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STUDIES ON THE PROPERTIES OF TRIPHOSPHOINOSITIDE PHOSPHOMONOESTERASE AND PHOSPHODIESTERASE OF RABBIT IRIS SMOOTH MUSCLE

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Summary

The rabbit iris smooth muscle has been shown to contain triphosphoinositide phosphomonoesterase (phosphatidyl-*myo*-inositol-4,5-bisphosphate phosphohydrolase, EC 3.1.3.36) and phosphodiesterase (triphosphoinositide inositoltrisphosphohydrolase, EC 3.1.4.11) activities. Under our experimental conditions about 77% of the phosphomonoesterase and 61% of the phosphodiesterase activities were localized in the particulate fraction. The kinetic properties of the enzymes in the microsomal fraction were examined. The enzyme preparation was specific to polyphosphoinositides; it did not attack phosphatidylinositol under the present assay condition. The effects of Ca^{2+} and Mg^{2+} were also studied. Although the microsomal enzymes did not require added divalent cations for their activities, both the phosphomonoesterase and phosphodiesterase were appreciably inhibited by 1 mM EDTA. Phosphodiesterase and phosphomonoesterase were stimulated by Ca^{2+} and Mg^{2+}, respectively.

The demonstration of triphosphoinositide phosphodiesterase in the iris muscle, coupled with the findings that this enzyme is activated by Ca^{2+} and is not influenced by acetylcholine add further support to our previous conclusion (J. Pharmacol. Exp. Ther. (1978) 204, 655—668; J. Neurochem. (1978) 30, 517—525) that an increased Ca^{2+} influx, following the interaction between the neurotransmitter and its receptor, could act to stimulate the phosphodiesterase, thus leading to increased triphosphoinositide breakdown and increased phosphatidic acid via increased diacylglycerol.

Abbreviations: triphosphoinositide, phosphatidylinositol 4,5-bisphosphate; diphosphoinositide, phosphatidylinositol 4-phosphate; EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*′-tetraacetic acid.

Introduction

Kai et al. [1], in 1968, compared the rates of synthesis and breakdown of triphosphoinositide with corresponding figures for acetylcholine. They observed that rates are of the same order in each case and that hydrolysis is more than 100 times as rapid as synthesis. In a later report these authors [2] concluded that while there is no evidence that triphosphoinositide can function as a transmitter like acetylcholine in the nerve, the enormous potential for its destruction is likely to be of physiological significance. Previously we reported that the neurotransmitters acetylcholine [3] and norepinephrine [4] at 0.05 mM and at short time intervals (<10 min) increase significantly the breakdown of triphosphoinositide in rabbit iris smooth muscle which was prelabeled with $^{32}P_i$. This 'triphosphoinositide effect' in response to acetylcholine and norepinephrine was blocked by atropine and phentolamine, respectively, thus indicating that this phenomenon is mediated through cholinergic muscarinic and α-adrenergic receptors. More recently we reported that the triphosphoinositide effect in response to acetylcholine [5] and norepinephrine [6] is dependent upon the presence of Ca^{2+} in the incubation medium. Based on these findings we suggested the sequence of events at the postsynaptic membrane as shown in Eqn. 1 below [4–6]:

$$\begin{aligned}&\text{Neurotransmitter} + \text{Receptor} \rightarrow \text{Neurotransmitter} \cdot \text{Receptor Complex}\\&\quad\rightarrow Ca^{2+}\text{ influx} \uparrow \rightarrow \text{Triphosphoinositide breakdown}\\&\quad\rightarrow \text{Changes in cation permeability} \rightarrow \text{Muscle response}\end{aligned} \qquad (1)$$

There are two enzymes involved in the catabolism of triphosphoinositide in animal tissues, namely triphosphoinositide phosphomonoesterase and phosphodiesterase [2]. The problem arose as to which of the two enzymes is stimulated by Ca^{2+}, and thus could be involved in the mechanism(s) underlying the neurotransmitter-stimulated breakdown of triphosphoinositide in the iris smooth muscle (Eqn. 1).

While there have been a number of studies on the properties and subcellular distribution of triphosphoinositide phosphomonoesterase and phosphodiesterase in brain [7–12], kidney [13–16], erythrocytes [17] and the Protozoan, Crithidia fasciculata [18], none have appeared on those of smooth muscle. Lack of information on these two enzymes in smooth muscle, coupled with the fact that their ionic requirements vary from tissue to tissue [7–18] prompted the present study in the iris muscle.

Materials and Methods

Materials

Diphosphoinositide and triphosphoinositide were prepared from bovine brain. A crude phosphoinositide fraction was prepared according to the method of Folch [19] and diphosphoinositide and triphosphoinositide were isolated by means of DEAE-cellulose column chromatography (Whatman DE-52, microgranular), as described by Hendrickson and Ballou [20]. This preparation gave one spot in two-dimensional thin-layer chromatography [3]. Phosphatidylinositol, 1,2-dipalmatin and cetyltrimethylammonium bromide were obtained

from Sigma Chemical Co., St. Louis, Mo. All other chemicals were of reagent grade.

Methods

Preparation of subcellular fractions from the rabbit iris muscle. Methods of homogenization, subcellular fractionation and monitoring of the purity of the preparations by means of electron microscopy and enzyme markers were essentially as was previously reported [21]. Briefly, the rabbit irises (approx. 4—6 g wet wt.) were rinsed with ice-cold buffered saline (pH 7.4). The muscle strips were blotted and immersed into chilled 0.25 M sucrose (pH 7.4) then minced with scissors. The minced tissue was then suspended in 10 vols. buffered 0.25 M sucrose and homogenized for 4 × 30 s using a Super Dispax Tissumizer Model SDT-182 (Takmar Co.) at maximum speed. The homogenate was filtered through two layers of cheese cloth, then centrifuged at 600 × *g* for 10 min in a refrigerated centrifuge. The nuclear pellet, which contained a considerable amount of cell debris was discarded. The supernatant was centrifuged at 10 000 × *g* for 30 min and the mitochondrial fraction, which was contaminated with non-mitochondrial membraneous fragments, was discarded. The supernatant obtained was then centrifuged at 110 000 × *g* for 90 min to give a fairly purified microsomal fraction [21] and a soluble fraction. The microsomal fraction was dialysed overnight against several changes of 10 mM Tris · HCl buffer (pH 7.4) to remove any inorganic phosphate present. In experiments where the aim was to investigate the effects of divalent cations on enzyme activity, the microsomal fraction was dialysed successively against 10 mM Tris · HCl buffer containing 1 mM EDTA or 1 mM EGTA and finally against buffer alone. This microsomal preparation was used in the following studies on the properties of triphosphoinositide phosphomonoesterase and phosphodiesterase.

To obtain a soluble and particulate fraction the homogenate was centrifuged at 110 000 × *g* for 90 min.

Assay of triphosphoinositide phosphomonoesterase and phosphodiesterase. Triphosphoinositide phosphomonoesterase, which hydrolyzes triphosphoinositide to diphosphoinositide and inorganic phosphate, was assayed by the method of Dawson and Thompson [7] in which the release of inorganic phosphate was taken as a measure of phosphomonoesterase activity. The reaction mixture contained 45 mM Tris · HCl buffer (pH 7.2), 1 mM $MgCl_2$, 0.6 mM triphosphoinositide, 0.72 mM cetyltrimethylammonium bromide, and 150—175 μg of the enzyme protein in a final volume of 0.5 ml. The reactants were equilibrated at 37°C for 5 min and the reaction started by the addition of a mixture of triphosphoinositide and cetyltrimethylammonium bromide. After 20 min incubation the reaction was stopped by the addition of 0.1 ml 5% (w/v) bovine serum albumin and 0.5 ml 10% (w/v) ice-cold $HClO_4$. The contents of the tubes were thoroughly mixed and kept in ice for 10 min, then centrifuged for 20 min in a refrigerated centrifuge and the clear supernatant analysed for inorganic phosphate according to the method of Bartlett [22].

Triphosphoinositide phosphodiesterase, which hydrolyzes triphosphoinositide to 1,2-diacylglycerol and inositol triphosphate, was assayed according to the method of Thompson and Dawson [8] in which the release of organic phosphate was taken as a measure of phosphodiesterase activity. Assay conditions

were the same as those used for the phosphomonoesterase except that $MgCl_2$ was omitted from the reaction mixture. Omission of Mg^{2+} was found to be necessary in order to obtain maximal phosphodiesterase activity. After 20 min incubation the reaction was stopped as described for the phosphomonoesterase assay. For estimation of total phosphate 0.25 ml supernatant was mixed with 0.15 ml 72% (w/v) $HClO_4$ and the mixture heated at 180° C for 1 h. Following dilution of the contents to 1 ml with water the phosphate was measured by the method of Bartlett [22]. Release of organic phosphate was calculated by subtracting inorganic phosphate from the total phosphate. Blank samples of substrate alone and enzyme alone released virtually no inorganic or organic phosphate when incubated under the same experimental conditions.

Protein was estimated by the method of Lowry et al. [23] using bovine serum albumin as a standard.

Qualitative analysis of 1,2-diacylglycerol as a product of triphosphoinositide hydrolysis. In order to show that hydrolysis of triphosphoinositide by the microsomal fraction yields 1,2-diacylglycerol instead of glycerolphosphorylinositol diphosphate, the lipid products and the remaining substrate were extracted with $CHCl_3/CH_3OH/HCl$ (400 : 200 : 1.5, v/v) and chromatographed on a silica gel-impregnated filter paper (Whatman SG-81) as described by Tou et al. [15]. Substrate and enzyme blanks accompanied each experiment. The chromatograms were dried for 30 min and the lipids detected by 0.015% Rhodamine 6G. The lipid spots on the chromatograms were then examined under an ultraviolet lamp.

Results

Distribution of triphosphoinositide phosphomonoesterase and phosphodiesterase in the rabbit iris muscle

A number of methods have been employed in the past for homogenization and subcellular fractionation of various tissues [24], however, none of these have been applied satisfactorily to smooth muscle. This could be due to the fact that smooth muscle cells, including the iris, are enmeshed in collagen and are quite difficult to homogenize in aqueous media with the conventional Teflon-glass homogenizers. Previously we reported that by using a Super Dispax Tissuemizer to disrupt the iris muscle we were able to prepare a fairly purified microsomal preparation [21]. The purity of the microsomal fraction was ascertained by means of electron microscopy and enzyme markers [21]. In contrast the nuclear and mitochondrial fractions were heavily contaminated. Therefore, in the present studies we used the microsomal fraction to investigate the properies of triphosphoinositide phosphomonoesterase and phosphodiesterase.

As can be seen from Table I about 77% of the total activity of triphosphoinositide phosphomonoesterase and 61% of that of the phosphodiesterase were recovered in the particulate fraction. However more than 22% of the total activity of the former and 39% of that of the latter were recovered in the soluble fraction. The specific activities of both enzymes were highest in the microsomal fraction. In general, the specific activity of the triphosphoinositide phosphodiesterase was 3—4 times as high as that of the phosphomonoesterase.

TABLE I

DISTRIBUTION OF TRIPHOSPHOINOSITIDE PHOSPHOMONOESTERASE AND PHOSPHODIESTERASE

Homogenization of the iris muscle and preparation of subcellular fractions were the same as described under Methods. The enzyme assay consisted of 45 mM Tris · HCl (pH 7.2), 0.6 mM triphosphoinositide, 0.72 mM cetyltrimethylammonium bromide and 160 μg of protein from the various subcellular fractions in a final volume of 0.5 ml. The phosphomonoesterase assay contained in addition 1 mM $MgCl_2$. The reaction was started by addition of a mixture of triphosphoinositide and cetyltrimethylammonium bromide and incubation continued for 20 min at 37°C. Inorganic and organic phosphates released during the reaction were determined.

Fraction	Protein (percent of homogenate)	Phosphomonoesterase		Phosphodiesterase	
		Specific activity *	Percent of total activity	Specific activity **	Percent of total activity
Homogenate	100	3.6	100	20.3	100
110 000 X *g* supernatant (soluble) ***	43.7	1.9	22.8	20.9	39.0
110 000 X *g* precipitate (particulate) ***	56.3	3.8	77.2	20.8	61.0
Microsomal		5.8	—	21.9	—

* nmol inorganic phosphate released/mg protein per min.

** nmol organic phosphate released/mg protein per min.

*** Obtained by centrifuging the homogenate at 110 000 X *g* for 90 min.

Nature of enzymic reactions

In the present study the release of organic and inorganic phosphates were taken as a measure of triphosphoinositide phosphodiesterase and phosphomonoesterase activities, respectively, in the microsomal fraction. Qualitative analysis of the lipid products in the reaction mixture by chromatography on a silica gel-impregnated filter paper revealed an increase in the release of 1,2-diacylglycerol from triphosphoinositide in the presence of the microsomal fraction (unpublished data), thus suggesting a phosphodiesteratic cleavage of triphosphoinositide under these conditions. Furthermore, when the lipid products in the reaction mixture were separated by means of two-dimensional thin-layer chromatography [3] there was a decrease in the concentration of triphosphoinositide and an increase in that of diphosphoinositide, thus indicating a phosphomonoesteratic cleavage of triphosphoinositide by the microsomal fraction.

Properties of the microsomal triphosphoinositide phosphomonoesterase and phosphodiesterase

Effect of substrate. The effect of various concentrations of triphosphoinositide on the enzyme activities were tested under standard conditions. The plots of triphosphoinositide phosphomonoesterase and phosphodiesterase as a function of triphosphoinositide concentration are shown in Figs. 1A and 1B, respectively. The activities of both enzymes increased as a function of substrate concentration and reached a maximum at about 0.45 mM. A Lineweaver-Burk plot of the data in Fig. 1 gave K_m values for triphosphoinositide phosphomonoesterase and phosphodiesterase of 90 and 120 μM, respectively (Figs. 1A and Fig. 1B). The V values for triphosphoinositide phosphomonoesterase and phosphodiesterase were 12.1 and 38.5 nmol phosphate released/mg per min, respec-

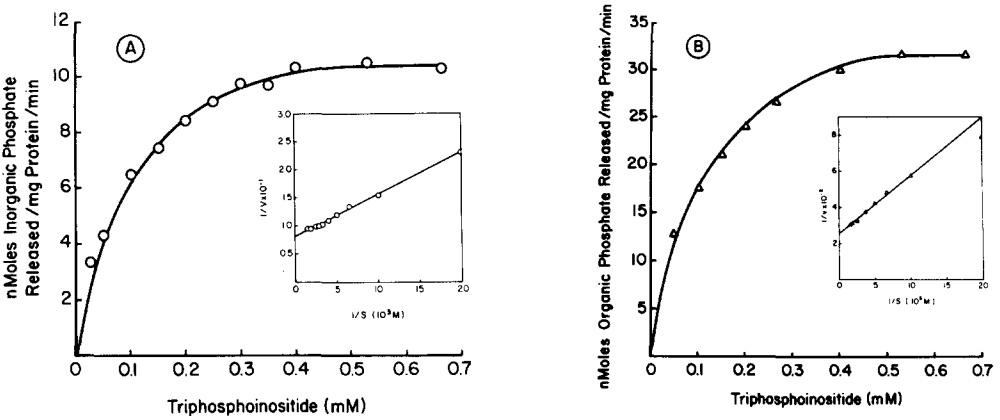


Fig. 1. (A) Triphosphoinositide phosphomonoesterase (○) and (B) phosphodiesterase (△) activities as a function of substrate concentration. The assay mixture consisted of 45 mM Tris · HCl (pH 7.2), 0.72 mM cetyltrimethylammonium bromide, 150 μg microsomal protein and various concentrations of triphosphoinositide as indicated in a final volume of 0.5 ml. The phosphomonoesterase assay contained in addition 1 mM $MgCl_2$. The reaction was started by addition of a mixture of triphosphoinositide and cetyltrimethylammonium bromide and incubation continued for 20 min at 37°C. Inorganic and organic phosphates released during the reaction were determined.

tively. In the presence of the detergent cetyltrimethylammonium bromide (at a detergent : triphosphoinositide molar ratio of 1.25) there was a maximal increase of triphosphoinositide phosphomonoesterase and phosphodiesterase activities of 22 and 14% of control, respectively (unpublished data). Higher concentrations of the detergent inhibited the triphosphoinositide phosphodiesterase appreciably.

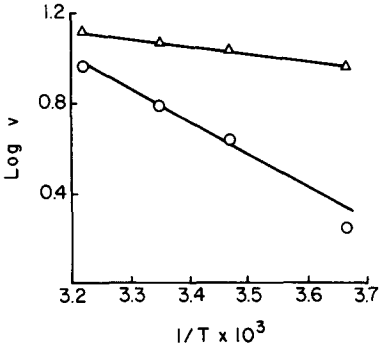
Effect of pH. Results of experiments in which triphosphoinositide was incubated at various pH values with the microsomal fraction showed that both triphosphoinositide phosphomonoesterase and phosphodiesterase to be most active at pH 7.2 (unpublished data). At pH 8.4 the phosphomonoesterase activity was 65% of that observed at pH 7.2. In contrast the phosphodiesterase activity remained constant between pH 7.4 and 8.4.

Effect of enzyme concentration. Under the standard assay conditions, hydrolysis of triphosphoinositide by the microsomal phosphomonoesterase and phosphodiesterase was a linear function of the amount of protein added to up to 200 and 300 μg, respectively.

Time course of the hydrolysis of triphosphoinositide. At optimum concentrations of triphosphoinositide and the enzyme protein the rate of reaction was linear up to 30 min then levelled off with time of incubation.

Activation energy. Optimum temperature for both enzymes was 37°C. Further increase in temperature to 60°C decreased the activities of triphosphoinositide phosphomonoesterase and phosphodiesterase by 40 and 27% of their maximal activities, respectively. The activation energies for triphosphoinositide phosphomonoesterase and phosphodiesterase were found to be 6.8 and 1.5 kcal/mol, respectively (Fig. 2).

Effects of Ca^{2+}, Mg^{2+} and other divalent cations on triphosphoinositide phosphomonoesterase and phosphodiesterase. Low concentrations of Ca^{2+} (10 μM) increased the activity of triphosphoinositide phosphodiesterase by 10%, and at



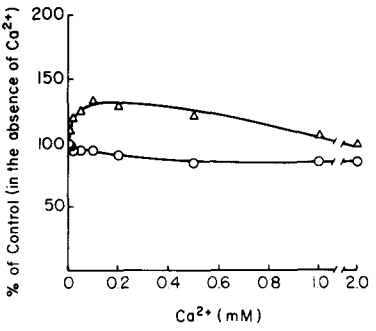
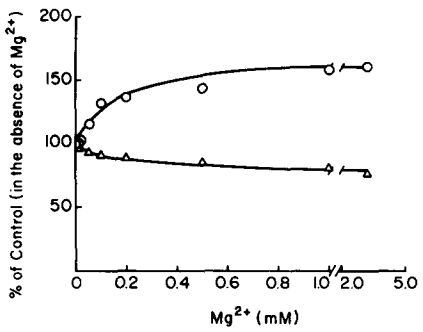


Fig. 2. Arrhenius plot of triphosphoinositide phosphomonoesterase (○) and phosphodiesterase (△) activities. The plots were constructed by determining the reaction rates of both enzymes at various temperatures. Logarithms of the initial velocities are plotted on the ordinate and the reciprocal of the absolute temperature on the abscissa. Conditions of assay were the same as described under Fig. 1.

Fig. 3. Effects of Ca^{2+} concentration on triphosphoinositide phosphomonoesterase (○) and phosphodiesterase (△) activities. Conditions of assay were the same as described under Fig. 1 except that no $MgCl_2$ was added in the phosphomonoesterase assay.

100 μM Ca^{2+} the activity of this enzyme increased by 32% (Fig. 3). Further increase in Ca^{2+} concentration resulted in a gradual decrease in the phosphodiesterase activity. While at low concentrations Ca^{2+} stimulated the phosphodiesterase activity, it exerted a 10—20% inhibitory effect on the phosphomonoesterase activity (Fig. 3). In contrast to Ca^{2+}, Mg^{2+} at concentrations from 0.1 to 1 mM increased the activity of triphosphoinositide phosphomonoesterase by 62% and inhibited the phosphodiesterase activity by 20% (Fig. 4). Neither of the enzymes showed an absolute requirement for Ca^{2+} or Mg^{2+} as both hydrolyzed triphosphoinositide in the absence of added divalent cations and also in presence of various concentrations of EDTA (Fig. 5). At 1 mM, EDTA inhibited the activities of triphosphoinositide phosphomonoesterase and phosphodiesterase by 22 and 52%, respectively (Fig. 5) and this was only partly



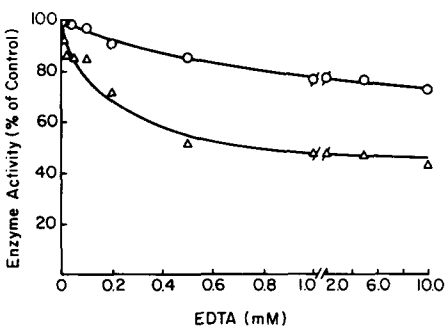


Fig. 4. Effects of Mg^{2+} concentration on triphosphoinositide phosphomonoesterase (○) and phosphodiesterase (△) activities. Conditions of assay were the same as described under Fig. 1.

Fig. 5. Effects of EDTA concentration on triphosphoinositide phosphomonoesterase (○) and phosphodiesterase (△) activities. Conditions of assay were the same as described under Fig. 1. EDTA was omitted from control incubations.

reversed by 2 mM Mg^{2+} and 2 mM Ca^{2+}, respectively. Other divalent cations such as Sr^{2+}, Ba^{2+}, Zn^{2+} and Mn^{2+} had no effect on phosphomonoesterase and phosphodiesterase activities thus indicating that the effects of Ca^{2+} and Mg^{2+} on these enzymes are specific.

Combined effects of Mg^{2+} and Ca^{2+} concentrations on triphosphoinositide phosphomonoesterase and phosphodiesterase. The inhibitory effect of 0.5 mM Ca^{2+} on the phosphomonoesterase was reversed by 0.1 mM Mg^{2+} and higher concentrations of this cation (2 mM) brought about a 79% increase in the activity of the phosphomonoesterase (Table II). Similarly the inhibitory effect of 1 mM Mg^{2+} on phosphodiesterase was relieved by 0.5 mM Ca^{2+} and further increase in concentration of the latter cation (1 mM) brought about a 17% increase in the activity of phosphodiesterase (Table III). However, when the concentrations of Ca^{2+} and Mg^{2+} were raised to 2 and 1 mM, respectively, there was a significant decrease in the activity of the phosphomonoesterase (Table III).

Effects of inhibitors and other agents on triphosphoinositide phosphomonoesterase and phosphodiesterase. Under standard assay conditions, cysteine, dithiothreitol and mercaptoethanol in the concentrations used resulted in varying degrees of stimulation of the phosphomonoesterase, but with little effect on the phosphodiesterase (Table IV). In contrast reduced glutathione and *p*-chloromercuribenzoate inhibited significantly the activities of both phosphomonoesterase and phosphodiesterase. Acetylcholine had no effect on either enzyme.

Substrate specificity of triphosphoinositide phosphomonoesterase and phosphodiesterase. As can be seen from Table V, diphosphoinositide could substi-

TABLE II

EFFECTS OF VARIOUS CONCENTRATIONS OF Mg^{2+}, IN THE PRESENCE OF 0.5 mM Ca^{2+}, ON THE ACTIVITIES OF TRIPHOSPHOINOSITIDE PHOSPHOMONOESTERASE AND PHOSPHODIESTERASE

The enzyme assay consisted of 45 mM Tris · HCl (pH 7.2), 0.6 mM triphosphoinositide, 0.72 mM cethyltrimethylammonium bromide, 150 μg microsomal protein, 0.5 mM $CaCl_2$ and varying concentrations of $MgCl_2$ in a final volume of 0.5 ml. In this experiment the microsomal fraction was dialysed successively against 10 mM Tris · HCl buffer 1 mM EDTA and finally against buffer alone. Both $CaCl_2$ and $MgCl_2$ were omitted from control incubations. The reaction was started by addition of a mixture of triphosphoinositide and cetylmethylammonium bromide and incubation continued for 20 min at 37°C. Inorganic and organic phosphates released during the reaction were assayed.

Additions		Enzyme activity (percent of control) *	
Ca^{2+} (mM)	Mg^{2+} (mM)	Phosphomonoesterase	Phosphodiesterase
0.5	—	85	135
0.5	0.01	89	126
0.5	0.02	92	118
0.5	0.05	94	115
0.5	0.1	102	112
0.5	0.2	112	106
0.5	0.5	121	99
0.5	1.0	148	94
0.5	2.0	179	89

* Control incubations were conducted in the absence of Ca^{2+} and Mg^{2+}.

TABLE III

EFFECTS OF VARIOUS CONCENTRATIONS OF Ca^{2+}, IN THE PRESENCE OF 1 mM Mg^{2+}, ON THE ACTIVITIES OF TRIPHOSPHOINOSITIDE PHOSPHOMONOESTERASE AND PHOSPHODIESTERASE

Assay conditions were the same as described under Table II except that the concentration of Mg^{2+} was constant at 1 mM and that of Ca^{2+} was variable.

Additions Ca^{2+} (mM)	Enzyme activity (percent of control) *	
	Phosphomonoesterase	Phosphodiesterase
—	188	68
0.01	188	68
0.02	190	68
0.10	191	79
0.5	189	100
1.0	186	117
2.0	130	110

* Control incubations were conducted in the absence of Ca^{2+} and Mg^{2+}.

TABLE IV

ACTION OF INHIBITORS AND OTHER SUBSTANCES ON TRIPHOSPHOINOSITIDE PHOSPHOMONOESTERASE AND PHOSPHODIESTERASE

Method of assay was exactly as described under Table I except that inhibitors and other substances were added as indicated.

Additions	Concentration (mM)	Enzyme activity (percent of control) *	
		Phosphomonoesterase	Phosphodiesterase
L-Cysteine	5	122	115
	10	119	107
Reduced glutathione	5	88	82
	15	60	51
Dithiothreitol	2	133	96
	5	138	101
Mercaptoethanol	2	122	96
	5	99	97
P-Chloromercuribenzoate	0.1	73	72
	0.5	62	56
Acetylcholine	0.05	100	98

* Controls were incubated in absence of inhibitors.

TABLE V

SUBSTRATE SPECIFICITY OF TRIPHOSPHOINOSITIDE PHOSPHOMONOESTERASE AND PHOSPHODIESTERASE

Method of assay was exactly as described under Table I except that 170 μg of microsomal protein and 0.6 mM of the various phosphoinositides were employed in this experiment.

Phosphoinositide	Phosphomonoesterase		Phosphodiesterase	
	Specific activity *	%	Specific activity **	%
Triphosphoinositide	6.3	100	21.7	100
Diphosphoinositide	5.1	81	6.4	29.4
Phosphatidylinositol	0	0	2.6	12

* nmol inorganic phosphate released/mg protein per min.

** nmol organic phosphate released/mg protein per min.

tute for triphosphoinositide as a substrate for triphosphoinositide phosphomonoesterase. In contrast both phosphatidylinositol and diphosphoinositide were poor substrates for the microsomal phosphodiesterase.

Discussion

Thompson and Dawson [7,8] reported that, in extracts of brain tissue, triphosphoinositide was hydrolyzed by a phosphomonoesterase and a phosphodiesterase. This finding was confirmed in other tissues. The observations reported above indicate that in smooth muscle triphosphoinositide is also degraded by a phosphomonoesterase and a phosphodiesterase. The results on the subcellular distribution of these enzymes in the iris muscle are consistent with those reported by others on brain [9—11,25] and kidney [15,16] that they are partly soluble and partly associated with membranes. Thus under the present experimental conditions both phosphomonoesterase and phosphodiesterase activities were found to be localized predominantly in the particulate fraction and in general the specific activity of the latter was four times as high as that of the former.

The apparent K_m values for the phosphomonoesterase and the phosphodiesterase were found to be 90 and 120 μM, respectively (Fig. 1). K_m values for the phosphomonoesterase in rat brain [12], guinea-pig brain [11] and kidney [13] were reported to be 25, 143 and 180 μM, respectively. Apparent K_m values of 1.6 mM for ox brain phosphodiesterase measured in the presence of cetyltrimethylammonium bromide [25] imply a lower affinity than observed in rat brain [26] and kidney [15] without the detergent. The activation energies for the iris muscle phosphomonoesterase and phosphodiesterase were 6.8 and 1.5 kcal/mol, respectively (Fig. 2). Lee and Huggins [14] reported the activation energies for kidney triphosphoinositide phosphomonoesterase and alkaline phosphatase as 7.7 and 3.5 kcal/mol, respectively. In contrast to a purified preparation of triphosphoinositide phosphomonoesterase, which had an optimum activity at 50°C, and the activity of which was not affected by temperatures up to 60°C [12] both phosphomonoesterase and phosphodiesterase of the iris muscle had temperature optima at 37°C and were considerably inactivate at 60°C. pH profiles for the phosphomonoesterase and phosphodiesterase of the iris muscle, measured in Tris · HCl buffer, revealed a maximal triphosphoinositide hydrolysis by both enzymes at pH 7.2. For brain phosphomonoesterase, the pH optimum was found to be around 7.2 [7,11,12] and for the kidney enzyme, the range was between 7.5 and 8.0 [13,16]. For brain phosphodiesterase the pH optimum was 7.2 [8,25] and for that of the kidney it was 6.7 [15]. The finding that at pH 7.2, phosphatidylinositol was not attacked by microsomal triphosphoinositide phosphodiesterase is in accord with the observations of others [8].

The data presented on the effects of Ca^{2+} and Mg^{2+} on the iris muscle triphosphoinositide phosphomonoesterase and phosphodiesterase indicate that these enzymes do not have an absolute requirement for either of the divalent cations (Figs. 3 and 4). Thus, although EDTA inhibited appreciably the activities of both enzymes some residual activity was still observed in the presence of this chelating agent (Fig. 5). However, Ca^{2+} stimulated the phosphodiester-

ase and inhibited the phosphomonoesterase and Mg^{2+} had an opposite effect on the two enzymes. Reports on the effects of Ca^{2+} and/or Mg^{2+} on the activities of triphosphoinositide phosphodiesterase and phosphomonoesterase in various tissues suggest that the requirement for these cations is dependent upon the tissue and methods employed in their preparation and assay. Thus Dawson and Thompson [7] reported that Ca^{2+} alone does not activate the phosphomonoesterase, however, in the presence of an optimum concentration of Mg^{2+}, it exerted a stimulatory effect on this enzyme. In brain [12] and kidney [16] Ca^{2+} was reported to stimulate the activity of this enzyme. In contrast phosphomonoesterase from kidney cortex was inhibited by Ca^{2+} [13]. In the protozoal system Ca^{2+} has no effect on this enzyme [18]. In general, the requirement for Mg^{2+} by the phosphomonoesterase has been shown in all of the above studies. In the protozoal system the phosphodiesterase was activated by Ca^{2+} and inhibited by Mg^{2+} [18]. In kidney [15] Mg^{2+} or Ca^{2+} depressed the phosphodiesterase activity. In the rabbit iris muscle the stimulatory effect of Mg^{2+} on triphosphoinositide phosphomonoesterase and of Ca^{2+} on phosphodiesterase appear to specific. Thus no other divalent cation has an effect similar to Ca^{2+} and Mg^{2+} on these enzymes. Further support for this conclusion is provided by the experiments in which the effects of both Ca^{2+} and Mg^{2+} were investigated. Thus inhibition of the phosphomonoesterase by 0.5 mM Ca^{2+} was relieved upon the addition of 0.1 mM Mg^{2+} whereas inhibition of the phosphodiesterase by 1 mM Mg^{2+} was overcome upon the addition of 0.5 mM Ca^{2+}, which could suggest a preferential requirement for Mg^{2+} by the former and for Ca^{2+} by the latter enzyme.

The experimental evidence for the possible relationship between cholinergic-muscarinic and α-adrenergic receptors and the metabolism of phosphatidylinositol [27] and triphosphoinositide [6] has recently been reviewed. The demonstration of triphosphoinositide phosphodiesterase in the iris muscle, coupled with the finding that this enzyme is activated by Ca^{2+} add further support to our previous conclusion that an increased Ca^{2+} influx, following the interaction between the neurotransmitter and its receptor (Eqn. 1), could act to stimulate triphosphoinositide phosphodiesterase, thus leading to increased phosphatidic acid via increased diacylglycerol [4—6]. In contrast to the observation of Torda [28] on the stimulatory effect of acetylcholine on brain triphosphoinositide phosphomonoesterase, this neurotransmitter exerted no effect on either the phosphomonoesterase or the phosphodiesterase of the iris microsomal fraction. Recently Griffin and Hawthorne [29] reported that the addition of the ionophore A23187 to synaptosomes prelabelled with $^{32}P_i$ caused an increase in Ca^{2+} influx, a release of transmitter and a rapid loss of label from triphosphoinositide and diphosphoinositide. Similarly Allan and Michell [30] showed a substantial Ca^{2+}-dependent loss of triphosphoinositide from human erythrocyte ghosts prepared in the presence of EDTA. These authors concluded that human erythrocyte membranes contain at their cytoplasmic surface a Ca^{2+}-activated phosphodiesterase that is active against both diphosphoinositide and triphosphoinositide.

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