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SEPARATION OF THREE CYCLIC-NUCLEOTIDE-PHOSPHODIESTERASES FROM BOVINE AORTA

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SUMMARY - Three distinct forms of cyclic-nucleotide-phosphodiesterases were separated and purified from the media layer of bovine aorta using two successive DEAE-cellulose column chromatographies. Form A hydrolyzed both cAMP and cGMP with similar K_m and V_{max} and was sensitive to the calcium dependent protein activator ; its activity on both substrates was inhibited by cIMP. Form B hydrolyzed specifically cGMP, was insensitive to the activator but was slightly stimulated in a proteinaceous medium ; it was inhibited by cIMP. Form C was specific for cAMP and was insensitive to the activator and to a proteinaceous medium ; it was poorly inhibited by cIMP.

INTRODUCTION - Multiple forms of cyclic nucleotide phosphodiesterases have been described in various tissues (for reviews see ref. 1 and 2). These forms differ in their kinetic properties, substrate specificity and sensitivity to endogenous or exogenous activators and inhibitors (3). We do not know whether the phosphodiesterases of intact cells are identical to the isolated enzymes. However, purification of phosphodiesterases is a prerequisite to the search for specific inhibitors. The finding of such compounds may in turn provide a clue to the understanding of the role of phosphodiesterases in regulating cyclic nucleotide levels. Although cAMP and cGMP may well be important regulators of vascular smooth muscle properties (4), few studies deal with the phosphodiesterases from these tissues (5, 6). Two forms using cAMP and cGMP respectively as prefered substrate have been separated from pig coronary arteries (6). The fact that a selective accumulation of cAMP could be produced in rat aorta by a phosphodiesterase inhibitor (7) suggests that the hydrolysis of cAMP and cGMP may be catalyzed by different enzymes in this organ. We report here that, in addition to two phosphodiesterase forms which were specific for cAMP and cGMP respectively, we have separated a third form which displayed no substrate specificity. Contrary to the first two forms this latter form was sensitive to a Ca^{2+} dependent endogenous protein activator. However its catalytic propertied differed from those of the

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activator-sensitive enzyme previously isolated by others (1).

MATERIAL and METHODS - Bovine aortas were obtained from a local slaughterhouse and kept until dissection (within 3 hours) at +4°C in an hypotonic medium containing 2 mM $MgCl_2$, 0.2 mM EGTA, 3 mM β mercaptoethanol and 20 mM Tris-HCl pH 7.5. The following operations were performed at 4°C. The media layer was dissected, 200 g were minced and homogenized using a glass potter homogenizer in 4 volumes of the hypotonic medium. The homogenate was filtered on gauze and centrifuged at 3 000 x g for 20 min. The supernatant was centrifuged at 105,000 x g for 60 min. The pellets were discarded and the final supernatant was precipitated by the addition of solid ammonium sulfate up to 60 % saturation. The pellet obtained by centrifugation at 10,000 x g for 20 min was resuspended in 20 ml hypotonic medium and dialyzed for 36 hours against 6 x 500 ml medium in order to eliminate ammonium sulfate. An aliquot of this preparation containing 600 mg protein was applied to a DEAE-cellulose column (3 x 23 cm) previously equilibrated with the extraction buffer. The enzymes were eluted from the column, using a linear gradient of sodium chloride (0.0 to 0.6 M ; with 2 mM $MgCl_2$, 0.2 mM EGTA, 3 mM β mercaptoethanol and 20 mM Tris-HCl pH 7.5 ; total volume, 2 000 ml). Protein was measured according to Lowry et al. (8) using bovine serum albumin as standard.

Phosphodiesterase activities were assayed using a modification (9) of the method of Thompson and Appleman (10). This method measures ^3H -products resulting from the successive incubations of ^3H -cyclic AMP or GMP with phosphodiesterase and with an excess of 5'nucleotidase. An anion exchange resin was used in a batch process to remove the residual substrate after the end of the enzyme reactions. In the experiments reported here, ^{14}C -adenosine or guanosine was added to each test tube before the resin in order to measure the recovery of the nucleoside and to correct each result for the binding of the reaction products to the resin. Routinely 1 µM substrate was used with 0.2 mM EGTA (basal activity) or with 0.2 mM $CaCl_2$ and 100 µg protein of calcium dependent activator prepared from bovine aorta according to the procedure of Cheung described for rat brain (11). Blanks were made with boiled aliquots accounting for the analytical interference of NaCl in eluted fractions. The calcium dependent protein activator activity was tested on boiled (1 min, 100°C) aliquots of the eluted fractions, using the activable phosphodiesterase preparation (peak A described below) obtained in previous experiments.

RESULTS. The 105,000 x g supernatant of bovine aorta displayed both cAMP and cGMP phosphodiesterase activities, with sensitivity to the calcium-dependent protein activator. Two peaks of cAMP hydrolyzing activity were resolved by DEAE-cellulose chromatography (fig. 1, lower graph). Peak A (fractions 50 to 80) had a slight activity but was highly activable. Peak C (fractions 95 to 120) exhibited a higher activity but was pratically insensitive to the activator. The intermediate fractions between peaks A and C (peak B, fractions 80 to 95) hydrolyzed cAMP poorly but were very active when cGMP was used as substrate (fig. 1, middle graph). Fractions 120 to 160 contained the totality of a protein activator with properties similar to those of the brain activator isolated by Cheung (11).

Peaks A, B and C were submitted to further purification by DEAE-cellulose column chromatography, using a different NaCl gradient for each enzyme preparation (fig. 2). Peak A of the first column led to a single

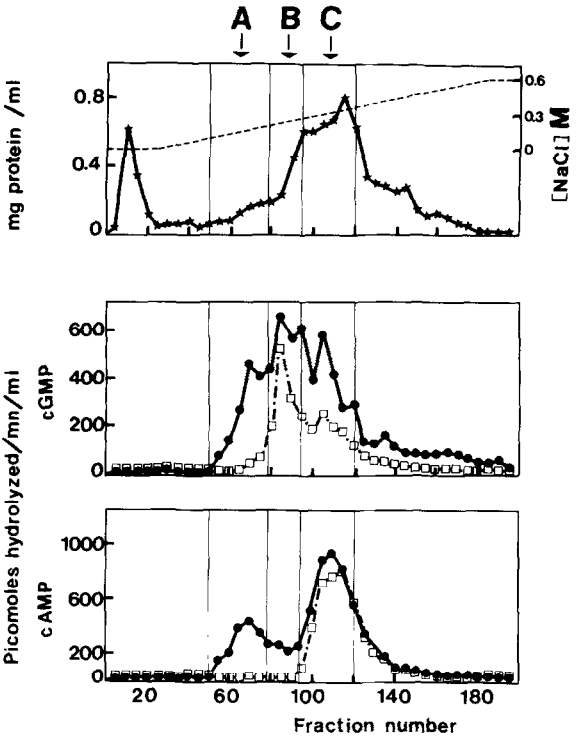

Fig. 1 - First DEAE-cellulose column chromatography of soluble phosphodiesterase preparated from the media layer of bovine aorta according to "Material and methods" - Activities were measured using as substrate 1 µM cGMP (middle plot) or cAMP (bottom plot), in absence (□) or in presence (●) of the calcium-dependent protein activator.

peak ("form A") with both activable cAMP and cGMP hydrolyzing activities. Peak B was resolved into two peaks, one (fractions 17 to 30) hydrolyzing both cAMP and cGMP, similar to peak A, and the other (from fraction 30) with specific cGMP activity ("form B"). A similar purification was obtained with peak C where fractions 15 to 35 contained both activities whereas following fractions ("form C") exhibited a specific cAMP phosphodiesterase activity. The activity of form A and, to a much lesser extent, the activity of form B, increased in the presence of the activator preparation and Ca^{2+} (fig. 1 and 2). However, the apparent activation of form B could be achieved in a proteinaceous medium (12-14) independently of the presence of Ca^{++} (data not shown). Form A was thus the only form sensitive to the Ca^{2+}dependent activator, whereas form B was only slightly stimulated by added proteins.

The kinetic properties of the purified phosphodiesterase forms were

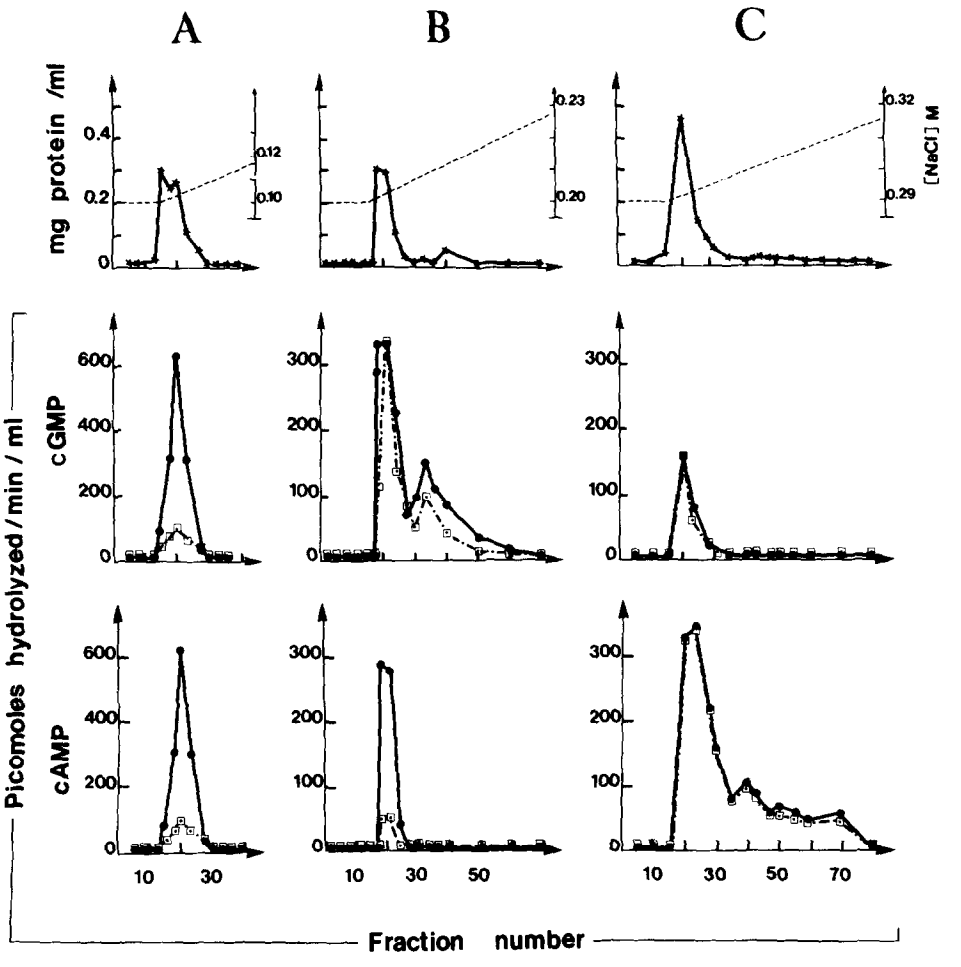

Fig. 2 - DEAE-cellulose column chromatographies of fractions A, B and C isolated from bovine aorta according to fig. 1 The collected tubes from the first chromatography (fig. 1) were pooled into 3 fractions A, B and C separately. There fractions were dialysed to eliminate NaCl, concentrated by diaflo ultrafiltration using an Amicon PM 30 membrane, and applied on DEAE-cellulose columns. The elutions were performed with NaCl linear gradients (see upper plots).

examined over a wide range of substrate concentrations (table 1). The three forms displayed non linear kinetics characterized by two apparent K_m values ("high" and "low"). This type of kinetics has already been observed on many phosphodiesterase preparations (for discussion see review ref. 1). The low apparent K_m values of the three forms were of the same order of magnitude, but the high apparent K_m values of forms B and C were higher than those of form A. The calcium-dependent protein activator in-

TABLE I

Kinetic parameters and inhibition by cyclic nucleotides of bovine aorta phosphodiesterases

Parameters*	Form A		Form B	Form C
	cAMP	cGMP	cGMP	cAMP
- Km (μM)				
without activator	15.0 *and* 1.7	13.0 *and* 2.2	100 *and* 7	70 *and* 3
with activator	7.0 *and* 1.6	6.5 *and* 1.4	-	-
- Vmax (picomoles/min/mg prot.)				
without activator	2,890 *and* 1,125	2,075 *and* 1,125	5,000 *and* 1,430	5,450 *and* 1,175
with activator	9,000 *and* 5,630	8,590 *and* 5,925	-	-
- I_{50} (μM) ***				
cyclic IMP	4	5	1	165
cyclic AMP	-	-	10,000	-
cyclic GMP	-	-	-	10,000

*The parameters were determined with purified fractions from the second chromatographies (fig. 2). Activities were measured as described in "Material and methods". Km and Vmax were extrapolated from Lineweaver and Burk plots : substrate concentrations ranging from 0.5 to 50 μM gave rise to two linear areas one between 0.5 μM and 5 μM substrate and the other between 10 μM and 50 μM substrate, thus allowing the calculation of two V_{max} and two appparent K_m for each enzyme.

***I_{50} is the concentration of compound required to give 50 % inhibition of phosphodiesterase activity. It was determined using 1 μM substrate and various concentrations of inhibitors it was calculated by interpolating two values of inhibition ranging from 35 to 75 %, against the logarithm of inhibitor concentration.

creased the V_{max} and decreased the high apparent K_m values of form A, which hydrolyzed both cAMP and cGMP with identical apparent K_m and V_{max}.

The inhibitory effects of cIMP, cAMP and cGMP were also tested on the purified forms (table 1). cIMP acted in the micromolar range to inhibit forms A and B, but was poorly effective on form C. It should be emphasized that it inhibited the hydrolysis of cAMP and cGMP by form A identically at a concentration which was not effective in inhibiting the hydrolysis of cAMP by form C. Form B and formC were respectively inhibited by the substrate of the other, but at very high concentrations only.

DISCUSSION - The three cyclic nucleotide phosphodiesterases that we obtained from bovine aorta may be compared to the enzymes previouly separated

from various tissues (1, 2), especially arteries (6).

Form A obtained here was activator-sensitive but its properties differed from those of the activator-sensitive enzyme which has been separated from many tissues (for review see ref. 1), including pig coronary arteries (6). This latter enzyme is often referred to as the "high K_m" phosphodiesterase since it exhibits linear kinetics, with a lower K_m for cGMP than for cAMP (in the range of 1 and 100 µM respectively), and a higher V_{max} for cAMP than for cGMP. In contrast, form A from bovine aorta was characterized by its lack of specificity for cAMP or cGMP as substrates. The finding that its activity on both substrates was inhibited by cIMP further documents the lack of specificity of form A for cyclic nucleotides, since cIMP has previously been shown to decrease specifically cGMP hydrolysis by guinea pig lung phosphodiesterase (15) and was indeed found here to be poorly effective in inhibiting the hydrolysis of cAMP by form C. Forms B and C were not activator-sensitive but showed a high degree of specificity for cGMP and cAMP respectively : Each of these forms not only selectively catalyzed the hydrolysis of its specific substrate, but also failed to be inhibited by the substrate of the other form except at very high concentrations. In addition, form B was for more sensitive to cIMP inhibition than form C. Thus, it may be concluded that the selectivity of the hydrolysis catalyzed by these two forms resulted from a selective affinity for cyclic nucleotides.

The presence of a phosphodiesterase with selective cAMP activity has been reported for every studied mammalian tissue, including blood vessels (6). This form is variously described as the "low K_m" or "negative cooperative" enzyme (1). The properties of form C described here were quite similar to those of this enzyme. As in other tissues, it was also found in membranous preparations from bovine aorta (6). On the other hand, a phosphodiesterase insensitive to the calcium dependent activator and showing selectivity for cGMP hydrolysis has only been separated from lung (12,13,14,16). This latter enzyme and our form B have quite similar properties.

With respect to vascular smooth muscle, it should be mentioned that two enzyme forms only were separated from pig coronary arteries (6). One had the properties of the activator-sensitive form with a lower K_m for cGMP than for cAMP. The other was relatively specific for cAMP and was this is comparable to our form C. We do not know whether the differences between these results and those reported here correspond to differences between pig coronary arteries and bovine aorta or to the differences in the separation procedures. It might be argued that form A appeared during our separation

procedure as the result of an interconversion phenomenon (17, 18) of form B, or of forms B and C, to A. However we verified that form A did not appear when the same procedure was applied to bovine lung. Lung of different species has been reported to be devoid of activator-sensitive phosphodiesterase (12, 14, 16). In this case we only obtained two forms which were quite similar to forms B and C (data not shown).

In conclusion, the separation and characterization of three phosphodiesterase forms from bovine aorta represents a step towards the analysis of the regulation of the cyclic nucleotide level in this tissue. The finding that, under the same conditions, two forms only (one specific for cAMP, the other for cGMP) could be obtained from lung whereas one additional form (activator-sensitive but showing no substrate specificity) was separated from aorta of the same species is consistent with the idea that different tissues contain various proportions of the different phosphodiesterases

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