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Properties of a Cyclic Nucleotide Phosphodiesterase of Amphibian Oocytes That Is Activated by Calmodulin and Calcium, by Tryptic Proteolysis, and by Phospholipids

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A calmodulin-Ca^{2+}-stimulated cyclic nucleotide phosphodiesterase (EC 3.1.4.17) which hydrolyzed both cGMP and cAMP has been purified about 2000-fold from ovaries of the amphibian *Xenopus laevis*. Gel filtration through Sephadex G-200 indicated a molecular weight of 140,000. A single, major protein band of molecular weight 66,000 was observed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In addition to the stimulation by calmodulin-Ca^{2+}, the enzyme was activated 5- to 10-fold by proteolysis and by certain phospholipids. Trypsin activation of the enzyme caused a reduction in the native molecular weight to 90,000 and a loss of the capacity to be stimulated by calmodulin-Ca^{2+} or by phospholipids. The phosphodiesterase was stimulated by low concentrations (0.1 μg/ml) of lysophosphatidylcholine and lysophosphatidylethanolamine. This response did not require calcium ions. Phosphatidylinositol, fatty acids, progesterone, and phospholipase C had little or no effect on activity. Simultaneous addition of 1 mM 2-chloro-10-(3-aminopropyl)phenothiazine and lysophosphatidylcholine to the enzyme did not diminish the stimulatory effect of the phospholipid. The activation of the enzyme by all three agents resulted in an increase in the maximum velocity of the reaction without significant modification of the apparent K_m values for cGMP (5 μM) or cAMP (30 μM). It was suggested that trypsin removed an inhibitory domain from the enzyme and that calmodulin and phospholipids interact with this same domain, eliminating its capacity to inhibit the active center of the enzyme.

Cyclic nucleotide phosphodiesterases (EC 3.1.4.17) that are activated by calmodulin and calcium have been found in many animal tissues. The enzymes from bovine brain and heart have been purified to homogeneity, and their characteristics have been studied in detail (for reviews see Refs. (1, 2)).

Since the induction of meiotic maturation of amphibian oocytes by progesterone is accompanied by a transient decrease of cAMP and a similarly transient increase of intracellular Ca^{2+} (3-6), several laboratories have looked for the possible involvement of a cyclic nucleotide phosphodiesterase activated by calcium through calmodulin in this process. Contrary to the initial finding of two other groups (7-8), work in our laboratory has demonstrated that *Xenopus laevis* oocytes also contain a major cyclic nucleotide phosphodiesterase species (PDE I)[2] that can be greatly stimulated by calmodulin and calcium (9). This

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[2] Abbreviations used: PDE-I, calmodulin-Ca^{2+}-stimulated cyclic nucleotide phosphodiesterase; SDS, sodium dodecyl sulfate; CAPP, 2-chloro-10-(3-aminopropyl)phenothiazine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; Hepes, 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid; LPC, lysophosphatidylcholine; CaM, calmodulin.

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observation has been confirmed by Miot and Erneux (10). Further work has determined that this enzyme can be activated by trypsin treatment (11), and that its activity is inhibited in the living oocytes (12).

The work presented in this report includes details of the extensive purification of oocyte PDE I, some physical and kinetic properties of the enzyme, and characteristics of its activation by tryptic proteolysis and by some phospholipids.

EXPERIMENTAL PROCEDURES

Materials. Mature female *X. laevis* were obtained from the South African Snake Farm, Capetown, R.S.A. [2,8-^{3}H]Adenosine 3′,5′-cyclic phosphate (32 Ci/mmol) and [8,5′-^{3}H]guanosine 3′,5′-cyclic phosphate (34 Ci/mmol) were purchased from New England Nuclear, Boston, Massachusetts, and were purified before use (13). Unlabeled nucleotides, cyanogen bromide-activated Sepharose 4B, Sephadex G-200 (superfine), trypsin (Type III), soy bean trypsin inhibitor (Type I-S), phospholipase C (Type XII), steroids, phospholipids, fatty acids, and molecular weight markers for gel filtration (cytochrome *C*, ovalbumin, bovine serum albumin, phosphorylase *b*, and alkaline phosphatase) were obtained from Sigma Chemical Company, St. Louis, Missouri.

DEAE-cellulose was from Whatman, Ltd., Kent, England. PM 30 membranes were from Amicon Corporation, Danvers, Massachusetts. Pure brain calmodulin was a gift from Dr. C. Klee, NIH, Bethesda, Maryland. 2-Chloro-10-(3-aminopropyl)phenothiazine was obtained from the Psychopharmacology Research Branch, National Institute of Mental Health, NIH, Bethesda, Maryland. Fluphenazine was a gift from Smith, Kline and French, Philadelphia, Pennsylvania.

Assay of cyclic nucleotide phosphodiesterase activity. A modified two-step procedure of Thompson *et al.* (13) was used. The standard incubation mixture used for the calcium and calmodulin-dependent activity contained, in a total volume of 200 μl, 10 mM Hepes, pH 7.6, 3 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 0.3 μM calmodulin, 0.5 mM $CaCl_2$, 5 μM [^{3}H]cAMP or 1 μM [^{3}H]cGMP, and the enzymatic fraction. Additional details and description of controls used routinely were published previously (11).

To measure the basal activity, the same mixture was used except that EGTA to a final concentration of 2 mM was added. Incubations were carried out at 30°C for 20 min, at which time less than 20% hydrolysis of the substrate was observed. For the measurement of enzyme activity in the presence of lipids and with [^{3}H]cAMP as substrate, 0.1 mM EGTA was added to the assay mixture and calmodulin and calcium ions were omitted, except where specified.

Purification of cyclic nucleotide phosphodiesterase I. Ovaries were removed surgically, and rinsed in a saline buffer containing 50 mM Hepes, pH 8.0, 5 mM $MgCl_2$, 3 mM 2-mercaptoethanol, 0.1 mM EDTA, and 0.1 mM EGTA (buffer A). All subsequent operations were at 4°C except where stated. Samples (150–200 g) of minced ovarian tissue were homogenized in small batches in buffer A using a motor-driven Potter-Elvehjem homogenizer. The material obtained was pooled and centrifuged at 27,000*g* for 20 min. The supernatant fraction was filtered through glass wool to remove a lipid layer and again centrifuged at 27,000*g* for 20 min. The supernatant fraction was used as enzyme source for further purification.

The extract was applied to a DEAE-cellulose (Whatman DE-52) column (1.5 × 30 cm) that had been equilibrated with 10 mM Hepes, pH 7.5, 5 mM $MgCl_2$, 3 mM 2-mercaptoethanol, 0.1 mM EDTA, and 0.1 mM EGTA (buffer B). The column was washed with 700 ml of buffer B and eluted with a linear salt gradient of 0 to 0.8 M sodium acetate in buffer B. The total gradient volume was 1.5 liters, and the elution was carried out at rate of 120 ml/h, collecting 7-ml fractions.

As published previously (9), PDE I elutes from such a column at a salt concentration of about 200 mM. The fractions with PDE I activity were pooled and concentrated by ultrafiltration using an Amicon PM 30 membrane, and stored at −80°C or processed further.

Affinity chromatography using calmodulin-Sepharose. The enzyme from the DEAE-cellulose column was adjusted to a final concentration of 0.5 mM $CaCl_2$ and passed through a 2-ml calmodulin-Sepharose column that had been equilibrated with buffer containing 40 mM Tris-HCl, pH 7.5, 50 mM NaCl, 3 mM $MgCl_2$, 0.2 mM $CaCl_2$, and 0.1 mM dithiothreitol (buffer C). The calmodulin-Sepharose resin had been prepared as described by Klee and Krinks (14). The column was washed with 20 ml of buffer C and subsequently with 10 ml of that buffer containing 0.2 M NaCl. The calmodulin-sensitive enzyme was eluted with a solution containing 40 mM Tris-HCl, pH 7.5, 2 mM EGTA, 1 mM $MgCl_2$, 0.1 mM dithiothreitol, and 0.2 M NaCl. The column flow rate was 12 ml/h and 1-ml fractions were collected. The active fractions were pooled and stored in 50% glycerol. Under these conditions the enzyme was stable for about 2 months at −80°C.

Electrophoretic fractionation of PDE I. Electrophoresis of purified fractions of PDE I was carried out in cylindrical tubes (1.4 × 11 cm) containing 6% polyacrylamide and 0.05% bisacrylamide, as described by Usunov *et al.* (15). The gels were prerun for 16 h with a buffer containing 50 mM Tris base, 0.38 M glycine, pH 8.8, 5 mM $MgCl_2$, 3 mM 2-mercaptoethanol, and 0.1 mM EGTA, with a constant current of 4 mA at 4°C. After loading the sample in 10% sucrose and

with bromphenol blue as marker, electrophoresis was performed at 4°C using 8 mA. The process was stopped when the marker dye had run through 90% of the gel, which took approximately 3.5 h. The gel cylinder was sliced into fractions (4 mm thick), which were ground up and then incubated with 1.5 ml of the buffer used for electrophoresis for 16 h at 4°C in order to elute the enzyme.

SDS electrophoresis. Electrophoresis in polyacrylamide gel slabs in the presence of SDS was run as described by Laemmli (16). A stacking gel of 3% acrylamide and a separating gel of 10% acrylamide were used. The electrophoresis was run with a constant current of 30 mA at room temperature. Proteins were stained with Coomassie blue R250.

Sephadex gel filtration. Gel filtration was performed with Sephadex G-200 superfine using a 1.5 × 66-cm column equilibrated with buffer A containing 0.1 M NaCl. The flow rate was 12 ml per hour and fractions of 2 ml were collected. Protein standards used were cytochrome *c* (M_r = 12,000), ovalbumin (M_r = 43,000), bovine serum albumin (M_r = 67,000), phosphorylase *b* (M_r = 94,000), and alkaline phosphatase (M_r = 160,000).

Trypsin treatment. PDE I in buffer B was incubated with 2 to 10 μg of trypsin/ml for 10 min at 30°C. The reaction was stopped by the addition of soy bean trypsin inhibitor at a concentration 5 times greater than the amount of trypsin used.

Protein determinations were performed routinely using the Coomassie G-250 dye binding method (17) with bovine serum albumin as standard.

RESULTS

Purification of cyclic nucleotide PDE I. High purification of PDE I can be achieved by chromatography on DEAE-cellulose followed by affinity chromatography on a calmodulin–Sepharose column. It has been demonstrated previously (9) that DEAE-cellulose chromatography of a crude cytosol fraction of *X. laevis* oocytes separates two fractions with cAMP phosphodiesterase activity. PDE I, which elutes between 180 and 210 mM sodium acetate, is greatly stimulated by calmodulin and calcium and hydrolyzes both cAMP and cGMP with similar efficiency. PDE II, which elutes with 350 mM salt, is not activated with calmodulin and calcium and is specific for cAMP.

The DEAE-cellulose fractions containing PDE I activity, concentrated by ultrafiltration, can be purified further by passage through a calmodulin–Sepharose column equilibrated with a calcium containing buffer. Figure 1 shows a typical elution pattern. It can be seen that a large fraction of the protein is not retained in this column. This unretained fraction has appreciable cyclic nucleotide phosphodiesterase activity which is not sensitive to calmodulin and calcium. This fraction corresponds to contamination of the DEAE-cellulose fractions with PDE II. This eluted protein would also contain any PDE I which had lost its capacity to be stimulated by calmodulin because of proteolysis occurring during the initial fractionation of the crude extract. Elution of the affinity column with a buffer containing EGTA results in the appearance of a small amount of protein which contains PDE I activity that is greatly stimulated by calmodulin and calcium. As seen in Table I, this step gives a purification of close to 300-fold, while the two combined steps give a purification of 2000-fold. The activity obtained after calmodulin–Sepharose purification is very labile but can be stabilized by 50% glycerol.

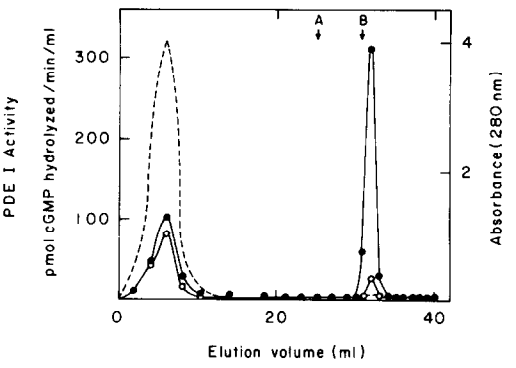

FIG. 1. Affinity chromatography of cyclic nucleotide phosphodiesterase I. PDE I (100 mg protein) obtained from DEAE-cellulose and adjusted to 0.5 mM $CaCl_2$ was applied to a 1 × 2.5-cm column of calmodulin–Sepharose. The column was washed with 20 ml buffer C, 10 ml of buffer C containing 0.2 M NaCl, and 10 ml of solution containing 40 mM Tris–HCl, pH 7.5, 2 mM EGTA, 1 mM $MgCl_2$, 0.1 mM dithiothreitol, and 0.2 M NaCl. The arrows indicate the change of eluting buffers. Aliquots of 10 μl were used for enzyme assays with [^{3}H]cGMP as substrate (basal activity, ○; calmodulin-Ca^{2+}-stimulated activity, ●). Protein was measured spectrophotometrically (- - -).

TABLE I

PURIFICATION OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE I FROM OVARY OF *Xenopus laevis*

Fraction	Volume (ml)	Total protein (mg)	Total units	Specific activity[a] (units/mg)	Yield (%)	Purification (-fold)
Extract	245	6080	225	0.037	100	1
DEAE-cellulose	150	396	104	0.262	46	7
CaM-Sepharose	2.2	0.12	41	743	18	2017

Note. Results are presented for 165 g of ovary.

[a] Activity was measured in the presence of Calmodulin/Ca^{2+} and with 1 μM [^{3}H]cGMP as substrate. One unit of enzyme is defined as the amount which hydrolyzes 1 pmol of cGMP/min.

Using nondenaturing conditions, PDE I activity can be recovered after polyacrylamide gel electrophoresis. This activity enters the gel approximately 1.2–1.5 cm under the conditions described under Experimental Procedures, and retains its capacity to be stimulated by calmodulin and calcium. However, the recovery of active enzyme is very low. Figure 2 shows the Coomassie blue staining pattern of an SDS-polyacrylamide gel which was used to fractionate the polypeptide chains present in the different fractions containing PDE I activity. A comparison of channels 1 (DEAE-cellulose) and 2 (calmodulin-Sepharose) demonstrates the high purification capacity of the affinity column. The principal band appearing in channel 2 is a polypeptide of approximately 66,000 Da. The other bands in that channel are minor and have molecular weights near 30,000. The active material recovered from nondenaturing electrophoresis (described below) gave a single, faint protein band of 66,000 Da (channels 3 and 6).

Activation of PDE I by proteolysis. As has been reported briefly (11), trypsin treatment of PDE I raises the basal activity of the enzyme to levels similar to those obtained with the calmodulin-Ca^{2+} complex. This proteolytic activation is accompanied by a loss of the capacity of the enzyme to be stimulated by calmodulin and calcium and to be retained on a calmodulin-Sepharose column. Subtilisin is slightly more effective than trypsin in stimulating PDE I activity but chymotrypsin is inactive in this respect (data not shown). As shown previously (11), using 2 μg/ml of trypsin, incubation times of up to 60 min do not cause a decrease in the proteolytic activation of PDE I.

The results presented in Figs. 3A and B demonstrate the effect of tryptic activation on the position of elution of PDE I from a Sephadex G-200 column. Using proteins of known molecular weight to calibrate this

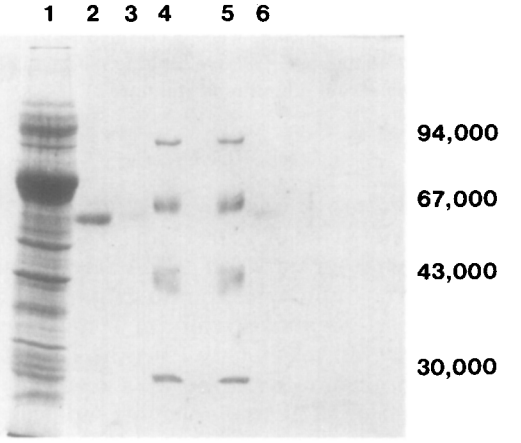

FIG. 2. SDS-gel electrophoresis of cyclic nucleotide phosphodiesterase I fractions on 10% polyacrylamide gels. Lane 1 contains 150 μg of protein from the DEAE-cellulose purification step; lane 2 contains 7 μg protein from the calmodulin-Sepharose affinity chromatography step; and lanes 3 and 6, 0.5 μg protein enzyme recovered from preparative gel electrophoresis as described under Experimental Procedures. Lanes 4 and 5 contain the protein standards phosphorylase *b* (M_r = 94,000), bovine serum albumin (M_r = 67,000), ovalbumin (M_r = 43,000), and carbonic anhydrase (M_r = 30,000). Gels were stained with Coomassie blue R-250.

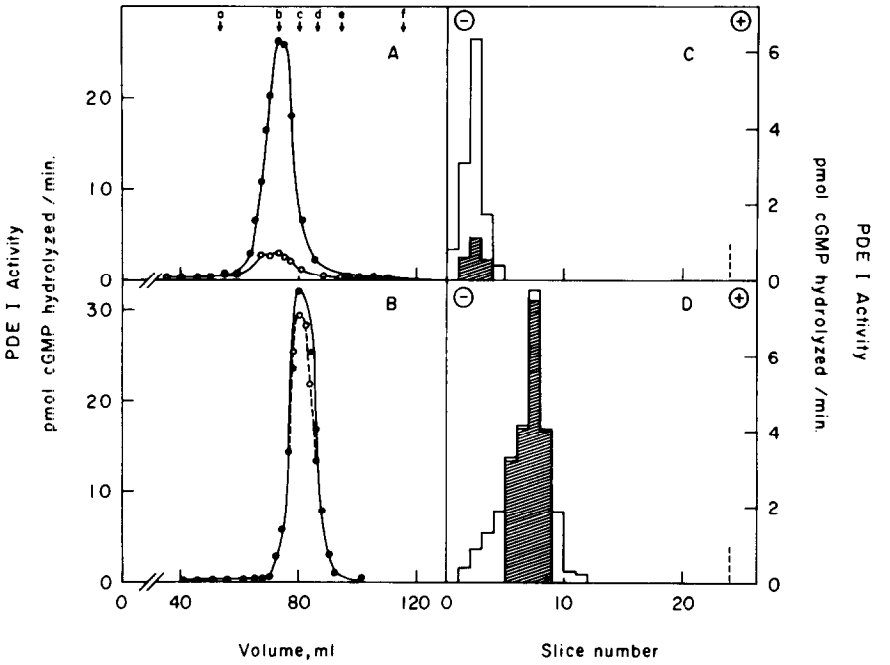

FIG. 3. The effect of trypsin on the behavior of cyclic nucleotide phosphodiesterase I on gel filtration and acrylamide gel electrophoresis. (A) A 2-mg sample of PDE I was applied to a 1.5 × 66-cm Sephadex G-200 column and eluted as given under Experimental Procedures. Two-milliliter fractions were collected, and assays were performed with 20 μl aliquots for basal (○) and calmodulin-Ca^{2+}-stimulated (●) activity, using [^{3}H]cGMP as substrate. The arrows indicate the void volume and the position of elution of protein molecular weight markers: a) void volume; b) alkaline phosphatase (M_r = 160,000); c) phosphorylase *b* (M_r = 94,000); d) bovine serum albumin (M_r = 67,000); e) ovalbumin (M_r = 43,000), and f) cytochrome *c* (M_r = 12,000). (B) A 2-mg sample of PDE I was incubated with 2 μg of trypsin for 10 min at 30°C, 10 μg soybean trypsin inhibitor added, and the resultant solution was subjected to gel filtration as in A. (C) A 200-μg aliquot of PDE I was analyzed by electrophoresis on a 6% polyacrylamide gel for 3.5 h at 4°C. The gel was sliced in 4-mm sections and eluted with 1.5 ml buffer B for 16 h at 4°C, and the resultant solutions were analyzed for PDE I activity using [^{3}H]cGMP as substrate (basal activity, cross-hatched bars; calmodulin-Ca^{2+}-stimulated activity, open bars). (D) A 200-μg aliquot of PDE I was incubated with 0.2 μg trypsin for 10 min at 30°C, 1 μg soybean trypsin inhibitor added, and the resultant solution was analyzed by gel electrophoresis as in C.

column, it can be estimated that the native PDE-I has an approximate molecular weight of 135,000–140,000, while the trypsin-treated enzyme is a 90,000-Da protein. It is also shown in Fig. 3B that the enzyme activated by proteolysis cannot be further stimulated by calmodulin and calcium. In Figs. 3C and D it is shown that trypsin also modifies the electrophoretic mobility of PDE I, increasing the capacity for migration of the activity into the gel. Again, the loss of response to calmodulin and calcium is illustrated by this experimental approach. It is unfortunate that the amount of protein recovered after trypsin treatment did not give a clear staining pattern in SDS-electrophoretic gels, and therefore no information could be obtained as to the size of the protomers after proteolysis.

Activation of PDE I by phospholipids. Some calmodulin-sensitive phosphodiesterases from mammalian tissues have been shown to be activated by specific phospholipids and fatty acids (18–20).

The effect of several lipids on oocyte PDE I was tested, as shown in Table II. It is clear that lysophosphatidylcholine and ly-

TABLE II

LIPID ACTIVATION OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE I

Addition	Phosphodiesterase activity [^{3}H]cAMP hydrolysis	
	pmol min mg protein^{-1}	-fold stimulation
Experiment 1		
None	58.8	—
Calmodulin (0.001 mg/ml)	251	4.3
Lysophosphatidylcholine (0.5 mg/ml)	277	4.7
Oleic acid (0.04 mg/ml)	52.9	0
Experiment 2		
None	88.3	—
Lysophosphatidylcholine (0.1 mg/ml)	383	4.3
Lysophosphatidylethanolamine (0.2 mg/ml)	372	4.2
Phosphotidylinositol (0.2 mg/ml)	149	1.7
Sodium stearate (0.15 mg/ml)	149	1.7
Sodium linoleate (0.15 mg/ml)	0	Inhibition
Experiment 3		
None	15.3	—
Progesterone (17 μM)	19.5	1.3
Progesterone (170 μM)	12.4	0
β-Estradiol (10 μM)	13.6	0
Testosterone (10 μM)	8.6	0
Hydrocortisone (10 μM)	0	Inhibition
Experiment 4		
None	71.5	0
Phospholipase C (5 U)	69.4	0
Phosphosphatidylcholine (0.1 mg/ml)	552	7.7
Lysophosphatidylcholine + Phospholipase C (0.1 mg/ml 5 U)	76.8	1.1

Note. Enzyme assays were performed using the calmodulin-Sepharose-purified PDE I in the presence of the indicated lipid or of the solvent used for its preparation, as described under Experimental Procedures, and using [^{3}H]cAMP as substrate. In experiment 2 lysophosphatidylethanolamine, sodium stearate, and sodium lineolate were prepared by a 5-min sonication in the incubation buffer immediately before use. In experiment 3, steroids were prepared as concentrated solutions in ethanol, and the final concentration of the solvent in the incubation mixtures was 0.01 to 0.1%. Phospholipase C was added at zero time.

sophosphatidylethanolamine cause a marked stimulation of the enzyme activity. On the other hand, phosphatidylinositol at a similar low concentration has a much smaller effect. Of the long-chain fatty acids tested, only sodium stearate had a significant positive effect. Sodium oleate had no effect while the linoleic acid salt caused a drastic inhibition of the enzyme. Progesterone and testosterone, which can induce oocyte maturation, had no significant effect, whereas cortisol also produced a marked inhibition. It is important to point out that the activation observed with lysophosphatidylcholine can be obtained in the absence of Ca^{2+} and that addition of calcium does not modify the values obtained (not shown).

Phospholipase C has been reported to stimulate the calmodulin-sensitive cyclic nucleotide phosphodiesterase of bovine brain (19). The basal activity of oocyte PDE I is not altered by the addition of phospholipase C (Table II, experiment 4). On

the other hand, as it would be expected, the presence of the lipase essentially eliminates the stimulation of PDE I caused by lysophosphatidylcholine.

The stimulation by lysophosphatidylcholine was studied further. Figure 4 shows the effects of different amounts of this phospholipid on the activity of PDE I. The activation of the enzyme reaches maximum values with 20 μg of lysophosphatidylcholine. No further increase in activity was observed when assays were carried out in the presence of both phospholipid and calmodulin and Ca^{2+}, showing that the stimulatory effects of both agents were not additive. However, in several experiments using different enzyme preparations, lysophosphatidylcholine was able to activate PDE I to levels that were 25–30% above the maximum values observed with calmodulin and calcium alone. Figure 4 also shows that a wide range of concentrations of lysophosphatidylcholine that give up to eightfold stimulation of the native PDE I

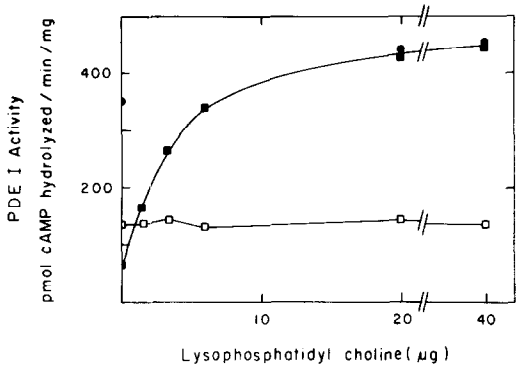

FIG. 4. The effect of lysophosphatidylcholine on the activity of cyclic nucleotide phosphodiesterase I. PDE I (9 μg) obtained after calmodulin-Sepharose purification was assayed with varying concentrations of lysophosphatidylcholine, using [^{3}H]cAMP as substrate, in the absence (■) and presence (●) of 0.3 μM calmodulin. PDE I (29 μg) was treated with trypsin (2.9 μg) for 10 min at 30°C, as described under Experimental Procedures, and applied to a 1-ml calmodulin-Sepharose column, and the enzyme which was not retained on the column was analyzed using varying concentrations of lysophosphatidylcholine (■) and [^{3}H]cAMP as substrate. The incubation with trypsin resulted in a 3.8-fold stimulation of activity as compared to the untreated enzyme.

do not have a significant effect on trypsin-activated PDE I.

Phenothiazines have been shown to inhibit calmodulin-activated enzymes (21) and, specifically, 2-chloro-10-(3-aminopropyl)phenothiazine (CAPP) has been demonstrated to abolish the stimulation of oocyte PDE I by calmodulin and calcium (9). Figure 5 compares the effects of two antipsychotic drugs, CAPP and fluphenazine, on the activity of PDE I in the presence of either calmodulin and calcium or lysophosphatidylcholine. In Fig. 5A it can be seen that CAPP fails to inhibit the enzyme activated by lysophosphatidylcholine at concentrations that abolish the calmodulin stimulation. Fluphenazine, on the other hand, as observed in Fig. 5B, behaves differently since it blocks the effect of both activators. However, while the inhibition of the enzyme activated by the phospholipid is gradual, the decrease in the calmodulin-stimulated activity has a sharp break typical of a titration curve.

Kinetic parameters in the activation of PDE I. Figure 6 shows Lineweaver–Burk plots of the effects of cAMP concentration on the activity of PDE I measured in the presence of lysophosphatidylcholine. For comparison, the activities observed in the presence and absence of calmodulin-Ca^{2+} are shown. The apparent K_m values for cAMP were essentially the same in all three experiments, 28 μM (basal), 26 μM (with lysophosphatidylcholine), and 33 μM (with calmodulin-Ca^{2+}). However, both activators increased the apparent V_{max} about fivefold.

Table III summarizes the results obtained with the three activation mechanisms of PDE I using cGMP as substrate. Again, the effect of the activators on the apparent K_m of the enzyme was not very significant, while in all cases the apparent V_{max} was increased by the activator.

DISCUSSION

To our knowledge, the work described above presents the first detailed study of a nonmammalian cyclic nucleotide phosphodiesterase that can be activated by calmodulin. It is of interest, therefore, to

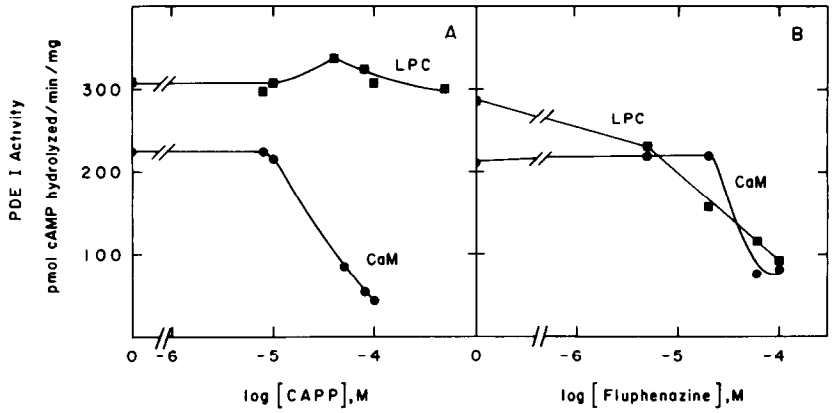

FIG. 5. The effect of phenothiazine drugs on the activity of cyclic nucleotide phosphodiesterase I in the presence of calmodulin-Ca^{2+} and lysophosphatidylcholine. (A) PDE I activity was measured in the presence of 0.3 μM calmodulin (●) or 2 μg lysophosphatidylcholine (■) with varying concentrations of 2-chloro-10-(3-aminopropyl)phenothiazine, and [^{3}H]cAMP as substrate. Assays were conducted with 9 μg of enzyme as given under Experimental Procedures. (B) PDE-I activity was measured as given in A with varying concentrations of fluphenazine-2 HCl.

compare the properties of this enzyme with those of cyclic nucleotide phosphodiesterases from bovine heart and brain, which have been thoroughly characterized.

The amphibian enzyme has a molecular weight of 140,000; the electrophoretic analyses suggest that the enzyme is probably a dimer constituted of two identical subunits of molecular weight approximately 66,000, which is the main protein band appearing in the SDS-electrophoretic gels of the most highly purified preparations. The mammalian enzymes are somewhat smaller; they have been shown to be dimers with a molecular weight of 120,000, having subunits of approximately 58,000-59,000 Da (22-24).

It is notable that the *X. laevis* enzyme is just as versatile as that obtained from mammalian tissues with respect to its ac-

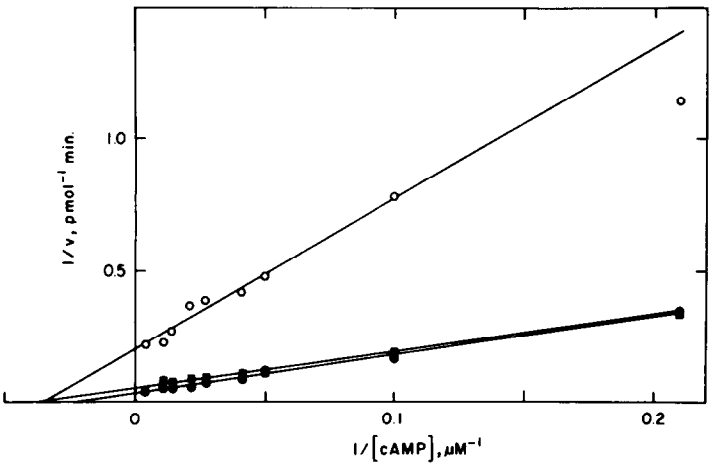

FIG. 6. Lineweaver-Burk plot of [^{3}H]cAMP hydrolysis catalyzed by cyclic nucleotide phosphodiesterase I. PDE-I activity was measured in the absence of activator (○) or in the presence of 4 μg lysophosphatidylcholine (■) or 18 μM calmodulin (●) using [^{3}H]cAMP as substrate. Assays were performed in triplicate using 9 μg of enzyme and 4.7 to 250 μM [^{3}H]cAMP.

TABLE III

KINETIC CONSTANTS OF PDE I IN THE PRESENCE OF ACTIVATORS

Activator or pretreatment	cGMP K_m (μM)	cGMP V (nmol min^{-1} mg^{-1})
Experiment 1		
None	5	2
Calmodulin/Ca^{2+} (18 nM)	2.9	25
Trypsin pretreatment	2.5	15
Experiment 2		
None	4.2	0.8
Lysophosphatidylcholine (0.1 mg/ml)	6.2	6.8

Note. Enzyme assays and trypsin pretreatment were performed using the calmodulin-Sepharose-purified enzyme and the standard assay conditions described under Experimental Procedures. Different enzyme preparations were used in these experiments.

tivation mechanisms since, in addition to calmodulin and calcium, this enzyme can be stimulated by proteolysis and by certain phospholipids. The degree of stimulation observed with this enzyme is, unfortunately, prejudiced by the lack of stability of the calmodulin-Sepharose-purified enzyme. The freshly eluted enzyme is routinely activated more than 15-fold with both substrates (Fig. 1), but this property is lost on prolonged storage at −80°, reaching a level of about 5-fold for cAMP and 10-fold for cGMP. This change is undoubtedly a result of an inactivation process.

Tryptic proteolysis causes a 35% decrease in the molecular weight of the amphibian PDE I, with a concurrent increase of its calcium-independent activity and a loss of its capacity to interact with calmodulin and calcium. This evidence fits a mechanism that has been proposed for the mammalian enzymes, in which proteolytic treatment would cause the removal of an inhibitory part of the enzyme (25, 26). This inhibitory structure would also be the portion of the molecule responsible for its interaction with calmodulin. Our previously reported findings that the trypsin treatment prevents the retention of the enzyme on calmodulin-Sepharose affinity columns and that calmodulin and calcium protect PDE I from tryptic activation lend additional support to this mechanism. Protection of mammalian PDE from tryptic attack by calmodulin has not been reported. Another difference is found in the fact that the amphibian enzyme is not inactivated by prolonged exposure to trypsin or other proteases, as is the mammalian enzyme (11).

The regulatory importance of the enzyme structure removed by trypsin is also demonstrated by the fact that PDE I, after proteolysis, does not respond to phospholipids that greatly enhance the activity of the native enzyme. The activation of the amphibian enzyme by phospholipids differs in some aspects from that observed with the mammalian enzymes. While there is consensus that lysophosphatidylcholine is a potent activator of PDE from several sources, the *X. laevis* PDE I is not appreciably activated by long-chain fatty acids, some of which have been shown to stimulate the bovine brain and human aorta enzymes (19, 20). Also, phosphatidylinositol gives only a small stimulation with the amphibian enzyme, while it has been reported that it is a strong activator of the porcine brain PDE (18). However, another report indicates that phosphatidylinositol is a poor activator of the bovine enzyme (19). The amphibian and mammalian enzymes are similar in that activation by lysophosphatidylcholine does not require Ca^{2+} and is not additive with the stimulation caused by calmodulin in the presence of Ca^{2+}.

The inhibition of calmodulin-sensitive enzymes by phenothiazine derivatives has been shown to be due to the binding of these drugs to the modulator protein (21). It is interesting that fluphenazine also inhibits PDE I activated by lysophosphatidylcholine. It seems possible that this effect might be due to hydrophobic interaction between the drug and the phospholipid. The inhibitory capacity, however, is not general to phenothiazines, as shown by the failure of CAPP to block phospholipid activation of PDE I under similar conditions.

With some other phosphodiesterases, activation by calmodulin or by phospho-

lipids has been observed to result in significant modification of the K_m values in addition to increases in the V_{max} of the reaction (2). However, the results obtained with oocyte PDE I using all three modes of activation of the enzyme indicate that their mechanism involves a large increase in the V_{max} of the reaction with only small variations in the apparent K_m for both cAMP and cGMP.

The properties of this enzyme have special interest because microinjection experiments have shown that PDE I, which is the major cyclic nucleotide phosphodiesterase activity detected *in vitro*, is inactive toward substrates introduced into living oocytes (12). Work in progress in this laboratory has demonstrated the existence in oocytes of a specific protein inhibitor that blocks PDE I activation (E. Jedlicki *et al.*, manuscript in preparation).

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