Soluble phosphatidylinositol phosphodiesterase in normal and denervated fast and slow muscles of the rat

Janis K. SHUTE and Margaret E. SMITH

Department of Physiology, Medical School, University of Birmingham, Birmingham B15 2TJ, U.K.

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Phosphatidylinositol phosphodiesterase activity was determined in cytosol prepared from rat slow (soleus) and fast (extensor digitorum longus) muscles. The substrate was prepared by incubation of sarcoplasmic reticulum with *myo*-[2-³H]inositol. The enzyme hydrolysed both membrane-bound and extracted phosphatidylinositol. The activity determined with the isolated phospholipid exhibited an optimum at pH 5.5. Ca²⁺ ions stimulated the activity. The enzyme specific activity was higher in cytosol prepared from soleus muscle than in that from extensor digitorum longus muscle. After section of the motor nerve, the activity of the enzyme increased in both muscles up to 36 h and then declined. A function for this enzyme in the control of acetylcholine sensitivity in muscle is discussed.

In our laboratory it was shown that the acetylcholine contracture and membrane-depolarization responses of intact rat skeletal muscles were increased by incubation of the muscles with purified bacterial phospholipases of the C type or with muscle cytosol (Watson *et al.*, 1976; Harborne *et al.*, 1978, 1984). One of these phospholipases was specific for PtdIns as substrate. Incubation of isolated sarcolemmal membranes with the purified enzymes or with muscle cytosol (Smith *et al.*, 1983) increased the capacity of the membranes to bind ¹²⁵I- α -bungarotoxin. The latter toxin binds irreversibly to the nicotinic acetylcholine receptor on skeletal muscles (Chang & Lee, 1963). The effect of the cytosol or enzymes was therefore probably to increase the number of 'available' acetylcholine receptors on the muscle or the sarcolemmal membranes. It is therefore possible that the acetylcholine receptor on skeletal muscle is associated with PtdIns in the plasma membrane. In the present study an attempt was made to see whether soluble PtdIns phosphodiesterase activity could be demonstrated in skeletal muscles of the rat. The muscles examined were the slow soleus, which exhibits acetylcholine receptors over its entire surface membrane, and the fast EDL, in which the receptors are confined to the endplate area (Miledi & Zelenka, 1966). After section of the motor nerve, skeletal muscles become hypersensitive to acetyl-

choline over their entire surface membrane (Albuquerque & McIsaac, 1970). Therefore denervated muscles were also examined for PtdIns phosphodiesterase activity. The substrate used was [³H]PtdIns, which had been synthesized on sarcoplasmic reticulum and which therefore probably resembled the endogenous substrate for the enzyme. The activity was determined with both membrane-bound and extracted PtdIns. Some of the properties of the enzyme are described. A brief report of part of this work has been published (Shute & Smith, 1983).

Methods

Surgical procedure

The lower hindlimb muscles of female Sprague-Dawley rats (200–250 g body wt.) were denervated unilaterally by sciatic-nerve section, high in the thigh. Diethyl ether anaesthesia was used and aseptic precautions were maintained.

Preparation of cytosol

The rats were killed by cervical dislocation, and the soleus and EDL muscles were carefully dissected out and minced on ice. The minced muscle was homogenized at 4°C in 0.3 M-sucrose/0.01 M-Tris/HCl buffer, pH 7.4, in a ratio of 1 g of muscle to 4 ml of buffer solution, with a Polytron (type PT 20 0D) homogenizer at setting 6. Homogenization was for 4 × 15 s periods with 30 s intervals between bursts. The homogenate was

Abbreviations used: PtdIns, phosphatidylinositol; EDL, extensor digitorum longus.

centrifuged at 105000g for 1 h in an MSE Super-speed 50 centrifuge, and the supernatant cytosol was decanted.

Phospholipase C determinations

The enzyme substrate was PtdIns, which had been synthesized on sarcoplasmic reticulum by the exchange reaction of Paulus & Kennedy (1960). Sarcoplasmic reticulum was prepared from the lower hindlimb muscle (mixed) of adult male Sprague-Dawley rats (300 g body wt.) by the method of Martonosi & Feretos (1964). The final membrane pellet was suspended in 0.3 M-sucrose/0.1 M-Tris/HCl, pH 7.4, at a concentration equivalent to 2 g original wt. of muscle/ml. The preparation (4.5 ml) was incubated immediately at 37°C for 1 h in a total volume of 5.0 ml containing 10 mM-Tris/HCl, pH 7.4, 1 mM-MnCl₂ and 30 μ Ci of *myo*-[2-³H]-inositol (sp. radioactivity 9.3–16.9 Ci/mmol). The reaction was stopped by dilution with 30 ml of ice-cold 100 mM-Tris/HCl, pH 7.4, and the labelled membranes were recovered by centrifugation at 28000g for 60 min. The labelled membrane preparation was washed three times by centrifugation and resuspension in 100 mM-Tris/HCl, pH 7.4. The final pellet was suspended in 0.3 M-sucrose/0.01 M-Tris/HCl, pH 7.4, at a concentration equivalent to 2 g original wt. of tissue/ml. This suspension was either stored at –20°C in small batches or used to prepare isolated [³H]PtdIns. In the latter case the [³H]PtdIns was extracted from the sarcoplasmic reticulum and purified on an alumina column by the method of Long & Owens (1962). The purified phospholipid was diluted with unlabelled PtdIns, which had been extracted and purified in the same way from whole muscle homogenates. The organic solvents were then removed *in vacuo* to leave an aqueous suspension of [³H]PtdIns (approx. 15–25 mM, sp. radioactivity 0.02–0.05 Ci/mol).

When the enzyme activity was determined with the membrane-bound substrate, the incubation mixture contained, unless otherwise stated, sarcoplasmic-reticulum protein (800 μ g/ml), sodium deoxycholate (250 μ g/ml), CaCl₂ (7.2 mM), Tris/maleate buffer, pH 7.4 (50 mM), and cytosol protein in a concentration of 400 μ g/ml. When the isolated PtdIns was used, it was incubated (unless otherwise stated) in a concentration of 1.4 mM with CaCl₂ (7.2 mM), Tris/maleate buffer, pH 5.5 (50 mM), and cytosol protein (100 μ g/ml). In both cases the final incubation volume was 0.5 ml, and incubations in duplicate were for 20 min at 37°C. Controls containing no enzyme were included for each set of test conditions. The reaction was stopped by addition of 2.0 ml of chloroform/methanol/1 M-HCl (500:500:3, by vol.), and the tubes were placed on ice. The solution was thoroughly mixed and the phases separated by centrifugation

(6000g). Samples (400 μ l) of the upper phase were added to FisoFluor 1 (4 ml) and counted for radioactivity in a liquid-scintillation counter. Quench corrections were made by the external-standard-ratio method. The water-soluble reaction products were identified by Dowex ion-exchange chromatography as described by Ellis *et al.* (1963), followed by high-voltage electrophoresis as described by Dawson & Clarke (1972).

Other determinations

Protein was determined by the biuret method (Weichselbaum, 1946) and lactate dehydrogenase by the method of Wroblewski & LaDue (1955).

Materials

myo-[2-³H]Inositol was obtained from Amersham International, Amersham, Bucks, U.K., and oleic acid and sodium deoxycholate were obtained from Sigma. FisoFluor 1 was obtained from Fisons Scientific Apparatus.

Results

PtdIns phosphodiesterase activity with isolated or membrane-bound PtdIns

Fig. 1(a) shows that when [³H]PtdIns was used as substrate for PtdIns phosphodiesterase the reaction rate reached a plateau at concentrations above 1.2 mM. However, with some preparations of the substrate the plateau was not reached until higher concentrations (up to 2.4 mM). This variation could have reflected differences in the size, shape or lamellar structure of the phospholipid liposomes in different preparations, or the presence of variable amounts of a minor non-radioactive contaminant.

The rate of reaction increased linearly with increasing amounts of cytosol up to 100 μ g of protein/ml. At the latter concentration the rate of reaction was constant for at least 20 min.

The water-soluble products of the reaction were *myo*-inositol 1,2-cyclic phosphate, *myo*-inositol 1-phosphate and glycerophosphoinositol, in the molar proportions approx. 2:1:1.

In other experiments sarcoplasmic-reticulum-bound PtdIns was used as substrate for the enzyme. The membrane preparation exhibited negligible lactate dehydrogenase activity, even in the presence of Triton X-100 (0.1%). Thus entrapment of soluble enzymes within the microsomal vesicles was negligible. Furthermore, no hydrolysis of the endogenous membrane [³H]PtdIns occurred in either the absence or the presence of Ca²⁺ unless the soluble enzyme was present.

Fig. 1(b) shows that the reaction rate with the membrane substrate was maximum at concentrations of the membranes above 100 μ g of protein/

Fig. 1. *Hydrolysis of PtdIns by soleus muscle cytosol* PtdIns phosphodiesterase activity was determined (a) with isolated PtdIns as substrate, at pH 5.5, in the presence of CaCl_2 (7.2 mM), and (b) with membrane-bound PtdIns as substrate, at pH 7.4, in the presence of CaCl_2 (7.2 mM) and sodium deoxycholate (125 μ g/incubation). The membrane additions indicated are per incubation. The results are typical of four experiments in each case.

incubation mixture. The reaction rate increased linearly with addition of cytosol up to 200 μ g of protein/incubation. At this concentration the rate was constant for at least 20 min. The major water-soluble product under these conditions was inositol 1-phosphate.

Effect of deoxycholate

When the membrane-bound substrate was used, enzyme activity could only be detected if sodium

Fig. 2. *Effect of sodium deoxycholate on PtdIns hydrolysis by soleus-muscle cytosol*

PtdIns phosphodiesterase activity was determined at pH 7.4 with isolated PtdIns ($\square$) or membrane-bound PtdIns ($\circ$) as substrate. The deoxycholate additions indicated are per incubation. The results are typical of six experiments in each case.

deoxycholate was present in the incubation mixture (Fig. 2). In the presence of 250 μ g of deoxycholate/incubation, precipitation was evident. Incubations routinely contained 125 μ g of deoxycholate. Irvine & Dawson (1978) observed a similar dependence on sodium deoxycholate for the activity of soluble PtdIns phosphodiesterase with membrane-bound substrate in rat brain.

Lapetina & Michell (1973) suggested that the detergent (at 2 mg/ml) stimulated PtdIns breakdown in rat cerebral-cortex homogenates via dissociation of endogenous PtdIns and membrane proteins. In the present experiments, however, the action of sodium deoxycholate at the concentrations used (250 μ g/ml) was unlikely to be due to dissociation of the substrate, since after sedimentation of labelled membranes that had been incubated with sodium deoxycholate (250 μ g/ml) no [^3H]PtdIns could be detected in the supernatant. Furthermore, electron micrographs of microsomal membranes incubated with this concentration of deoxycholate showed that the membranes were structurally intact.

When the isolated substrate was used, the activity at neutral pH, in the absence of sodium deoxycholate, was considerable and sodium deoxycholate (125 μ g/incubation) caused only a slight stimulation of the activity (Fig. 2). Higher concentrations of the detergent were inhibitory. These results are in contrast with those of Irvine & Dawson (1978), who observed a marked stimulation of the activity with sodium deoxycholate.

Effect of oleic acid

Oleic acid was added to both the purified and the membrane-bound substrates as small batches of a

Fig. 3. Effect of oleic acid on PtdIns hydrolysis by soleus-muscle cytosol

PtdIns phosphodiesterase activity was determined at pH 7.4 with isolated PtdIns ($\square$) or membrane-bound PtdIns ($\circ$) as substrate, in the absence of sodium deoxycholate. The results are typical of two experiments in each case.

3 mM solution in chloroform. The chloroform was removed under a stream of O_2/CO_2 (19:1) before addition of the other components of the incubation mixture. When membrane-bound substrate was treated with oleic acid in this way, and then centrifuged for 1 h at 28000g, there was no radioactivity in the supernatant. The chloroform was not therefore solubilizing the substrate.

Fig. 3 shows that oleic acid caused a marked stimulation of the enzyme activity when the membrane-bound substrate was used. This was at concentrations one order of magnitude lower than those reported to stimulate the hydrolysis by brain cytosol of PtdIns bound to rat liver microsomal fractions (Irvine *et al.*, 1979). Oleic acid (60 nmol/incubation) caused a slight stimulation of enzyme activity with the isolated PtdIns as substrate. This contrasts with the 8-fold stimulation of the activity of the enzyme from rat brain by oleic acid at this concentration seen by other workers (Irvine *et al.*, 1979).

Metal-ion dependency

The enzyme activity determined with either the purified substrate (Fig. 4a) or the membrane-bound substrate (Fig. 4b) was stimulated by Ca^{2+} ions. The effect was specific for this bivalent cation, in that $CaCl_2$ could not be replaced by $MnCl_2$, $MgCl_2$ or $ZnCl_2$.

Fig. 4(a) shows that the activity of the enzyme in cytosol prepared from soleus-muscle cytosol was greater than that in EDL-muscle cytosol. The enzyme from both sources displayed optimum activity with concentrations of $CaCl_2$ higher than 4 mM when the purified substrate was used. When the membrane-bound substrate was used (Fig. 4b), the enzyme in soleus-muscle cytosol showed a similar requirement for Ca^{2+} .

Fig. 4. Effect of Ca^{2+} on the activity of PtdIns phosphodiesterase in muscle cytosol

(a) The activity in soleus ($\bullet$) and EDL ($\circ$) muscle cytosol was determined at pH 5.5 with the isolated PtdIns substrate. (b) The activity in soleus cytosol was determined at pH 7.4 with the membrane-bound substrate in the presence of sodium deoxycholate. The results are typical of six experiments in each case.

Effect of pH

Fig. 5(a) shows the pH-activity curve for PtdIns phosphodiesterase in soleus and EDL cytosols with isolated PtdIns as substrate. Two pH optima were apparent, at pH 5.5 and pH 7.4. Soleus cytosol was more active than EDL cytosol at the pH optima. Fig. 5(b) shows the pH-activity curve obtained when the membrane-bound substrate was used to

Fig. 5. Effect of variation in pH on PtdIns phosphodiesterase activity in muscle cytosol

(a) Activity in cytosol prepared from soleus (●) and EDL (○) muscles was determined at pH 5.5 with the isolated PtdIns substrate. (b) Activity in cytosol from soleus muscle was determined at pH 7.4 with the membrane-bound PtdIns substrate in the presence of sodium deoxycholate. The results are typical of three experiments in each case.

assay the enzyme activity in soleus cytosol. Again two optima were indicated, one at pH 7.8 and another at a more acid pH value. However, with respect to the latter, the detergent in the incubation mixture was precipitated at pH values below 6.2,

Fig. 6. Effect of motor-nerve section on the activity of PtdIns phosphodiesterase in muscle cytosol

The enzyme activity was determined in soleus (●) and EDL (○) muscle cytosols at various times after denervation. Values marked * are increased significantly ($P < 0.05$; Student's t test) above those obtained for normally innervated muscles (zero-time values). The enzyme activity was determined at pH 5.5 with the isolated PtdIns substrate. Each value represents the mean of results from at least three animals at each time after denervation. The bars represent $\pm$ S.D.

which prevented the enzyme from being activated at the lower pH values under these conditions.

Effect of motor-nerve section

Fig. 6 shows the activity of PtdIns phosphodiesterase in soleus and EDL muscle cytosols at various times after section of the motor nerve. The activity increased in cytosol from either muscle, up to 36 h after motor-nerve section. Thereafter the activity declined. The activity in the soleus cytosol was higher than that in EDL cytosol in each series of experiments.

Discussion

PtdIns phosphodiesterase activity was demonstrated in skeletal-muscle cytosol. The properties of this enzyme were similar in many respects to those described for soluble PtdIns phosphodiester-

ases in other mammalian tissues (for a review see Shukla, 1982).

Substrates

PtdIns endogenous to skeletal muscle was used to determine the enzyme activity. Appreciable enzyme activity was detected with the isolated PtdIns substrate in the absence of deoxycholate or oleic acid, and these substances only caused a slight stimulation of the enzyme activity. This is in contrast with the results obtained by other workers, who used yeast and soya-bean PtdIns (Irvine & Dawson, 1978; Irvine *et al.*, 1979) as substrate. Arachidonic acid stimulates PtdIns hydrolysis (Irvine *et al.*, 1979), and the results may therefore reflect the high proportion of arachidonic acid in mammalian (White, 1973), but not in yeast (Trevelyan, 1966) or soya-bean (Galliard, 1973), PtdIns.

Sodium deoxycholate (250 µg/ml) stimulated the enzyme activity when the membrane-bound substrate was used. Since PtdIns has been assigned to the inner surface of membrane vesicles (Nilsson & Dallner, 1977), the effect of deoxycholate was possibly to render the membrane vesicles more permeable to the enzyme, thereby facilitating PtdIns hydrolysis.

Products

The water-soluble products detected when the isolated PtdIns was used as substrate at pH 5.5 were inositol monophosphate, *myo*-inositol 1,2-cyclic phosphate and glycerophosphoinositol. When the membrane-bound PtdIns was used as substrate for the enzyme, at pH 7.4, the only product detected was inositol monophosphate. As glycerophosphoinositol is not hydrolysed at this acid pH value (Dawson & Hemington, 1977), the inositol phosphates were the products of PtdIns phosphodiesterase activity.

pH optima at both 5.5 and 7.4 were seen when the enzyme activity was determined with the isolated substrate. Two pH optima have been reported for PtdIns phosphodiesterase in other tissues (Shukla, 1982). When the membrane-bound substrate was used with the muscle cytosol enzyme, two pH optima were again indicated. In this case, however, the relative activities of the enzyme at acid and neutral pH values simply reflected the fact that sodium deoxycholate precipitated out of the incubation medium at pH values below 6.2.

Effect of Ca^{2+}

The Ca^{2+} -dependency of soluble PtdIns phosphodiesterase in skeletal muscle resembles that for such enzymes in other tissues (Shukla, 1982). Millimolar concentrations of Ca^{2+} were required for maximum stimulation of the enzyme activity,

whether the isolated or the membrane-bound substrate was used. This may partially reflect binding of Ca^{2+} to the acidic phospholipid substrate (Hauser & Dawson, 1968), which would decrease the available free Ca^{2+} in the incubation medium. However, Ca^{2+} bound in this way would be displaced by H^+ between pH 3.0 and 6.5 (Quinn & Dawson, 1972). The effect of Ca^{2+} on the enzyme activity is therefore probably a direct effect on the enzyme rather than an indirect effect on the substrate.

Intracellular concentrations of Ca^{2+} in muscle are low (0.1 µM) and rise to only approx. 10 µM in skeletal-muscle cells during contraction (Schaub & Watterson, 1981). Hence the enzyme is unlikely to be active inside the cell under physiological conditions. However, it was shown in previous work (Harborne *et al.*, 1984) that this enzyme leaks from muscles incubated *in vitro* and that the medium containing the released enzyme increases the acetylcholine sensitivity of intact muscles. Furthermore, purified bacterial phospholipases of the C type mimicked the effect of the medium (Harborne *et al.*, 1978, 1984). It is therefore possible that the enzyme can act under certain circumstances in the extracellular fluid, where the Ca^{2+} concentration is approx. 2.0 mM.

Fast and slow muscles

The specific activity of PtdIns phosphodiesterase was higher in cytosol prepared from the slow soleus muscle than in that from the fast EDL muscle. This difference was observed in determinations under saturating conditions with respect to Ca^{2+} and substrate concentrations. Interestingly, the soleus muscle is more sensitive to acetylcholine than is EDL.

Effect of denervation

The activity of PtdIns phosphodiesterase in both soleus and EDL muscles 24–36 h after denervation was significantly greater than in normally innervated muscles from rats of the same body weight. The increase in enzyme activity occurred at a time when muscles undergo the most rapid increase in chemosensitivity (Jones & Vrbova, 1974; Harborne & Smith, 1982). It is therefore possible that the enzyme is involved in the control of acetylcholine sensitivity at this early time after denervation. Such a mechanism has been proposed for the early appearance of acetylcholine receptors after denervation of rat diaphragm (Olek & Robbins, 1981).

General comments

The hydrolysis of inositol phospholipids accompanies the interaction of membrane receptors with a variety of hormones and neurotransmitters in

many tissues (for a review see Michell *et al.*, 1981). It has been suggested, however (Hawthorne & Pickard, 1979), that triphosphoinositide (PtdIns 4,5-bisphosphate) breakdown is the primary response to receptor stimulation. It is unclear whether the same enzyme can hydrolyse both PtdIns and polyphosphoinositides. Bovine myocardial PtdIns phosphodiesterase has been isolated in several forms, each capable of hydrolysing both PtdIns and polyphosphoinositides (Low & Weglicki, 1983). However, Downes & Wusteman (1983) reported a separate phosphodiesterase in rat parotid glands which degrades polyphosphoinositides. It is not known whether skeletal muscle contains a phosphodiesterase which is specific for PtdIns. Soleus-muscle cytosol fraction has also been shown to degrade polyphosphoinositide, and this activity was also stimulated by Ca^{2+} (J. K. Shute & M. E. Smith, unpublished work). Thus the possibility that polyphosphoinositide may be the physiological substrate cannot be ruled out. However, it is noteworthy that the PtdIns-specific enzyme isolated from *Staphylococcus aureus*, which mimicked the action of muscle fractions which contained PtdIns phosphodiesterase, does not hydrolyse polyphosphoinositides (Shukla, 1982).

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