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Inositol 1,4,5-trisphosphate activity in membranes isolated from amphibian skeletal muscle

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The hydrolysis of [^{3}H]inositol 1,4,5-trisphosphate by a soluble fraction and by isolated transverse tubule and sarcoplasmic reticulum membranes from frog skeletal muscle was studied. Transverse tubule membranes displayed rates of hydrolysis several-fold higher than those of sarcoplasmic reticulum and soluble fraction; K_m and V_{max} were 25.2 μM and 44.1 nmol/mg/min, respectively. Transverse tubule membranes sequentially hydrolyzed inositol trisphosphate to inositol bisphosphate, inositol 1-phosphate and inositol, indicating that these membranes have inositol bis- and monophosphatases in addition to inositol trisphosphatase.

Excitation/contraction coupling; Inositol phosphate; Sarcoplasmic reticulum; Transverse tubule

1. INTRODUCTION

The mechanism of excitation/contraction coupling in skeletal muscle is not known, although mechanical or chemical coupling mechanisms, the latter involving messenger molecules, have been proposed [1]. The mechanical hypothesis states that the voltage sensor molecule in the transverse tubule (T-T) undergoes a conformational change that is transmitted through the feet of the terminal cisternae to activate sarcoplasmic reticulum (SR) calcium release [2]. On the other hand, in analogy to its function in other cell systems, where inositol 1,4,5-trisphosphate (IP_3) plays a role as intracellular messenger coupling external stimuli to calcium release from non-mitochondrial stores [3], IP_3 has been postulated as a chemical messenger linking T-T depolarization to calcium release from SR ([4–7], reviewed in [8]).

This proposal is supported by the following observations: (i) IP_3 induces calcium release [9,10] and contraction [4–9] in skeletal muscle fibers, made permeable by skinning, peeling or rupture; (ii) IP_3 induces calcium release both in intact muscle [11] and in isolated heavy SR vesicles [6,12]; (iii) IP_3 activates calcium channels in SR vesicles fused to planar lipid bilayers [13,14]; (iv) the lipid precursors and enzymes responsible for IP_3 production are present in T-T membranes [15–19]; and (v) electrical stimulation of muscle fibers increases the intracellular IP_3 concentration [4,20] a few milliseconds after stimulation [20].

According to this hypothesis, relaxation of skeletal muscle is dependent on an IP_3 phosphatase (IP_3-ase) that would remove the IP_3 released by electrical stimulation [4]. While some indirect evidence was initially presented supporting this proposal [4], the presence of an IP_3-ase in skeletal muscle has been disputed. Significant IP_3-ase activity was demonstrated in muscle extracts [5] and in isolated membranes [21] by two independent laboratories, but not by others [22]. Milani et al. [21] found the IP_3-ase activity in rabbit skeletal muscle to be membrane bound and also present in soluble form; the highest specific activity was found in a microsomal fraction enriched in T-T membranes.

In this work, we compared the rates of hydrolysis of IP_3 in purified T-T and SR membranes isolated from frog skeletal muscle. We found that there is an IP_3-ase activity localized mainly in T-T membranes, and that in this membrane fraction, the resulting inositol bisphosphate (IP_2) is further hydrolyzed to inositol 1-phosphate (IP) and inositol.

2. MATERIALS AND METHODS

2.1. *Materials*

The chemicals used were purchased from Merck, Sigma Chemical Co. and Fluka. [^{3}H]IP_3 (1 Ci/mmol), [^{3}H]inositol 1,4-bisphosphate (1 Ci/mmol), [^{3}H]inositol 1-phosphate (1 Ci/mmol) and [^{3}H]myoinositol were obtained from Amersham Corp. IP_3 was from Calbiochem.

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2.3. *Isolation of muscle membrane fractions*

Highly purified T-T and SR membranes were isolated from frog (*Caudiverbera caudiverbera*) skeletal muscle as described [23]. Briefly, using differential centrifugation a 100000 × *g* pellet and supernatant were obtained. The microsomes present in the 100000 × *g* pellet were further resolved into SR and T-T membranes by sucrose density gradient fractionation. Protein was measured [24] using bovine serum albumin as standard.

2.3. *IP_3-ase assay*

IP_3-ase activity was measured at 25°C, in a final volume of 0.1 ml of a solution containing (mM): 100 KCl, 50 Hepes-Tris, pH 7.2, 0.5 $MgCl_2$, calcium-adjusted to pCa 6 with 1 EGTA plus 0.91 $CaCl_2$, and 0.05–0.1 mg of protein. The reaction was started by addition of [^{3}H]IP_3 (40000 cpm), diluted with unlabeled IP_3 to a specific activity of 10000 cpm/nmol, and stopped by addition of 0.2 ml of 20% trichloroacetic acid. The samples were centrifuged, the supernatants were extracted 4 times with 1 ml of diethyl ether saturated with water, and were neutralized with NH_4OH prior to further analysis.

2.4. *Separation of [^{3}H]inositol phosphates by Dowex-1 anion-exchange chromatography or HPLC*

[^{3}H]Inositol phosphates were separated on 1.0 ml columns of Dowex 1 x-8 (formate form) as described [25]. The columns were calibrated with a standard mixture of [^{3}H]inositol phosphates. The chloride form of Dowex 1 x-8 was transformed into the formate form with successive washings with 1 N HCl at 100°C, 1 N formic acid at 60°C, 1 M sodium formate, 0.1 M formic acid and water.

HPLC analysis was performed by the method of Irvine et al. [26], using a 25 × 4.6 mm Partisil SAX-10 column (Whatman). Before injection, each sample was mixed with 0.1 μmol each of AMP, ADP and ATP, which were detected by absorption at 260 nm, and served as reference markers. The columns were calibrated with a standard mixture of [^{3}H]inositol phosphates.

3. RESULTS AND DISCUSSION

Measurements of [^{3}H]IP_3 hydrolysis in soluble fractions and in SR and T-T fractions isolated from frog skeletal muscle showed that IP_3-ase activity was ubiquitous. At 25°C and 20 μM [^{3}H]IP_3, the hydrolysis rates of the soluble fraction, SR and T-T membranes were ≤0.1, 1.2 ± 0.1 (2), and 22.0 ± 2.7 (9) nmol/min/mg of protein, respectively. These results indicate that, as has been demonstrated in several cell systems [25], in skeletal muscle the enzyme is mostly present in membrane-bound form. Furthermore, the IP_3-ase activity is highly enriched in the T-T membranes, that displayed about 20-fold higher activity than SR membranes, and a V_{max} value of 44.1 ± 2.2 nmol hydrolyzed per mg of protein per min (Fig. 1). Likewise, in rabbit muscle a membrane fraction containing a mixture of SR and T-T membranes displays higher IP_3 phosphatase activity than SR [20], with a reported V_{max} of about 20 nmol/min/mg of protein at 30°C. These results indicate that, in contrast to other reports [26], skeletal muscle has significant IP_3 phosphatase activity, comparable to that of other tissues [20], and that this enzyme is predominantly localized in the transverse tubule system. Furthermore, the activity of SR is so low that it might reflect residual contamination with T-T membranes.

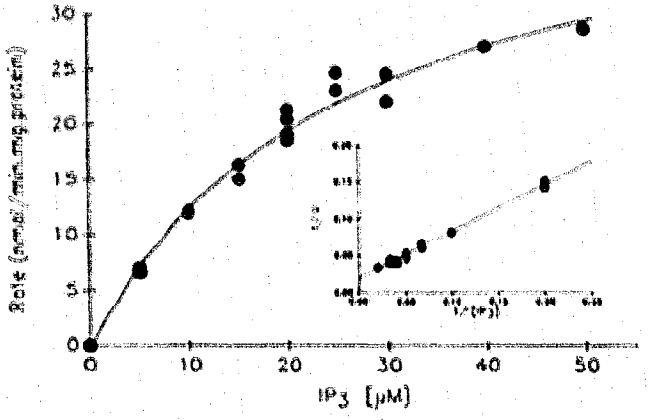

Fig. 1. Hydrolysis rates of isolated transverse tubule membranes as a function of substrate concentration. The amounts of [^{3}H]inositol 1,4,5-trisphosphate hydrolyzed were determined by HPLC for concentrations ≤20 μM, and by separation in Dowex columns for higher concentrations (see section 2).

Milani et al. [21] reported a K_m value for IP_3-ase in rabbit muscle in the range of 15–18 μM. We have measured a somewhat higher K_m value in the T-T fraction, 25.2 ± 2.6 μM for frog muscle (Fig. 1). Both the enzyme present in rabbit [21] as well as the enzyme present in frog skeletal muscle, have an absolute requirement for Mg and are completely inhibited by 0.1 mM $CdCl_2$ (not shown).

We studied the time course of [^{3}H]IP_3 degradation and the appearance of [^{3}H]inositol phosphate derivatives in T-T membranes. We found that [^{3}H]IP_3 hydrolysis led first to the formation of [^{3}H]IP_2, followed later by the sequential appearance of [^{3}H]IP and [^{3}H]inositol; all the initial [^{3}H]IP_3 had been converted to [^{3}H]inositol after 10 min incubation (Fig. 2). In the

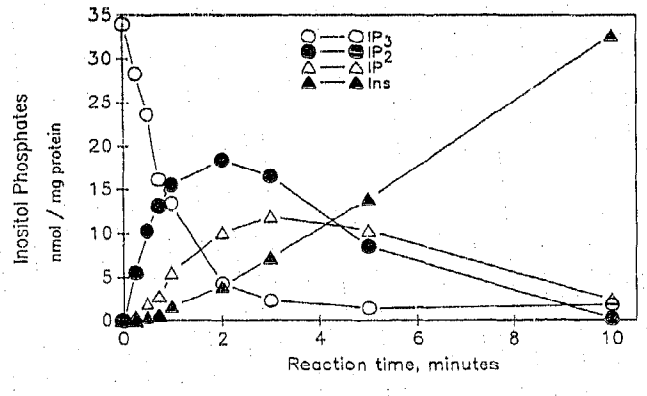

Fig. 2. Time course of hydrolysis of [^{3}H]inositol 1,4,5-trisphosphate by isolated transverse tubule membranes at 20 μM IP_3. To isolate and identify the inositol phosphates, separation in Dowex columns was used (see text). The results represent average values of two different membrane preparations.

case of SR membranes, hydrolysis proceeded at a considerably slower rate (data not shown); even after 20 min incubation there was still a mixture of inositol trisphosphate and bisphosphate, with low but significant amounts of inositol phosphate and inositol. These results demonstrate that T-T membranes possess measurable IP_2 and IP phosphatase activities, in addition to IP_3-ase activity. Our findings contrast with those reported in rabbit skeletal muscle [21], where only IP_3-ase activity was found. This difference might be due to the fact that the membranes used by Milani et al. [21] have lower specific activity than the T-T membranes used in this work, so that the lower amounts of inositol phosphate and inositol produced might have escaped detection.

If we assume (i) that during a twitch the concentration of IP_3 in the triadic space reaches 1 μM (a value higher than the threshold for opening calcium channels at resting p*Ca* [8,28]), (ii) that the volume of the triadic space is about 1.7×10^{-17} liters/μm^2 of T-tubule surface [16], and (iii) that relaxation takes place in 50–200 ms [29], then an IP_3-ase activity in the range of $0.85–3.4 \times 10^{-22}$ mol/s/μm^2 of T-T surface would be required to remove all the IP_3 released. Assuming that there are 10^6 phospholipid molecules per μm^2 of membrane surface [30] and 2 μmol phospholipid per mg of protein [23], these values can be converted to 6–24 nmol/mg of protein per min. The V_{max} rates measured are above this range. At 1 μM IP_3, the physiologically relevant concentration, using HPLC to measure IP_3 hydrolyzed, we determined a rate of hydrolysis of 1.2 ± 0.1 (2) nmol/mg per min, lower than given above but still in a range adequate to account for relaxation. Furthermore, it is possible that the isolated membrane preparations might have lost regulatory factors, i.e. phosphorylation [31], that might increase the activity in vivo. Thus the IP_3-ase activity may be directly involved in the mechanism of physiological relaxation, and unquestionably plays a role in returning the IP_3 levels to the resting values [4,5]. In support of this proposal, it has been reported that microsomes isolated from swine which are susceptible to malignant hyperpyrexia are deficient in IP_3-ase activity, and that the myoplasmic concentrations of IP_3 and calcium are higher than in normal swine [32]; these results were interpreted as indicative that the raised myoplasmic calcium ion concentration of hyperexic muscle is caused by the deficiency in IP_3-ase activity.

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