Studies on the soluble phosphodiesterases of chicken gizzard smooth muscle

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In this study we describe the identification of four soluble forms of cyclic nucleotide phosphodiesterase from chicken gizzard smooth muscle. These isoenzymes were separated from one another by ion-exchange chromatography on DEAE-cellulose and by calmodulin–Sepharose affinity chromatography. Each form migrates as a single discrete band when it is electrophoresed on non-denaturing polyacrylamide gels and stained for phosphodiesterase activity. Each form is also eluted as a single peak on gel-permeation chromatography, giving apparent $M_r$ values of 114000, 116000, 122000 and 59000. All four enzymes have apparent $K_m$ values in the 0–20 µM range, although their relative specificities for cyclic AMP and cyclic GMP differ. Two of the forms bind to calmodulin in a Ca$^{2+}$-dependent manner; however, only one is activated by calmodulin. The interaction of the second calmodulin-binding form with calmodulin is disrupted by the papaverine derivative verapamil without significantly altering the hydrolytic activity of the enzyme.

Many studies have suggested that the regulation of contraction of smooth muscle may be affected by the concentrations of cyclic nucleotides present in the tissue (see reviews by Bar, 1974; Andersson et al., 1975; Diamond, 1978; Kramer & Hardman, 1980; Hardman, 1982). A number of regulatory mechanisms in smooth muscle, including the Ca$^{2+}$-translocating system (Nishikori et al., 1977; Bhalla et al., 1978) and myosin light-chain kinase (Adelstein et al., 1978; Hartshorne & Mrwa, 1982; Bhalla & Sharma, 1982), may be under the influence of cyclic nucleotides through the mediation of cyclic nucleotide-dependent protein kinases. A full understanding of the regulation of contraction of smooth muscle will therefore require an understanding of the parameters controlling cyclic nucleotide concentrations in this tissue.

In the present study we have investigated the properties of the enzyme in smooth muscle which is responsible for the degradation of cyclic nucleotides, i.e. 3':5'-cyclic nucleotide phosphodiesterase (EC 3.1.4.17). Since many of the molecular mechanisms of contraction in smooth muscle have been elucidated with gizzard muscle (see, e.g., Ebashi et al., 1975; Sobieszek & Small, 1976; Hartshorne et al., 1977; Adelstein et al., 1978), we have chosen to study the characteristics of phosphodiesterase in this tissue. Several other investigations have shown that in vascular smooth muscle, as in many other tissues, there are multiple forms of phosphodiesterase (Wells et al., 1975; Hidaka et al., 1978; Ilien et al., 1978; Murtaugh & Bhalla, 1979; Keravis et al., 1980). Using chicken gizzard muscle we have identified four forms of the enzyme in the supernatant fraction of tissue homogenates. These isoenzymes differ in their cyclic nucleotide specificities, their kinetics, their calmodulin-dependence, their interactions with verapamil and their electrophoretic mobilities.

Materials and methods

Materials

Cyclic [8-3H]AMP (27 Ci/mmol) and cyclic [8-3H]GMP (6.3 Ci/mmol) were purchased from ICN Pharmaceuticals (Irvine, CA, U.S.A.). Alkaline phosphatase from (type IIIS, Escherichia coli), 5'-nucleotidase (from Crotalus atrox venom), soya-bean trypsin inhibitor, phenylmethylsulphonyl fluoride, leupeptin and antipain were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Dowex AG 1 X8 (formate form) was purchased from Bio-Rad Labs (Rockville Center, NY, U.S.A.). DEAE-cellulose (DE-52) was purchased from Whatman (Clifton, NJ, U.S.A.). Sepharose 4B and 6B were purchased from Pharmacia (Piscataway, NJ, U.S.A.). Verapamil was a gift from Knoll Pharmaceutical Co. (Whippany, NJ, U.S.A.). Chickens were purchased from Mayflower Poultry (Cambridge, MA, U.S.A.).
Calmodulin was prepared from bovine brain and from chicken gizzard by fluphenazine-Sepharose affinity chromatography as described by Head et al. (1982).

Enzyme preparation

All procedures were performed at 4°C unless otherwise noted. Chickens (11-week-old females) were killed by decapitation and the gizzards immediately removed. Surface connective tissue and fat were cleaned from the muscle and the tough inner lining was removed. Cleaned gizzard muscle (150 g) was homogenized in 400 ml of 20 mM-Tris/HCl, pH 7.8, containing 15 mM-2-mercaptoethanol, 1 mM-MgCl₂, 1 mM-imidazole, 1 mM-EGTA, 5 mM-Na₃, 0.1 mM-phenylmethanesulphonyl fluoride and 80 mM-NaCl. The unbound material was washed through with 1 litre of the equilibration buffer and the column then eluted with a linear 80–620 mM-NaCl gradient in a total volume of 1200 ml of the same buffer. The column flow rate was maintained at 70 ml/h, and 9 ml fractions were collected.

Calmodulin-Sepharose affinity chromatography

Appropriate samples from the eluate of the anion-exchange-chromatography column were pooled and adjusted to 10 mM-CaCl₂. The sample was applied to a column (2 cm x 10 cm) of calmodulin-Sepharose equilibrated in 40 mM-Tris/HCl, pH 7.8, containing 15 mM-2-mercaptoethanol, 3 mM-MgCl₂, 2 mM-CaCl₂, 0.1 mM-phenylmethanesulphonyl fluoride, 5 mM-Na₃ and 50 mM-NaCl, and the column clamped off for 30 min. The unbound material was washed through with 125 ml of the equilibration buffer containing an additional 150 mM-NaCl. Elution in the presence of varapamil was achieved with a further 125 ml of this wash buffer including 500 μM-Verapamil. Elution with EGTA was performed also with the wash buffer but including 5 mM-EGTA in place of CaCl₂. The column flow rate was maintained at 120 ml/h, and 9 ml fractions were collected.

Gel-permeation chromatography

This was done in a 1.5 cm x 90 cm column of Sepharose 6B equilibrated in 20 mM-Tris/HCl, pH 7.6, containing 1 mM-MgCl₂, 1 mM-EGTA, 100 mM-KCl, 0.1 mM-phenylmethanesulphonyl fluoride, 5 mM-Na₃, 15 mM-2-mercaptoethanol and bovine serum albumin (0.1 mg/ml). The column was calibrated with proteins of known Mr; thryoglobulin (670000), ferritin (420000), catalase (232000), aldolase (158000), bovine serum albumin (68000), ovalbumin (45000), chymotrypsinogen (25000), myoglobin (17000). Samples (1 ml) were applied and 1 ml fractions collected at a column flow rate of 10 ml/h.

Phosphodiesterase assays

Phosphodiesterase activity was determined by the two-stage procedure of Boudreau & Drummond (1975), by using 45 s of boiling to terminate the phosphodiesterase reaction (Thompson et al., 1974) and AG1-X8 (formate form) to separate the final reaction products.

Tritiated cyclic AMP and cyclic GMP were
purified before use by chromatography on AG 1 X8 resin (formate form). A 2 ml column of resin was equilibrated in 0.1 M-formic acid. Then 200 μCi of tritiated cyclic AMP (27 Ci/mmol) or cyclic GMP (6.3 Ci/mmol) were applied to the column, and unbound material was washed through with 10 ml of the equilibration buffer. Cyclic AMP was subsequently eluted with 2 M- and cyclic GMP with 4 M-formic acid. Purified samples were adjusted to pH 7.6 and stored at -20°C for use within 1 month of purification.

All enzyme samples were dialysed before assay against a dialysis medium of 20 mM-Tris/HCl, pH 7.6, containing 15 mM-2-mercaptoethanol, 1 mM-MgCl2, 5 mM-NaNO3, 0.1 mM-phenylmethanesulphonylfluoride and 50 mM-NaCl. Bovine serum albumin (0.5 mg/tube) was routinely added to all assays. Control assays showed that none of these components or additives at these concentrations affected the measurement of enzymic activity. CaCl2 (0.1 mM) or EGTA (1 mM) was included in the incubation medium when testing for activation of phosphodiesterase by endogenous calmodulin. Added calmodulin (15 μg/tube) was included in the incubation medium when testing for the presence of calmodulin-dependent isoenzymes in the absence of endogenous calmodulin. In preliminary studies we found that the effects of gizzard and bovine brain calmodulin on gizzard phosphodiesterase were qualitatively and quantitatively the same. In the studies described here, we have therefore routinely used the bovine brain protein because of its ready availability in quantity.

Assays of the eluates from the DEAE-cellulose and affinity columns were performed at 5 μM substrate concentrations. Assays to determine inhibition by verapamil were performed at 1 μM substrate. When testing for verapamil inhibition of the calmodulin-activated enzyme, the calmodulin concentrations were lowered to 15 ng/assay tube. This gave 75% of maximum activation of the enzyme and avoided the possible masking of inhibition by supramaximal concentrations of calmodulin. In kinetic studies substrate concentrations ranged from 0.5 to 100 μM; 200 000 c.p.m. of tracer was added per tube, and blank values averaged approx. 3% of the total counts. Total hydrolysis did not exceed 20% of the substrate present.

Protein determination

Protein concentrations were determined with fluorescamine, by the method of Udenfriend et al. (1972) as modified by Sims & Carnegie (1975), with bovine serum albumin as a standard.

Detection of phosphodiesterase on non-denaturing polyacrylamide gels

Enzyme samples were electrophoresed on polyacrylamide slab gels (17 cm × 5.5 cm × 2 mm) in a continuous buffer system of 25 mM-Tris/80 mM-glycine, pH 8.6. The gels contained 10% acrylamide at a bisacrylamide/total acrylamide ratio of either 1:15 or 1:39 (as indicated in the text), and 40% (v/v) glycerol. The gels were prerun at 20 mA for 1 h before application of samples. Samples included 0.01% Bromophenol Blue as tracking dye. When it was established that no rapidly migrating forms of the enzyme were present, the gels containing the 1:39 ratio were routinely run for 1 h after emergence of the Bromophenol Blue. Gels containing the 1:15 ratio were run for a further 2 h. Electrophoresis was run at a constant current of 15 mA.

Staining for phosphodiesterase activity was based on the method of Goren et al. (1971). After electrophoresis the gels were washed for 30 min in 0.1 M-Tris/maleate, pH 7.0, and subsequently incubated overnight in 80 mM-Tris/maleate, pH 7.0, containing 2.5 mM-MgSO4, 3 mM-Pb(NO3)2, 2 mM-cyclic AMP or -cyclic GMP, with 1 unit of alkaline phosphatase/ml. Gels were then washed for 2 h in distilled water, stained with 5% (w/v) (NH4)2S for 2 min and destained in distilled water.

Enzyme stability

Separated isoenzymes could be stored without detectable loss of activity or change in electrophoretic mobility for up to 3 weeks at 4°C in 20 mM-Tris/HCl, pH 7.8, containing 15 mM-2-mercaptoethanol, 1 mM-MgCl2, 5 mM-NaNO3, 0.1 mM-phenylmethanesulphonylfluoride, 50 mM-NaCl and bovine serum albumin (1 mg/ml). If 30% (v/v) ethylene glycol was included in the medium (Thompson et al., 1979a), the enzyme activity was stable at -20°C for several months.

Reproducibility

The four enzymic forms described in this study were each shown to be consistently present over many preparations. The elution profiles (Figs. 1 and 3) and electropherograms (Figs. 2, 4 and 5) are typical results. The kinetic and other data presented have been found to be reproducible by using at least two separate enzyme preparations in each case.

Results

Anion-exchange chromatography

Fractions eluted from the anion-exchange column showed two peaks of phosphodiesterase activity when assayed in the presence of cyclic AMP (Fig. 1). These were designated peak I and peak II respectively. Peak I was eluted at 150 mM-NaCl and, although insensitive to Ca2+ on its own, it was activated 2.5-fold in the presence of Ca2+ and added exogenous calmodulin. The peak-II enzyme was eluted at 220 mM-NaCl and was insensitive to Ca2+ or calmodulin. The endogenous calmodulin in this
preparation was eluted as a single peak at 320 mM-NaCl, as assessed by alkaline gel electrophoresis, with no detectable overlap with the phosphodiesterase-containing fractions.

Enzymic assays of the same eluate fractions in the presence of cyclic GMP showed only one peak of activity, corresponding to the peak-I enzyme for cyclic AMP. This activity was increased 3-fold in the presence of Ca$^{2+}$ and calmodulin.

Electrophoresis on non-denaturing gels of fractions from the anion-exchange column showed several bands when stained for phosphodiesterase activity. Gels stained for cyclic AMP hydrolysis showed the peak-I enzyme to comprise two slowly migrating forms, whereas peak II showed a single, more rapidly migrating, form (Fig. 2).

When stained for cyclic GMP hydrolysis, the gels showed a very similar pattern, including the rapidly migrating band corresponding to the peak-II enzyme. This form of enzyme was not detected by the routine assay procedure at 5 μM substrate because of the very low activity of the enzyme against cyclic GMP at this concentration. However, the activity against cyclic GMP at 2 mM, as used in gel incubations, is far higher (see below, under ‘Kinetic properties of enzymic forms’).

If the peak-I or peak-II enzymes were re-applied separately to the DEAE-cellulose column, each was found to be re-eluted in the salt gradient as a single peak at the same ionic strength as in the original column, indicating that there was no interconversion between the enzymes of the two peaks under these conditions.

**Calmodulin-Sepharose affinity chromatography of peak-I enzyme**

Peak-I enzyme from the anion-exchange column was pooled and adjusted to 10 mM-CaCl$_2$. A portion was applied to a calmodulin-Sepharose affinity column as described in the Materials and methods section. After unbound material had been washed through, the column was eluted with a buffer containing EGTA in place of Ca$^{2+}$. When assayed for cyclic AMP phosphodiesterase activity, the unbound material was found to contain enzyme that was insensitive to Ca$^{2+}$ or calmodulin (Fig. 3a). The EGTA eluate contained enzyme that could be activated approx. 2.5-fold by added calmodulin in the presence of Ca$^{2+}$ (Fig. 3b).
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Fig. 3. Elution profiles of phosphodiesterase activity from the calmodulin–Sepharose affinity column assayed in the presence of 100 μM-CaCl₂ and purified bovine brain calmodulin (∇), or in the presence of 1 mM-EGTA (▲)
(a) Unbound material passing through the column; (b) elution with 5 mM-EGTA of bound activity without prior verapamil elution; (c) elution with 500 μM-verapamil of bound activity; (d) elution with 5 mM-EGTA after verapamil elution of bound activity. For all assays 5 μM-cyclic AMP was used as substrate.

A second portion of the peak-I enzyme was also applied to a calmodulin–Sepharose affinity column, and unbound enzyme was washed through with Ca²⁺-containing buffer, as above. The column was then eluted with Ca²⁺ wash buffer including 500 μM-verapamil. This step eluted an enzyme which, like the unbound form, was also insensitive to Ca²⁺ or calmodulin (Fig. 3c). Subsequent elution of the column with buffer containing EGTA liberated enzyme that could then be activated approx. 6.5-fold by calmodulin in the presence of Ca²⁺ (Fig. 3d).

We are designating the unbound enzyme from the affinity columns as form Ia, the enzyme eluted by verapamil form Ib, and that form eluted by EGTA after the verapamil elution Ic.

After dialysis, both forms Ib and Ic were found to bind to calmodulin affinity columns when re-applied separately. This indicates that each enzyme has a calmodulin-binding site and also that dialysis adequately removes verapamil from form Ib. Form Ic could only be eluted from the column by EGTA, whereas form Ib could be eluted by either verapamil or EGTA. Verapamil-gradient elution of form Ib from the calmodulin affinity column showed the enzyme to be eluted as a broad peak at verapamil concentrations ranging from 130 to 280 μM, with a maximum at approx. 200 μM.

Peak-II enzyme from the anion-exchange column remained unbound when applied to the calmodulin–Sepharose column.

To determine whether any form of phosphodiesterase might bind in a non-specific manner to Sepharose matrices, we also applied the enzymes to a column of bovine serum albumin covalently bound to Sepharose. None of the enzymic forms were bound by this resin.

As described above, the peak-I enzyme from the anion-exchange-chromatography column migrated as two bands when stained for phosphodiesterase activity after electrophoresis on 10% polyacrylamide gels with 1:39 bisacrylamide/acrylamide ratio (Fig. 4, gel a). Electrophoresis of the affinity-column-purified enzymic forms showed Ia to correspond to the upper band (Fig. 4, gel b), and Ib and Ic to co-migrate at the position of the lower band (Fig. 4, gels c and d). Although forms Ib and Ic could not be distinguished under these conditions, they could be resolved by increasing the bisacrylamide/acrylamide ratio to 1:15 (Fig. 5). The enzyme eluted from the affinity column by EGTA
without a prior verapamil elution contained both forms Ib and Ic (Fig. 5, gel e).

Gel-permeation chromatography

The molecular weights of the four forms of gizzard phosphodiesterase were determined by gel-permeation chromatography on Sepharose 6B, as described in the Materials and methods section. The apparent \( M_r \) values of the peak-I enzymes were all similar: form Ia 114 000, form Ib 116 000, form Ic 122 000. Form II had an apparent \( M_r \) of 59 000. The lower \( M_r \) of the form-II enzyme is consistent with the more rapid migration of this enzyme on non-denaturing gel electrophoresis.

Kinetic properties of enzymic forms

The rates of hydrolysis of cyclic AMP and cyclic GMP were determined at a range of substrate concentrations for each of the forms of phosphodiesterase. Form Ic was assayed in the presence and absence of calmodulin. Data were analysed graphically by the Hanes plot. Linear relationships were obtained for all but two of the ten data sets. The hydrolysis of cyclic AMP by form-Ia enzyme and the hydrolysis of cyclic GMP by the form-II enzyme were biphasic in the substrate range studied (Figs. 6a and 6c).

The apparent \( K_m \) and \( V_{max} \) values determined for the various isoenzymes with the two substrates are shown in Table I. At substrate concentrations approaching physiological values, e.g. 1 \( \mu \)M, the two forms not bound by the calmodulin affinity column (Ia and II) showed different relative activities against cyclic AMP and cyclic GMP. The form-II enzyme showed a 20-fold higher activity towards cyclic AMP than towards cyclic GMP, whereas the form-Ia enzyme had 5-fold higher activity towards cyclic GMP.

The two enzymes binding to the calmodulin affinity column (Ib and Ic) each showed a 2–3-fold preference for cyclic GMP over cyclic AMP in the absence of \( \text{Ca}^{2+} \) and calmodulin (at 1 \( \mu \)M substrate). Although the form-Ib enzyme was able to bind to calmodulin–Sepharose in a \( \text{Ca}^{2+} \)-dependent manner, its activity was unaffected by \( \text{Ca}^{2+} \) or calmodulin. The form-Ic enzyme was activated by calmodulin in the presence of \( \text{Ca}^{2+} \). However, the mechanism of activation of form Ic by calmodulin appeared to differ, depending on the substrate. With cyclic GMP as substrate, calmodulin principally changed the \( K_m \) of the enzyme, having a more modest effect on \( V_{max} \).

At 1 \( \mu \)M-cyclic GMP this was reflected as an 11-fold activation by calmodulin. With cyclic AMP as substrate, calmodulin altered mainly the \( V_{max} \), leaving the \( K_m \) little changed. At 1 \( \mu \)M-cyclic AMP the enzyme was activated 4-fold by calmodulin.
Fig. 6. Hanes plots of kinetic data for enzyme forms Ia and II
(a) Form Ia with cyclic AMP as substrate; (b) form Ia with cyclic GMP as substrate; (c) form II with cyclic GMP as substrate; (d) form II with cyclic AMP as substrate. Continuous lines represent the best fit determined by linear regression of the data shown. \( \nu \) is initial velocity, in nmol/min per mg.
Table 1. Summary of kinetic data for all four forms of phosphodiesterase

Values were calculated from linear regressions of experimentally determined data. For enzymic forms showing linear kinetics, the linear regression gave correlation coefficients >0.99. For enzymes showing biphasic kinetics, pairs of numbers are quoted representing values determined for each phase. The point of departure of one phase from the other was determined by inspection. $V(1 \mu M) =$ rate of hydrolysis at 1 $\mu M$ substrate concentration.

<table>
<thead>
<tr>
<th>Form</th>
<th>$K_m$ (\mu M)</th>
<th>$V_{max}$ (nmol/min per mg)</th>
<th>$V(1 \mu M)$ (nmol/min per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cyclic AMP</td>
<td>Cyclic GMP</td>
<td>Cyclic AMP</td>
</tr>
<tr>
<td>Ia</td>
<td>174.6</td>
<td>2.9</td>
<td>78.4</td>
</tr>
<tr>
<td></td>
<td>5.5</td>
<td>425.8</td>
<td>9.0</td>
</tr>
<tr>
<td>Ib (+ Ca$^{2+}$-calmodulin)</td>
<td>16.1</td>
<td>0.6</td>
<td>517.5</td>
</tr>
<tr>
<td>Ic (+ EGTA)</td>
<td>13.4</td>
<td>10.3</td>
<td>98.4</td>
</tr>
<tr>
<td>II</td>
<td>1.8</td>
<td>11.2</td>
<td>4.0</td>
</tr>
</tbody>
</table>

Hence, under these conditions form Ic showed a 6-fold preference for cyclic GMP over cyclic AMP.

Cyclic GMP did not activate the hydrolytic activity of any of the enzymic forms against cyclic AMP when assayed at 1 $\mu M$ or 5 $\mu M$-cyclic AMP with cyclic GMP present at concentrations in the range 0.05–1 $\mu M$.

Effects of verapamil on enzymic activity

The effect of increasing concentrations of verapamil on the activities of the four phosphodiesterases are shown in Fig. 7. The rate of hydrolysis of cyclic AMP by form II was found to be the most sensitive, with an IC50 (concentration required to give 50% inhibition) of approx. 30 $\mu M$ (Fig. 7). With cyclic GMP as substrate only weak inhibition was observed with any of the forms, none having an IC50 of less than 500 $\mu M$.

Although form Ib could be eluted from the calmodulin-Sepharose affinity column by 200 $\mu M$-verapamil, the drug had only slight effect on the activity of the enzyme against either cyclic AMP or cyclic GMP at this concentration, and failed to reach 50% inhibition even at 1 mM.

The activity of the calmodulin-dependent enzyme (Ic) was little affected by verapamil in the presence or absence of calmodulin, with either cyclic AMP or cyclic GMP as substrate.

Discussion

We have reported here the separation and partial characterization of four forms of cyclic nucleotide phosphodiesterase from the cytosolic fraction of chicken gizzard muscle. Three of the forms co-eluted from anion-exchange chromatography, but were further resolved by chromatography on calmodulin-Sepharose. Alkaline non-denaturing gels stained for phosphodiesterase showed single discrete bands of activity corresponding to each of the isoenzymic forms.

The relationship of these forms to the multiple forms of phosphodiesterase described by others in vascular smooth muscle and in other tissues remains to be determined. However, certain similarities are evident on the basis of the results already obtained.

The high-salt elution of the gizzard form-II enzyme from anion-exchange chromatography and the $M_r$ and kinetic properties of this form appear to correspond most closely to the $M_r$-60000 high-affinity cyclic-AMP-specific enzyme of kidney (Thompson et al., 1979b; Epstein et al., 1982a). The
biphasic kinetics of the enzyme with cyclic GMP and the verapamil-sensitivity observed in the present study have also been reported for the kidney enzyme (Epstein et al., 1982a,b). Similar forms of phosphodiesterase have been identified in vascular smooth muscle (Wells et al., 1975) and other tissues (see review by Beavo et al., 1982). Although the $M_t$ of form II is approximately half that of the peak-I enzymes, we have found no evidence that it is able to dimerize to generate any of the peak-I forms.

The $K_m$ values of the calmodulin-activatable gizzard enzyme (Ic) appear to correspond more closely to those of the high-affinity class of calmodulin-sensitive phosphodiesterases, as reported by Purvis et al. (1981) in rat testis, Smoake et al. (1981) in liver, Andrenyak & Epstein (1982) in chick heart and Vandermeers et al. (1983) in rat pancreas, rather than the low-affinity form found in bovine brain (Klee et al., 1979; Morrill et al., 1979; Sharma et al., 1980) and heart (Ho et al., 1977; LaPorte et al., 1979). The precise changes in kinetic properties observed in the present study with changes in substrate and calmodulin do not correspond exactly to those described previously for the high- or low-affinity enzymes. The $M_t$ determined for the gizzard form-Ic enzyme is similar to that reported for bovine brain calmodulin-dependent phosphodiesterase (Klee et al., 1979; Morrill et al., 1979; Sharma et al., 1980) and the $P_{II}$ form of this enzyme from rat pancreas (Vandermeers et al., 1983).

The form-Ia enzyme, which is eluted from the anion-exchange column in 'peak I' and is unbound from the calmodulin–Sepharose column, may be related to the carotid-artery 'type B' enzyme described by Murtaugh & Bhalla (1979). In addition to being eluted from DEAE-cellulose at low ionic strength, both enzymes have a marked preference for cyclic GMP at low substrate concentrations. However, the biphasic kinetics found in the present study with cyclic AMP were not described for the carotid enzyme.

The relationship of the form-Ib enzyme to the other forms of phosphodiesterase, in gizzard or in other tissues, is uncertain. This enzyme binds to calmodulin in a Ca$^{2+}$-dependent manner, but is not activated by calmodulin. Previous studies have reported proteolysis of calmodulin-dependent enzyme to produce an enzymic form which is maximally activated and insensitive to calmodulin (Tucker et al., 1981). Although we cannot yet preclude this as a possible source of the form-Ib enzyme, we have attempted to minimize proteolysis by inclusion of proteinase inhibitors in our preparations and have observed no conversion of the calmodulin-activated form-Ic enzyme into form Ib during prolonged storage after isolation. Keravis et al. (1980) have reported that the calmodulin-activatable form of coronary-artery phosphodiesterase can reversibly aggregate to give a poorly activatable enzyme, and Vandermeers et al. (1983) have also described the formation of a higher-$M_t$ form of the pancreas enzyme on storage. We believe that such effects are unlikely to lead to the presence of our form-Ib enzyme, since, as noted, we see no evidence of interconversion between forms Ib and Ic, and the $M_t$ values of the two enzymes are similar.

Although verapamil is principally recognized as a blocker of slow Ca$^{2+}$ channels in the sarcolemma of heart and smooth muscle, it has previously been shown to affect the activity of phosphodiesterase in several tissues (Epstein et al., 1982b). In the present study we have shown that, in addition to inhibiting the form-II enzyme, verapamil selectively disrupts the interaction of the Ib form of the enzyme with calmodulin. Calmodulin has been shown to expose a hydrophobic region on binding Ca$^{2+}$, and this region has been suggested as a site of interaction of calmodulin with target enzymes (LaPorte et al., 1980; Tanaka & Hidaka, 1980). It is possible therefore that the Ib enzyme binds to this region of calmodulin through weak hydrophobic interactions, which are disrupted by the lipophilic verapamil, whereas the binding of the form-Ic enzyme, through strong hydrophobic or other interactions, remains unaffected by the drug. Binding of Ca$^{2+}$ antagonists to calmodulin has previously been reported (Bostrom et al., 1981; Johnson et al., 1982). Differences in the relative hydrophobic affinities of two forms of bovine brain calmodulin-dependent phosphodiesterase have also been noted by Gopalakrishna & Anderson (1983). Whether the interaction of calmodulin with the gizzard Ib enzyme has any physiological importance remains to be determined.

The concentrations of verapamil observed to inhibit the form-II enzyme and to elute the form-Ib enzyme from calmodulin–Sepharose are higher than those expected to bring about the pharmacological actions of the drug. However, studies by Lullman et al. (1979) and Pang & Sperelakis (1983) have shown that heart muscle and ileal smooth-muscle cells are able to accumulate verapamil against low extracellular concentrations. Although most of the pharmacological effects of verapamil probably result from action at the plasma membrane, it remains possible that intracellular concentrations of the drug in some cell types could be sufficient to affect phosphodiesterase activity.

Whether the presence or absence of various cofactors or subunits could account for some of the isoenzyme forms of phosphodiesterase described in this study, or whether one form might be a phosphorylated derivative of another (Sharma et al., 1980; Marchmont & Houslay, 1981), remains to be
determined. However, it is evident that each form has distinct kinetic properties and may therefore have a distinct physiological role.

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