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Phospholipase C activity in membranes and a soluble fraction isolated from frog skeletal muscle

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Highly purified triads and transverse tubules, as well as a soluble fraction isolated from frog skeletal muscle, hydrolyze exogenous phosphatidylinositol 4,5-bisphosphate forming inositol 1,4,5-trisphosphate with maximal rates in the range 0.5–1 nmol/mg per min at pCa 3. Sarcoplasmic reticulum membranes present a minor activity. The hydrolysis rates in triads were 0.072 ± 0.015 nmol/mg per min at pCa 7, increasing to 0.263 ± 0.026 nmol/mg per min at pCa 5 with 1.0 mM Mg and 0.1 mM substrate. The phospholipase C activity of isolated transverse tubules at pCa 3 was 0.570 ± 0.032 nmol/mg per min. Since triads contain 10% transverse tubules, and correcting for the small contribution of sarcoplasmic reticulum, the calculated phospholipase C activity of transverse tubules at pCa 3 is about 10-times higher than the observed values, suggesting loss of activity during isolation. The activation by calcium was also observed in a soluble fraction and was neither replaced nor inhibited by magnesium. No effect of GTP analogs on phospholipase C activity was detected.

Introduction

Many aspects of inositol 1,4,5-trisphosphate (IP_3) metabolism in skeletal muscle have been described following the proposal of IP_3 as a possible chemical messenger in excitation-contraction (E-C) coupling [1,2]. While the mechanism of E-C coupling has not yet been solved, biochemical studies on muscle cells and on isolated muscle membranes have demonstrated that the metabolic machinery necessary for the formation and removal of IP_3 is present in skeletal muscle. Basically, phosphatidylinositol 4,5-bisphosphate (PIP_2), the lipid precursor of IP_3, is synthesized in transverse tubule (T-tubule) membranes only, as demonstrated both in rabbit and frog skeletal muscle [3–8]. Likewise, the phosphatase that degrades IP_3 to metabolites which do not release calcium occurs mostly in the particulate fractions, with the highest specific activity in T-tubule membranes [6,9,10]. The hydrolysis of PIP_2 by phospholipase C (PLC) is a key step in IP_3 formation. Studies in rabbit skeletal muscle have reported hydrolytic activity distributed in soluble and triad fractions [7]. In the present work, we have studied PLC activity in amphibian skeletal muscle where many physiological and biochemical studies on IP_3 have been carried out. Besides, methods to obtain highly purified T-tubules, sarcoplasmic reticulum (SR) and triads from this tissue are available [11,12]. Our aim was to characterize the PLC activity of skeletal muscle under physiological ionic conditions, with special emphasis on the regulation by Ca^{2+} and the possible involvement of G proteins. Part of this work has been presented in abstract form [13].

Materials and Methods

Materials

[^{3}H]PIP_2 (spec. act. 8.8 Ci/mmol) was purchased from Dupont New England Nuclear. PIP_2 and phosphatidylserine (PS), both from bovine brain, were obtained from Sigma, phosphatidylethanolamine (PE) from Avanti-Polar Lipids and anion-exchange resin AG 1-X8 (100–200 mesh, chloride form) from Bio-Rad. All the glassware used in the PIP_2 hydrolysis assays was silanized with dimethylchlorosilane from Fluka.

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Abbreviations: BSA, bovine serum albumin; DHP, dihydropyridine; E-C, excitation-contraction; IP_3, inositol 1,4,5-trisphosphate; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PS, phosphatidylserine; SR, sarcoplasmic reticulum; T-tubule, transverse tubule.

Other chemicals were obtained from Sigma and from Merck.

Isolation of muscle soluble and membrane fractions

T-tubules and SR membranes were isolated from frog skeletal muscle (*Caudiverbera caudiverbera*) as described [11], with the only modification that the KCl concentration was reduced from 0.6 M to 0.15 M. The PLC enzymes are described as peripheral proteins and in fact, it has been reported that 0.6 M KCl dissociates the PLC activity of triads from rabbit skeletal muscle [7]. Triads were obtained in an isotonic medium containing 0.15 M KCl by the procedure described by Hidalgo et al. [12]. Briefly, the homogenate was subjected to differential centrifugation and triads were obtained as a 17000 $\times g$ pellet after 30 min centrifugation. The soluble fraction was collected as the 100000 $\times g$ supernatant following 60 min centrifugation. Triads, homogenate and soluble fraction were frozen in solid CO_2 after adding 0.25 M sucrose, and were stored at −80°C. PLC activity was retained for a month.

Phospholipase C activity assay

Phospholipase C activity was assayed with exogenous [^{3}H]PIP_2 sonicated with PE and PS at a 1:2:2 molar ratio. This lipid combination results in the highest PLC activity as compared to a series of different lipid compositions and molar proportions [14], and has been used in the study of PLC activity in rabbit skeletal muscle [7]. The assay was carried out at 25°C in a final volume of 0.1 ml of a solution containing 50 mM Hepes-Tris (pH 6.8), 100 mM KCl, 1.0 mM EGTA or HEDTA and 0.1 mM [^{3}H]PIP_2 (25000 cpm/nmol). The incubation time was 10 min, since initial experiments showed that [^{3}H]PIP_2 hydrolysis was linear up to this time. The reaction was started by the addition of 0.1 mg of protein and was terminated by the addition of 10% TCA and 0.1% BSA. After centrifugation, aliquots of the resulting supernatant were either assessed for radioactivity or separated on Dowex-chloride columns for the identification of inositol phosphates [17]. The radioactivity in blanks, where TCA was added before the protein, was less than 2% of the total radioactivity and was subtracted from the PLC assays. Experiments performed in the presence of 2 mM cholate and 20 mM deoxycholate showed no differences with respect to experiments carried out without detergents. A computer program was used to calculate free Ca^{2+} and Mg^{2+} concentrations [15]. The actual free Ca^{2+} concentrations were measured with a calcium electrode (Selectrode, Radiometer). [^{3}H]PIP_2 conversion to [^{3}H]PIP and/or [^{3}H]PI was assessed after TLC separation of the organic phase of assays where the reaction was stopped by addition of acidified chloroform/methanol [3].

TABLE I

Distribution of [^{3}H]PIP_2 hydrolytic activity in frog skeletal muscle

[^{3}H]PIP_2 hydrolytic activity was measured in the standard medium with 0.5 mM Mg^{2+} at pCa 4. Data represent mean ± S.E. of four different preparations.

Fraction	Specific activity (nmol [^{3}H]PIP_2 hydrolyzed/mg per min)	Activity recovered (%)
Homogenate	0.87 ± 0.03	100
Soluble fraction	0.92 ± 0.09	20.9 ± 1.3
Triads	1.06 ± 0.28	0.30 ± 0.18

Results and Discussion

[^{3}H]PIP_2 hydrolytic activity was recovered associated to particulate and soluble fractions (Table I), as has been found in most cell types [16]. The three fractions we analyzed presented similar specific activities. That is, there was no enrichment in the soluble or the triad fractions with respect to the total homogenate. To monitor the products of [^{3}H]PIP_2 hydrolysis, triads and soluble fraction were assayed with 1 mM Mg^{2+} at several free Ca^{2+} concentrations. After incubation, TLC of the phosphoinositides was performed, showing that most of ^{3}H-associated radioactivity was recovered as [^{3}H]PIP_2, with no radioactivity being detected in the fraction corresponding to PIP or PI standards (data not shown). This result indicates that the concentration of substrate did not decrease during the assay due to PIP_2 phosphatases. For the analysis of the water-soluble products of PIP_2 hydrolysis, assays included 0.1 mM $CdCl_2$ which inhibits completely frog skeletal muscle IP_3 phosphatase [10]. Under these conditions the only product formed was IP_3 (data not shown).

We examined [^{3}H]PIP_2 hydrolysis by highly purified T-tubules and SR membranes to compare them with triads. At pCa 3, the hydrolysis rates in T-tubules and triads were very similar (Table II). At pCa 7, both fractions presented hydrolysis rates much lower than at pCa 3. The SR values represent combined results from

TABLE II

PLC activity in membrane fractions from frog skeletal muscle

PLC activity was measured in the standard medium with 0.5 mM Mg^{2+}. Data represent mean ± S.E., the number of membrane preparations studied is given in parentheses.

Membrane fraction	pCa	PLC activity (nmol/mg per min)
Triads	3	0.660 ± 0.104 (4)
	7	0.069 ± 0.009 (3)
T-tubules	3	0.570 ± 0.032 (3)
	7	0.041 ± 0.039 (2)
SR	3	0.063 ± 0.012 (4)

longitudinal and heavy SR membranes since their PLC activities were very similar. The SR activity measured at pCa 3 was about 10% of the activity found in T-tubules in the same conditions (Table II). Binding assays of [^{3}H]nitrendipine showed that SR membranes presented a contamination with T-tubules lower than 1%; thus the activity found in SR membranes indicates that they actually contain some [^{3}H]PIP_2 hydrolytic activity. SR membranes phosphorylate endogenous PI to PIP but do not form PIP_2 [3–8]. It is well-known, though, that the three forms of PLC catalyze the hydrolysis of PI, PIP and PIP_2 [18]. Thus, the PLC activity found in SR might correspond to an enzyme that hydrolyzed primarily either PI or PIP, a possibility we are currently investigating.

The triads used in this study contain about 10% T-tubules, as indicated by their density of dihydropyridine and total ouabain-binding sites (9.5 and 19.2 pmol/mg of protein, respectively; 12). This membrane fraction also contains a high density of ryanodine-binding sites (8.9 pmol/mg of protein [12]). In frog skeletal muscle, most T-tubules membranes are associated to SR terminal cisternae [19], so the majority of the isolated T-tubules have the same origin as the T-tubules forming part of triads. Considering the results shown in Table II, and that triads contain 10% T-tubules, we calculate that the PLC activity of T-tubules associated in triads should be about 6.0 nmol [^{3}H]PIP_2 hydrolyzed/mg per min, 10-times higher than the activity presented by isolated T-tubules (Table II). There are two main differences in the procedures used to obtain triads and T-tubules that might explain this result. Triads were obtained in the presence of a combination of proteinase inhibitors, while T-tubules were not; besides, triad isolation takes considerably less time than the isolation of T-tubules; both factors may contribute to lose activity in the T-tubules. An alternative explanation would be that the triad structure helped to stabilize the hydrolytic activity or prevented the dissociation of the PLC enzyme from the membranes.

The three types of PLC enzymes described in the literature hydrolyze PIP_2 in a Ca^{2+}-dependent manner [18]. We found that both particulate and soluble PLC activities were Ca^{2+} dependent, with virtually no activity at Ca^{2+} concentrations lower than pCa 7 (Fig. 1). In both fractions, PLC activity increased significantly from pCa 7 to pCa 5, which are approximately the free Ca^{2+} concentrations of resting and activated muscle, respectively. The triad fraction activities in this Ca^{2+} range were higher than those measured in the soluble fraction. This result indicates that the PLC activity present in triads cannot be accounted for by trapped soluble enzyme. Besides, the assays were carried out in the absence of detergents; thus trapped enzyme would not be detected since the T-tubules forming parts of triads

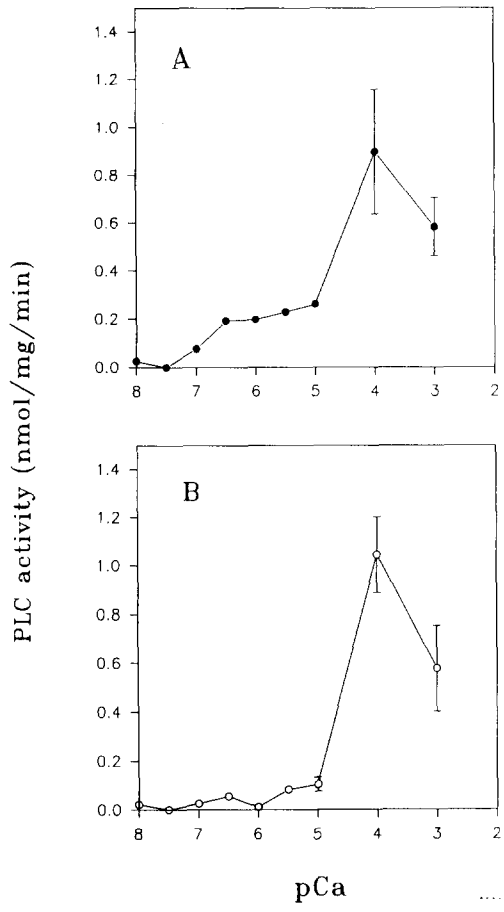

Fig. 1. Calcium dependence of PLC activity. Triads (A) and soluble fraction (B) were incubated under standard conditions with various concentrations of calcium in the presence of 1 mM Mg^{2+}. Values with error bars represent means ± S.E. of 3–7 separate experiments. At pCa 7 and pCa 5 in triads, the standard errors were less than the size of the symbol. The rest of the values are the mean of duplicates in one experiment. The increase in activity observed with calcium changing from pCa 7 to pCa 5 was significant in triads ($P < 0.0001$, non-paired Student's t-test) and in soluble fraction ($P < 0.05$). At pCa 7, the activity in the triads was significantly higher ($P < 0.01$) that the activity measured in the soluble fraction.

are sealed vesicles [12]. PLC activity increased at pCa 4 and was maintained relatively unchanged at pCa 3. The lipid vesicles precipitated at pCa 2 and no activity could be measured.

The effect of Ca^{2+} on PLC activity was studied at 1.0 mM Mg^{2+}, the physiological free Mg^{2+} concentration of frog skeletal muscle [20]. It is well known that Mg^{2+} can affect PLC activity and, thus, we performed assays in the presence of 0.5–2.0 mM Mg^{2+} either at a fixed pCa of 5 or 7. PLC activity was independent of Mg^{2+} in this concentration range, as shown in Table III. Besides, these results indicate that the effect of Ca^{2+} on PLC activity is specific and is not due to non-specific charge screening effects that might increase the availability of PIP_2 to the enzyme. The essential role of Ca^{2+} in the control of PLC activity has been clearly demonstrated in a study with short-acyl-

TABLE III

Effect of magnesium on PLC activity of triads

The results are expressed as mean ± S.E., the number of experiments is given in parentheses.

$[Mg^{2+}]$ (mM)	PLC activity (nmol/mg per min)	
	pCa 7	pCa 5
0.5	0.073 ± 0.010 (3)	0.256 ± 0.018 (4)
1.0	0.072 ± 0.015 (5)	0.263 ± 0.026 (3)
2.0	0.087 ± 0.014 (3)	0.240, 0.227 (2)

chain inositol phospholipids in the monomeric state such that the steps preceding catalysis, related to the aggregated state of the substrate, could be eliminated [21].

It has been described that the activity of PLC in triads from rabbit skeletal muscle is also activated by increasing Ca^{2+} in the physiological range [7]. It presents, though, some differences with the PLC activity from frog triads reported here. The enzyme of rabbit muscle is maximally active between pCa 5 and pCa 4 decreasing at higher Ca^{2+} concentrations, and is inhibited half-maximally by 1 mM Mg^{2+} [7]. Since the experimental conditions in both studies were very similar, it is likely that the differences found are species-specific. Another difference we have found between PLC from both species refers to the effect of ionic strength on the dissociation of PLC activity from particulate fractions. While 0.6 M KCl dissociates PLC activity of triads from rabbit skeletal muscle [7], we found in the present work that T-tubules and SR membranes isolated from frog skeletal muscle in the presence of 0.15 M KCl, displayed the same activities as T-tubules and SR membranes isolated in the presence of 0.6 M KCl (data not shown).

In previous work [6] we reported endogenous $[^{32}P]PIP_2$ hydrolysis in T-tubules isolated in 0.6 M KCl. In those experiments where PI was phosphorylated to $[^{32}P]PIP$ and $[^{32}P]PIP_2$ by $[^{32}P]ATP$, we found that while the levels of $[^{32}P]PIP$ remained relatively constant, the levels of $[^{32}P]PIP_2$ decreased steadily with time and this decay was blocked by 1.0 mM neomycin [6]. In experiments with exogenous $[^{3}H]PIP_2$ which were assayed in very similar conditions to the phosphorylation experiments, we determined that 1.0 mM neomycin abolished completely PLC activity (not shown). Therefore, based on the present results we infer that the decrease of endogenous $[^{32}P]PIP_2$ levels we had observed in T-tubules previously was due to PLC activity. However, the PLC activity determined with endogenous PIP_2 is much lower than with exogenous substrate.

An important aspect of the studies of PLC activity is to determine whether G proteins are involved in its regulation. We performed several experiments with triads obtained from three separate preparations, using PLC assay solutions that contained 0.1 mM GTPγS, 1.0 mM Mg^{2+} at pCa 7. No statistically significant changes in PLC activity were obtained after addition of GTPγS. G-protein-mediated PLC activation is either sensitive to pertussis toxin (PTX) or insensitive, in which case a Gq class of α subunits would be involved [22,23]. Some of the α subunits of the Gq family contain tightly-associated GDP which exchanges very slowly with GTPγS, but they are activated with $[AlF_4]^-$ [23]. Thus, we performed experiments with $[AlF_4]^-$ in assays with triads containing 10 mM NaF and 20 μM $AlCl_3$. In these assays EGTA buffers were omitted, since EGTA chelates aluminum [24]. Under these conditions, $[AlF_4]^-$ had no effect on PLC activity (not shown). The triads and T-tubule membranes isolated from frog that we used present several G-proteins, as determined by ADP-ribosylation and immunological studies (Carrasco, M.A., Sierralta, J. and de Mazancourt, P., data not shown). Among them, possible PLC-related PTX-sensitive and Gq proteins, detected by antibodies that recognize both αq and α11, are present. With respect to PLC identification in skeletal muscle, a study in rat has identified PLC γ_2 in this tissue [25]. Another study in rat skeletal muscle with specific antibodies directed to the subtypes β_1, γ_1 and δ_1, has shown that a low level of PLC β_1 could be detected, while higher amounts of γ_1 and δ_1 were present [26]. If a similar distribution occurs in frog skeletal muscle, GTP analogs would not show effect when the overall PLC activity of the three subtypes is measured since only PLC β is affected by G proteins [18]. It is interesting that PLC activity of triads from rabbit skeletal muscle, measured at several Ca^{2+} concentrations, also did not respond to GTP analogs [7]. Recently, a phosphoinositide-specific PLC from rabbit skeletal muscle has been partially purified but it has not been identified yet [27].

Originally, it was proposed that PLC activity would be controlled by T-tubule membrane depolarization [1,2]. It has been reported that the total IP_3 content in amphibian and mammalian skeletal muscle was in the range 1.2–2.5 μM under resting conditions, increasing significantly with tetanic stimulation [28]. However, an alternative explanation would be that the depolarization released IP_3 bound to T-tubule membranes [29]. In this regard, recent results have shown that T-tubules and triads isolated from frog and rabbit skeletal muscle, contain high amounts of IP_3 which represent a significant fraction of the total IP_3 content of muscle [12].

Whatever the mechanism(s) of activation of PLC in skeletal muscle, it is clear that an increase in Ca^{2+} in the physiological range stimulates its activity. We have demonstrated previously, in T-tubules from frog skele-

tal muscle, that PIP kinase, the enzyme that synthesizes PIP_2, is activated by Ca^{2+} in the same range as PLC activity [5]. Thus, the increase in intracellular Ca^{2+} from pCa 7 to pCa 5 not only would activate PLC, but also PIP_2 formation leading to increased IP_3 production.

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