,4,5-triphosphate [Ins(1,4,5)P_3] as second messenger in a variety of cell systems (Berridge and Irvine, 1984; Hokin, 1985; Majerus *et al.*, 1986; Berridge, 1987; Majerus *et al.*, 1988).

The cleavage of phosphoinositides by phospholipase C into diacylglycerol and inositol phosphates is the initial reaction following cell stimulation

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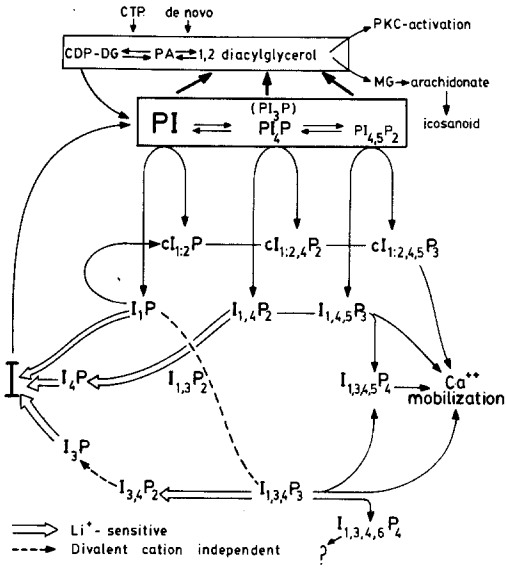

Fig. 1. Metabolic pathways for inositol phosphates. The membrane-bound lipid components are shown in boxes at the top: PI phosphatidylinositol; PI3P, phosphatidylinositol 3-phosphate; PI4P, phosphatidylinositol 4-phosphate; $PI_{4,5}P_3$, phosphatidylinositol (4,5)-bisphosphate; PA, phosphatidic acid; and CDP-DG, cytidine diphosphate diacylglycerol. On the lower levels are shown the inositol phosphates: I, inositol; and P, phosphate; the numbers preceding P represent the positions of the phosphate groups, and those following P represent the number of phosphate groups. PKC, protein kinase C; and MG, 2 monoacylglycerol. Modified from Majerus *et al.* (1988).

(Fig. 1). Both diacylglycerol (Nishizuka, 1984) and Ins(1,4,5)P_3 (see above reviews) act as second messengers. Diacylglycerol activates protein kinase C and, upon further hydrolysis by phospholipases, yields arachidonic acid, a precursor in several metabolic pathways. Ins(1,4,5)P_3 causes calcium release from intracellular stores in a variety of cell systems, including tumor and transformed cells, white cells, platelets, secretory cells, liver, kidney, and nerve cells (for specific references, see Berridge, 1987, and Sekar and Hokin, 1987). In addition, Ins(1,4,5)P_3 induces calcium release in smooth muscle (Somyo *et al.*, 1988), cardiac muscle (Nosek *et al.*, 1986), crustacean muscle (Rojas *et al.*, 1987), and skeletal muscle (see below).

As research on the chemistry of inositol phosphates develops, knowledge about the role of other inositol phosphates on cellular processes is beginning to emerge (Majerus *et al.*, 1988). In addition to Ins(1,4,5)P_3, other inositol phosphates have been detected and, with this knowledge, the complexity of phosphoinositide metabolism has increased (Fig. 1). Which of these compounds participate in cellular calcium mobilization is currently under active scrutiny. Furthermore, it has been described recently (Whitman

et al., 1988) that, in addition to phosphatidylinositol 4-phosphate (PI4P), nerve cells phosphorylate phosphatidylinositol (PI) to yield phosphatidylinositol 3-phosphate (PI3P) (Fig. 1). The metabolism of PI3P is not known at present.

Ins(1,4,5)P_3 and Excitation–Contraction Coupling in Skeletal Muscle

A role for Ins(1,4,5)P_3 as a chemical messenger in excitation–contraction (E–C) coupling in skeletal muscle was proposed by Vergara *et al.* (1985) and Volpe *et al.* (1985). According to these authors, transverse-tubule (T-tubule) depolarization triggers Ins(1,4,5)P_3 production by stimulation of the phospholipase C that hydrolyzes phosphatidylinositol (4,5)-bisphosphate [PI(4,5)P_2]. The Ins(1,4,5)P_3 released from the T-tubule membrane would rapidly diffuse through the triadic junctional space and, upon reaching the sarcoplasmic reticulum (SR) membrane, would open Ins(1,4,5)P_3-sensitive calcium channels, leading to massive calcium release. The resulting increase in intracellular calcium concentration allows actin–myosin interaction, with the ensuing muscle contraction. According to this model, relaxation takes place by the action of an Ins(1,4,5)P_3-phosphatase that would decrease the augmented Ins(1,4,5)P_3 level to its resting value.

The model outlined above is based on several experimental observations, although some negative findings make it still controversial. For the sake of clarity, we discuss first the experimental observations in favor of and against a role of Ins(1,4,5)P_3 as a chemical messenger obtained from physiological experiments, and then we discuss the biochemical evidence supporting this model.

Studies in Intact and Skinned Muscle Fibers

Although not strictly physiological, the first evidence that PI metabolism might be involved in E–C coupling comes from the experiments of Novotny and coworkers (1978, 1983). These authors reported that K^+ depolarization of whole frog sartorius muscle incubated with ^{32}P leads to an increase in PI production relative to the controls; no information regarding PI4P or PI(4,5)P_2 formation was provided.

Vergara *et al.* (1985) and Volpe *et al.* (1985) reported a few years later that addition of Ins(1,4,5)P_3 to skinned muscle fibers induces contraction. In addition, Vergara *et al.* (1985) found that short tetanic stimulation produces an increase in Ins(1,4,5)P_3 levels with respect to resting muscles, that inhibitors of Ins(1,4,5)P_3 hydrolysis increase the sensitivity of the fibers to Ins(1,4,5)P_3, and that neomycin, an inhibitor of PI(4,5)P_2 hydrolysis, blocks E–C coupling.

Several additional reports in the following years have described contraction of skinned skeletal muscle fibers on exposure to Ins(1,4,5)P_3 (Donaldson *et al.*, 1987; Nosek *et al.*, 1986; Vergara *et al.*, 1987; Blinks *et al.*, 1987). However, Lea *et al.* (1986) and Isac *et al.*, (1988) reported no effect, and Walker *et al.* (1987), using a caged compound that, upon laser flash photolysis, releases Ins(1,4,5)P_3, found that the contractile response of skinned frog fibers was too slow to be of physiological relevance and that high concentrations of the caged compound were needed to elicit a response.

On the other hand, microinjection of Ins(1,4,5)P_3 into intact fibers induces contraction according to Vergara *et al.* (1987), but Blinks *et al.* (1987; see also Hannon *et al.*, 1988) found an effect on detubulated fibers only.

The concentration of Ins(1,4,5)P_3 needed to produce an effect varies according to different authors. Rojas *et al.* (1988) studied the effect of Ins(1,4,5)P_3 on calcium release detected as aequorin-induced light signals in frog skinned fiber bundles; they received significant signals at final Ins(1,4,5)P_3 concentrations as low as 0.2 μM. Much higher concentrations were reported to be needed under different experimental conditions by Walker *et al.* (1987). The calcium dependence of the Ins(1,4,5)P_3 effect (see below) may account for this apparent difference in sensitivity, but other explanations may be valid as well. Walker *et al.* (1987) did not control for by-products of the photolytic process that may interfere with the contractile response, nor did they measure the actual Ins(1,4,5)P_3 concentration and its homogeneity at the site of action. While the kinetics for contraction described by Walker *et al.* (1987) show a time to peak of tens of seconds, Rojas *et al.* (1988) reported a time to peak on the order of 100 msec, apparently limited by diffusion of the agonist, since caffeine or calcium evoked a response with similar kinetics. Furthermore, Ins(1,4,5)P_3 at low concentrations presumably produces a fast release of calcium, as indicated by the fast contraction measured in skinned fibers after Ins(1,4,5)P_3 microinjection (Vergara *et al.*, 1985, 1987; Donaldson, 1986; Donaldson *et al.*, 1987; Nosek *et al.*, 1986). Vergara *et al.* (1987) observed contraction 33 msec after microinjection of Ins(1,4,5)P_3 (one frame in the video recording used to visualize contraction), and Rojas *et al.* (1988), as mentioned above, observed a delay of 130 msec between Ins(1,4,5)P_3 addition and increase in aequorin light emission, the minimal time allowed by their experimental conditions. Thus, increasing the time resolution of the experimental measurements following Ins(1,4,5)P_3 addition is an absolute requirement to establish whether the delay is compatible with the physiological response times that, depending on temperature, are 0.5–4.0 msec for calcium release and a few milliseconds more for muscle contraction (Vergara and Delay, 1986).

Contradicting the results shown by Vergara *et al.* (1987), Blinks *et al.* (1987) claim that the response to Ins(1,4,5)P_3 in skeletal muscle is not

seen in intact fibers, but is present only in "detubulated" fibers (after a glycerol-induced osmotic shock); their interpretation is that Ins(1,4,5)P_3 acts at the T-tubule membrane and not at the level of SR. We found occasionally that skinned fiber bundles are insensitive to Ins(1,4,5)P_3 even in the presence of calcium; we think that a plausible explanation for these observations is that the Ins(1,4,5)P_3 receptor complex, under certain conditions, may not be readily accessible to cytoplasmic Ins(1,4,5)P_3 in intact fibers (and sometimes in skinned fibers) or that other factors may regulate the interactions that lead to the opening of the calcium release channels. It is interesting to note in this regard that Donaldson *et al.* (1988) studied the relationship between T-tubule membrane potential and Ins(1,4,5)P_3-induced calcium release in rabbit skinned skeletal muscle fibers. They reported that the T-tubule–SR junction remains functionally intact after skinning and that the membrane potential across the T-tubule membrane can be changed by modifying the composition of the solution bathing the fiber. Only fibers with depolarized T-tubule showed consistent responses following addition of 0.5 μM Ins(1,4,5)P_3 (Donaldson *et al.*, 1988). These results suggest that the effect of Ins(1,4,5)P_3 can be controlled by the membrane potential at the T-tubule level. Whether the accessibility of the SR receptor to Ins(1,4,5)P_3 is controlled by the T-tubule potential remains to be established by direct experimental tests of Ins(1,4,5)P_3 binding as a function of membrane potential.

The amount of calcium released by Ins(1,4,5)P_3 from SR is usually lower than that released by high concentrations of caffeine (Rojas *et al.*, 1988). The Ins(1,4,5)P_3-induced release of calcium requires a calcium concentration of $\geqslant 10^{-7}$ M in the medium, while caffeine induces calcium release regardless of the presence of calcium. Thus, there are conditions in which the fibers respond to caffeine, but not to Ins(1,4,5)P_3. The calcium dependence of the Ins(1,4,5)P_3 response might explain some of the negative results obtained with skinned fibers in the presence of EGTA (Lea *et al.*, 1986; Isac *et al.*, 1988).

The light signal obtained when submaximal doses of Ins(1,4,5)P_3 and caffeine are added together to the skinned fibers is much higher than the sum of the individual signals produced by Ins(1,4,5)P_3 or caffeine alone (Fig. 2). These observations suggest that Ins(1,4,5)P_3 and caffeine act on different sites of the calcium release pathway.

The receptor site has the highest sensitivity to the Ins(1,4,5)P_3 derivative, since the light response is less sensitive to Ins(1,3,4)P_3 and Ins(2,4,5)P_3. The sensitivity to inositol 1,4,5,6-tetrakis phosphate and to inositol bisphosphate is certainly much less evident.

A chemical transmitter in E–C coupling requires that a measurable period of time elapses between T-tubule depolarization and calcium release. Vergara and Delay (1986) were able to measure simultaneously, in a stimu-

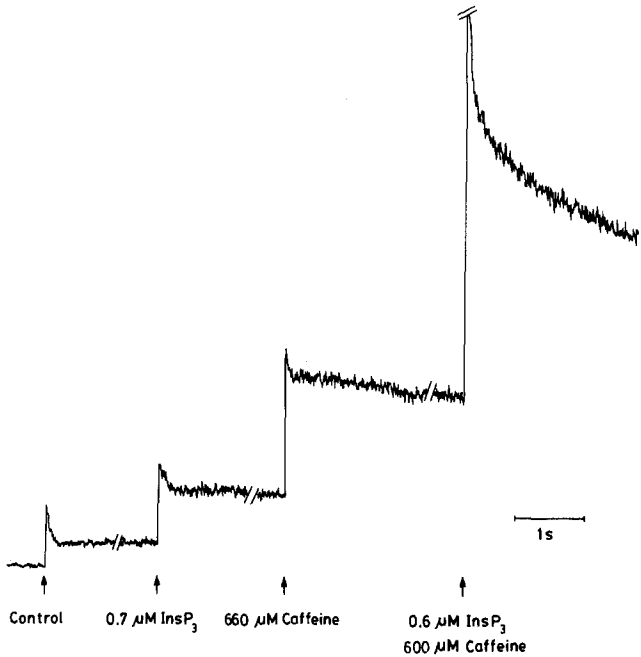

Fig. 2. Aequorin light signal from a from sartorius skinned fiber bundle incubated in 130 mM sodium glutamate, 2.5 mM ATP-sodium, 3 mM $MgCl_3$, and 5 mM Hepes Na, pH 7.2, at room temperature. The fiber bundles were preincubated in the same solution containing 10^{-5} M Ca^{2+} for 60 min. The initial volume of the chamber was 200 μl and each arrow represents addition of 20 μl saline (control) plus the drugs needed to reach the indicated concentration (C. Rojas and E. Jaimovich, to be published).

lated frog muscle fiber, the optical signals for both T-tubule depolarization, sensed with a potentiometric dye, and calcium release, measured with a calcium-sensitive dye. After subtracting the time needed for propagation of the electric signal, they found a lag ("triadic delay") ranging from ~ 0.5 msec at 25.6°C to ~ 4 msec at 4°C. Both the magnitude of this delay and its marked temperature dependence ($Q_{10} = 2.7$ between 10 and 20°C) suggest that E–C coupling may be mediated by a series of chemical reactions (Vergara and Delay, 1986; Vergara *et al.*, 1987).

Calcium Release from Isolated SR Vesicles

Experiments carried out with isolated SR vesicles to investigate whether $Ins(1,4,5)P_3$ produces calcium release have led to conflicting reports.

Volpe *et al.* (1985) found that addition of μM $Ins(1,4,5)P_3$ to SR vesicles isolated from the terminal cisternae induced rapid calcium release. Several negative reports followed (Scherer and Ferguson, 1985; Adunyah and Dean, 1986; Mikos and Snow, 1987; Palade, 1987). The reasons for these

discrepancies remain to be established, although the origin of the SR vesicles used to study calcium release might explain some negative results. Thus, Scherer and Ferguson (1985) used light SR vesicles that presumably lacked the calcium channels primarily involved in E–C coupling, and Adunyah and Dean (1986) provide no details regarding the origin of their SR vesicles.

Single-Channel Experiments

Heavy SR vesicles isolated from rabbit skeletal muscle fuse into planar lipid bilayer membranes and display a calcium channel with many of the physiological and pharmacological characteristics of the calcium release pathway of SR (Smith *et al.*, 1985, 1986). Furthermore, the ryanodine receptor

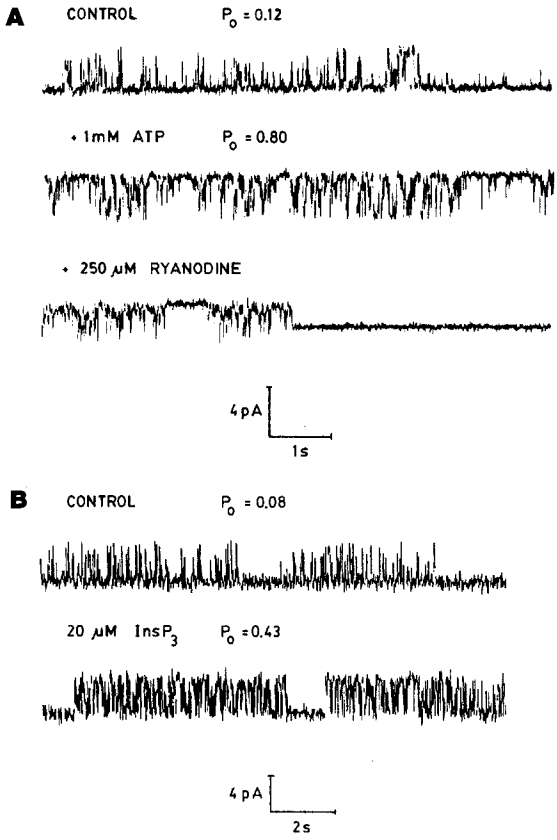

Fig. 3. Records of single-channel activity obtained by fussion of heavy SR vesicles from frog muscle into planar bilayers (Suárez-Isla *et al.*, 1988). The probability of opening (P_o) is indicated for the control record and after addition of (A) ATP and ATP plus ryanodine, and (B) Ins(1,4,5)P_3.

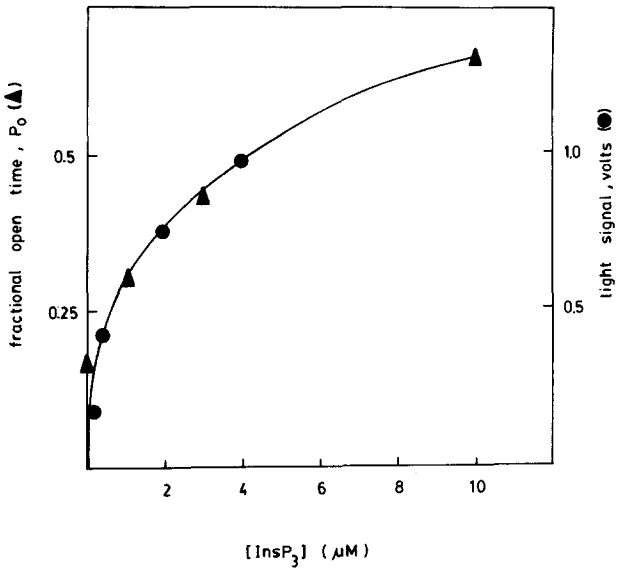

Fig. 4. Probability of single-channel opening (P_o) in bilayers and aequorin light signal in skinned fiber bundles plotted as a function of Ins(1,4,5)P_3 concentration. From R. Bull, J. J. Marengo, V. Irribarra, and B. A. Suárez-Isla, to be published, and from C. Rojas and E. Jaimovich, to be published.

purified from the terminal cisternal regions of SR has been reconstituted into bilayers as a calcium channel with similar characteristics as the calcium channel of the native SR vesicles (Imagawa *et al.*, 1987; Lai *et al.*, 1988; Hymel *et al.*, 1988). Thus, it is generally accepted that the purified ryanodine receptor protein is the calcium release channel of SR. Morphological evidence indicates, furthermore, that the ryanodine receptor is identical to or forms part of the feet (Inui *et al.*, 1987; Lai *et al.*, 1988) that link the SR and the T-tubule membrane at the level of the triadic junction (Franzini-Armstrong, 1970).

Heavy SR membranes isolated from frog skeletal muscle have a calcium channel with the same properties as the calcium release channel described in SR isolated from rabbit skeletal muscle (Suárez-Isla *et al.*, 1988). Thus, the channel is activated by μM calcium and mM ATP, is blocked by ruthenium red and lanthanum, is modulated by magnesium, and decreases its single-channel conductance following addition of 250 μM ryanodine (Fig. 3A). The same properties have been described for the calcium channel studied in SR vesicles (Smith *et al.*, 1986) or for the purified ryanodine receptor isolated from rabbit skeletal muscle and fused into lipid bilayers (Imagawa *et al.*, 1987; Lai *et al.*, 1988). In addition, we have found (Fig. 3B) that the calcium channel from frog SR is activated by μM Ins(1,4,5)P_3 (Suárez-Isla *et al.*,

1988). The activation by Ins(1,4,5)P_3 shows a striking dependence on calcium concentration; no activation was observed at pCa = 8, and maximal sensitivity to Ins(1,4,5)P_3 was obtained at pCa = 7 or 6. Increasing calcium concentration decreased the sensitivity of the Ins(1,4,5)P_3 response. At pCa = 4.4, much higher Ins(1,4,5)P_3 concentrations are needed to activate the channel (Suárez-Isla *et al.*, 1988). It is interesting to note in this regard that the response of skinned fibers showed a dependence of Ins(1,4,5)P_3 concentration at pCa = 7 that is similar to that observed with the channel (Fig. 4); in both cases, no response was obtained at pCa = 8.

Stimulation of the calcium channel by Ins(1,4,5)P_3 was also found in SR vesicles from rabbit skeletal muscle (Suárez-Isla *et al.*, 1988). In contrast, other reports indicate a lack of Ins(1,4,5)P_3 sensitivity for the calcium channel present in SR isolated from skeletal (Smith *et al.*, 1986) and cardiac (Rouseau *et al.*, 1986) muscle.

Biochemical Studies

A crucial assumption for a role of Ins(1,4,5)P_3 in E–C coupling in skeletal muscle is the presence in the T-tubule membrane of the lipid precursors of Ins(1,4,5)P_3 (Fig. 1).

Experiments in whole muscle fibers have shown that PI synthesis increases following K^+ depolarization (Novotny *et al.*, 1983). Furthermore, lipid extracts of whole muscle cells incubated with [^{3}H]inositol contain labeled PI, PI4P, and PI(4,5)P_2 (Vergara *et al.*, 1987), indicating that significant amounts of these lipids are synthesized in muscle cells. The soluble inositol phosphate derivatives of the these phosphoinositides are also found in muscle cells at rest, and it has been reported that, following a brief tetanic stimulation, the levels of Ins(1,4,5)P_3 increase with respect to the resting controls (Vergara *et al.*, 1985).

Experiments with isolated SR and T-tubule membranes have shed further light on PI metabolism in skeletal muscle. Thus, PI kinase, the membrane-bound enzyme that phosphorylates PI to PI4P, is present both in SR and T-tubule membranes isolated from frog (Hidalgo *et al.*, 1986; Carrasco *et al.*, 1988) and rabbit (Varsanyi *et al.*, 1986; Carrasco *et al.*, 1988) skeletal muscle. In contrast, PI4P kinase, the membrane-bound enzyme responsible for the production of PI(4,5)P_2, is present in only T-tubule membranes (Hidalgo *et al.*, 1986; Varsanyi *et al.*, 1986; Carrasco *et al.*, 1988). Thus, the metabolic machinery responsible for the synthesis of the direct precursor of Ins(1,4,5)P_3 is restricted to the T-tubule membrane, indicating that all of the Ins(1,4,5)P_3 produced following muscle depolarization originates from breakdown of the PI(4,5)P_2 localized in the T-tubule membranes.

The rates of PI4P and $PI(4,5)P_2$ formation by T-tubules from frog muscle (600 and 480 pmol/min/mg protein, respectively) are higher than the values (159 and 32 pmol/min/mg protein) reported by Varsanyi *et al.*, (1986) for a triad-enriched preparation from rabbit muscle. It is likely that PI4P formation in their triad-enriched preparation represented mostly SR PI kinase activity, whereas, in the T-tubule preparation from frog muscle used by Hidalgo *et al.* (1986), SR contamination was negligible.

Most (90%) of the T-tubule vesicles used by Hidalgo *et al.* (1986) are sealed with the cytoplasmic side out, making it likely that both PI and the kinases that form PI4P and $PI(4,5)P_2$ are present in the cytoplasmic side of the T-tubule membranes *in vivo*. Vergara *et al.* (1985) have calculated that release into the triadic space of 100 molecules of $Ins(1,4,5)P_3/\mu m^2$ T-tubule surface would be sufficient to raise the concentration of $Ins(1,4,5)P_3$ in this region to 10 μM. It is important to point out that this calculation is restricted to a triadic space of 1.7×10^{-17} liters/μm^2 T-tubule surface, and not to the entire intracellular volume of the muscle fiber.

We find levels of $PI(4,5)P_2$ of up to 60 pmol/μmol lipid phosphorus (120 pmol/mg protein). Assuming that there are 10^6 molecules phospholipid/μm^2 membrane surface (Janiak *et al.*, 1979), 60 pmol $PI(4,5)P_2/\mu$mol lipid phosphorus corresponds to 60 molecules of $PI(4,5)P_2/10^6$ molecules of phospholipids, or 60 molecules of $PI(4,5)P_2/\mu m^2$ T-tubule membrane. Hydrolysis of all this $PI(4,5)P_2$ to yield $Ins(1,4,5)P_3$ would increase the concentration of $Ins(1,4,5)P_3$ to 6 μM in the triadic space, a value that is sufficient to elicit contractures in skeletal muscle fibers (Vergara *et al.*, 1985; Donaldson *et al.*, 1987). Furthermore, it seems likely that the levels of $PI(4,5)P_2$ formed by the isolated vesicles represent only a fraction of the amounts formed *in vivo*, since the supply of ATP was limited in the *in vitro* studies. If this was the case, even higher concentrations of $Ins(1,4,5)P_3$ could be reached following hydrolysis of $PI(4,5)P_3$.

Both phosphoinositide kinases of muscle membranes are regulated by calcium, although they exhibit different calcium dependences (Carrasco *et al.*, 1988). Phosphorylation of PI to PI4P is independent of calcium in a range of concentrations from 10^{-9} to 10^{-6} M, and is progressively inhibited to 10% of the maximal values by increasing calcium to $\geqslant 10^{-4}$ M ($K_{0.5} = 5 \times 10^{-6}$ M). In contrast, phosphorylation of PI4P to $PI(4,5)P_2$ is maximal at calcium concentrations $> 2 \times 10^{-6}$ M and decreases to 30% of maximal values at calcium concentrations of $\leqslant 2 \times 10^{-7}$ M ($K_{0.5} = 10^{-6}$ M).

The regulation of PI4P and $PI(4,5)P_2$ formation by increasing calcium from pCa = 7 to pCa = 5 might have important physiological implications. If the behavior of the isolated membranes reflects the *in vivo* situation, in resting muscle (pCa = 7) the levels of $PI(4,5)P_2$ present, which represent only 20–30% of the maximal values obtainable at pCa < 6, would be probably

sufficient to release enough $Ins(1,4,5)P_3$ during one or a few twitches. Following calcium release from SR and calcium entry from the extracellular space, via opening of voltage-dependent T-tubule calcium channels (Sanchez and Stefani, 1978; Fosset *et al.*, 1983; Schwartz *et al.*, 1985; Rios and Brum, 1987), the calcium concentration in the triadic space would increase to pCa < 6. This increase in calcium levels would stimulate $PI(4,5)P_2$ formation, ensuring an adequate supply of this lipid for repetitive stimulation.

Two enzymes that play a key role in the model that postulates $Ins(1,4,5)P_3$ as chemical messenger in E–C coupling are the phospholipase C specific for $PI(4,5)P_2$ and the $Ins(1,4,5)P_3$ phosphatase. The latter has been found both in soluble form and in membrane-bound form (Milani *et al.*, 1988). The enzyme, which is present in T-tubule and in SR membranes, is enriched in the T-tubule membranes. We have confirmed the presence of $Ins(1,4,5)P_3$ phosphatase in both membranes and its higher activity in the T-tubule system (Table I). The $Ins(1,4,5)P_3$ phosphatase activity reported by Milani *et al.* (1988) and that measured by us in T-tubule membranes from frog muscle display the highest activities so far reported for the membrane-bound form of the enzyme in other tissues (Milani *et al.*, 1988). Furthermore, its higher activity in the T-tubule membrane suggests a functional role associated with this particular location. Such a role implies, however, that the enzyme is regulated so that the relatively high K_m value ($\sim 20\,\mu M$) reported by Milani *et al.* (1988) would be lower *in vivo* during muscle relaxation.

Much less information is available regarding phospholipase-C activity in isolated muscle membranes. Partially purified T-tubule membranes hydrolyze exogenous $[^3H]PI(4,5)P_2$; this reaction is stimulated by 0.1 mM Ca and 10 mM NaF (Salviati *et al.*, 1988). Our own experiments indicate that endogenous ^{32}P-labeled $PI(4,5)P_2$ in T-tubule membranes from frog muscle is hydrolyzed with rates on the order of 10 pmol/mg protein/min at 25°C. This reaction is inhibited by 1 mM neomycin. We have also found hydrolysis

Table I. Specific Activity of $Ins(1,4,5)P_3$-Phosphatase in Membrane Fractions from Skeletal Muscle[a]

Membrane fraction	Specific activity (nmol/min/mg protein)	Ref.
Light SR + T-tubule (rabbit)	13.5 ± 2.2 (8)	Milani *et al.* (1988)
Heavy SR (terminal cisternae, rabbit)	2.7 ± 0.7 (7)	Milani *et al.* (1988)
Light SR (frog)	8.2	Our work[b]
T-tubule (frog)	29.5 ± 4.8 (2)	Our work[b]

[a]The specific activity determined by Milani *et al.* (1988) was measured at 30°C using 50 μM $Ins(1,4,5)P_3$; our determinations were carried out at 25°C using 40 μM $Ins(1,4,5)P_3$.
[b]X. Sánchez, M. A. Carrasco, E. Jaimovich, and C. Hidalgo, unpublished observations.

of exogenously added [^{3}H]PI(4,5)P_2 by T-tubule membranes. The highest activity that we have measured with endogenous substrate, 10 pmol/mg protein/min, corresponds to 5 pmol/μmol lipid Pi/min. Assuming 10^6 molecules of phospholipid/μm^2 T-tubule surface (Janiak *et al.*, 1979), this activity can be converted to 10^{-4} molecules of PI(4,5)P_2 hydrolyzed/μm^2 T-tubule surface/msec. This value is at least 10^4 times lower than that needed to produce enough Ins(1,4,5)P_3 to raise its concentration to 0.1 μM in the triadic space following one twitch. One important observation is that this enzyme seems markedly labile, so that the activity decays to almost negligible levels a few hours after membrane isolation. Thus, the low values of activity that we found might reflect a partially inactivated phospholipase C. We are currently investigating the factors that regulate this activity.

Discussion

The experimental observations obtained so far show that all of the biochemical pathways required for Ins(1,4,5)P_3 production and degradation are present in skeletal muscle. Moreover, PI(4,5)P_2, the direct lipid precursor of Ins(1,4,5)P_3, is synthesized in the T-tubule membrane, as required by the model proposed by Vergara *et al.* (1985) and Volpe *et al.* (1985). In addition, Ins(1,4,5)P_3 causes calcium release and contraction in skinned fibers, and opens the calcium release channels of SR, although, as discussed above, these results are not generally accepted. Furthermore, short tetanic stimulation leads to an increase in Ins(1,4,5)P_3 levels; whether this is the case for a single twitch remains to be established.

In order to establish a physiological role for Ins(1,4,5)P_3 as a chemical messenger in E–C coupling, one important question needs to be answered: how does the T-tubule membrane release Ins(1,4,5)P_3 in a time range of 1–2 msec following depolarization? It was originally proposed in this regard that depolarization activates either the phospholipase C that hydrolyzes PI(4,5)P_2 (Vergara *et al.*, 1985) or a GTP-binding protein that in turn would activate PI(4,5)P_2 hydrolysis (Di Virgilio *et al.*, 1986; Volpe *et al.*, 1986). Neither of these hypotheses has been tested experimentally, and other alternatives can be considered, as discussed below.

Another important question concerns Ins(1,4,5)P_3 removal following repolarization. The model originally proposed (Vergara *et al.*, 1985; Volpe *et al.*, 1985) assigned this role to an Ins(1,4,5)P_3 phosphatase. According to Milani *et al.* (1988) and our own results, this reaction seems to be fast enough to account for the physiological relaxation rates, but the apparent K_m of this enzyme in isolated membrane fractions is too high to account for a physiological role.

An alternative view is that Ins(1,4,5)P_3 could be already present in the T-tubule membrane, bound to high-affinity sites. Following T-tubule depolarization, the affinity to Ins(1,4,5)P_3 would decrease, leading to its release into the triadic space. On repolarization, Ins(1,4,5)P_3 would rapidly bind again to the T-tubule membrane. This model would certainly account for fast on-and-off physiological responses, but it must be tested experimentally to ascertain its validity.

Any model regarding Ins(1,4,5)P_3 as chemical messenger will have to account for the discrepancies reported regarding the effects of Ins(1,4,5)P_3 on whole muscle or in SR vesicles. The Ins(1,4,5)P_3 receptor and the calcium release channel of SR might be functionally associated in the intact muscle fiber. This complex would not be readily accessible to cytoplasmic Ins(1,4,5)P_3, so the effect would be more easily detected in detubulated fibers or in skinned fibers. The complex would be maintained in isolated SR membranes, so the effect of Ins(1,4,5)P_3 can be seen in vesicles fused into bilayers, but isolated SR vesicles may have this complex modified so it is possible to obtain vesicles with channels that are not sensitive to Ins(1,4,5)P_3. In fact, we occasionally see calcium channels in bilayers that fail to respond to Ins(1,4,5)P_3 (Suárez-Isla *et al.*, 1988). More experiments are needed to establish the existence of such a complex.

In conclusion, a role for Ins(1,4,5)P_3 in skeletal muscle function is highly likely, but whether Ins(1,4,5)P_3 is the physiological messenger in E–C coupling has not yet been established. Clearly, further experimental tests are required to settle the issue.

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Note Added at Proof

Liu *et al.* (1989) have confirmed the activation of the SR calcium channel by Ins(1,4,5)P_3 and have further described activation of the purified ryanodine receptor-calcium channel by 5–20 μM Ins(1,4,5)P_3 (Liu, Q. I., Lai, A., Xu, L., Jones, R. V., LaDine, J. K. and Meissner, G. (1989). *Biophys. J.* **55**, 85a).

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