Isoelectric-focusing patterns of cyclic nucleotide phosphodiesterase from rat heart

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1. Isoelectric focusing on a flat gel bed of the rat heart cytosolic fraction resolved cyclic nucleotide phosphodiesterase activity into several forms, characterized by their substrate specificity, kinetic constants and dependence towards Ca^{2+} and calmodulin. A peak of pI 4.9 displayed 20 times more affinity for cyclic GMP than for cyclic AMP and was markedly inhibited by EGTA. A less substrate-specific form, only slightly sensitive to EGTA inhibition, focused at pH 5.45. Several overlapping peaks detected between pH 5.55 and pH 6 specifically hydrolysed cyclic AMP, with non-Michaelian kinetics; these peaks were insensitive to Ca^{2+} chelation. 2. Isoelectric focusing did not dissociate enzyme-calmodulin complexes, as none of the resulting peaks was activatable by calmodulin plus Ca^{2+} . 3. Some new information on rat cardiac phosphodiesterase is obtained with this technique, which is convenient for routine analytical studies of phosphodiesterase, as well as for preparative purposes.

Cyclic nucleotide phosphodiesterase (EC 3.1.4.17) exists in multiple forms in virtually every tissue examined (Wells & Hardman, 1977), but the number and characteristics of the isolated forms may sometimes be influenced by conditions and techniques of separation used (Van Inwegen *et al.*, 1976). Therefore the comparison of fractionation patterns and of properties of the enzymic forms obtained by means of various procedures is of major interest for a better elucidation of the actual state of phosphodiesterase in the cell, as has been emphasized by many authors (e.g. Thompson *et al.*, 1979a). We have been interested for several years in heart phosphodiesterase, since it is a possible target for some cardioactive drugs (Kline & Buckley, 1972; Némóz *et al.*, 1978; Prigent *et al.*, 1979). Mammalian heart phosphodiesterases have been investigated by several groups, who generally employed classical techniques. Most studies concerned the bovine heart enzyme, which was analysed by DEAE-cellulose chromatography (Hrapchak & Rasmussen, 1972; Donnelly, 1976; Ho *et al.*, 1976), gel filtration and electrophoresis (Goren & Rosen, 1972). Affinity chromatography on immobilized inhibitors was also attempted (Mohindru *et al.*, 1978). However, discrepancies appeared between the various results reported. The rat heart phosphodiesterase has been principally studied by DEAE-cellulose chromatography

(Appleman & Terasaki, 1975; Terasaki & Appleman, 1975; Donnelly, 1978). We have undertaken the study of this enzyme by isoelectric focusing, a technique relatively rarely employed in phosphodiesterase study. The main examples of its use are rat brain enzyme fractionation on a column device (Pledger *et al.*, 1974, 1975; Minneman, 1976). To our knowledge a single report of isoelectric focusing on a flat gel bed of rat brain phosphodiesterase exists (Lindl & Chapman, 1976). We chose this latter process since, besides greater convenience, it also permits either preparative or analytical work. Similarities and discrepancies between isoelectric forms and the DEAE-cellulose forms described by Appleman & Terasaki (1975), Terasaki & Appleman (1975) and Donnelly (1978) are considered.

Experimental

Chemicals

Cyclic [^3H]AMP (20–30 Ci/mmol), cyclic [^3H]GMP (10–30 Ci/mmol), [^3H]adenosine (20–25 Ci/mmol), [^3H]guanosine (5–15 Ci/mmol) and [^{14}C]inosine (577 mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Snake (*Ophiophagus hannah*) venom, unlabelled cyclic nucleotides and EGTA were pur-

chased from Sigma Chemical Co. AG1X2 resin (200–400 mesh) was obtained from Bio-Rad Laboratories. Sephadex G-75 was from Pharmacia Fine Chemicals. Ampholines PAG plates and Ampholine carrier ampholytes were supplied by LKB Produkter.

Preparation of a cytosolic fraction of rat heart muscle

Male Sprague–Dawley rats weighing about 250 g were killed by decapitation. The hearts were removed and perfused with 0.32 M-saccharose in 1 mM-Tris/HCl buffer (pH 7.4) to remove blood. The fresh muscle was homogenized with a glass/glass Potter homogenizer in 3 vol. (v/w) of the above buffer (an iso-osmotic medium was used for homogenization to decrease an eventual release of proteinases caused by lysis of lysosomes). The homogenate was immediately centrifuged at 20 000 g for 15 min. The supernatant was then centrifuged at 100 000 g for 1 h. The 100 000 g supernatant was stored at -20°C .

Isoelectrofocusing procedures

Analytical polyacrylamide plates. LKB plates, pH 3.5–9.5 or pH 4–6.5, received 400 μl of heart preparation (3 mg of protein). Sample droplets were applied directly on the gel surface. The plate temperature was maintained at 4°C . The migration distance was 9.5 cm. After completion of the focusing, the plate was sliced into 2.5 mm-wide bands, the pH of which was measured. The bands were then ground in 2.5 ml of 160 mM-Tris/HCl buffer (pH 8), kept at 4°C overnight, and the phosphodiesterase activity of the suspension was assayed.

Preparative gel plates. The gel was prepared in distilled water with 5 g of extensively washed Sephadex G-75, 3.5 ml of Ampholines pH 4–6 and 1.5 ml of Ampholines pH 5–8, layered on a 12.5 cm $\times$ 26 cm plate; 25–50 mg of protein was applied. The migration was along the large dimension, at 4°C . At the end of the experiment, the gel was sliced into 8 mm-wide bands. The pH of each band was determined, and the proteins were eluted from the gel with 5 ml of 160 mM-Tris/HCl buffer (pH 8). The eluates were stored at -20°C and used as enzyme source for characterization studies.

Phosphodiesterase assay

Cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase activities were assayed by a two-step radioisotopic procedure based on that described by Thompson *et al.* (1974), as modified by Boudreau & Drummond (1975). In preliminary control experiments, we made sure of the validity of this procedure in our experimental assay conditions. (i) The

acidification of resin slurry (AG1X2 resin suspended in 15 mM-acetic acid for cyclic AMP phosphodiesterase assay and in 115 mM-formic acid for cyclic GMP phosphodiesterase assay) allowed a good recovery of nucleosides, in the same range of magnitude for both inosine (85%) and adenosine (75%), and slightly better for guanosine (95%). These nucleoside recoveries were systematically measured by using radiolabelled adenosine, inosine and guanosine in all experimental assay conditions, and were shown not to vary significantly within an experiment and between experiments at fixed pH and ionic-strength conditions. Especially, cyclic nucleotide concentrations in assays, ranging from 0.25 to 100 μM , did not significantly modify recoveries of nucleosides. Phosphodiesterase activities were systematically corrected for these recoveries. (ii) In control experiments, with phosphodiesterase of crude rat heart supernatant and 0.25 μM -cyclic AMP, a good agreement was observed, up to 40% substrate hydrolysis, between results obtained by means of the acidic-resin batch method and with polyethyleneimine–cellulose t.l.c. of the assay medium performed as described by Böhme & Schultz (1974), which is considered as a highly reliable reference method. In the above assay conditions, the formation of inosine by contaminating adenosine deaminase activity, which became no longer negligible when substrate hydrolysis was higher than 15% (inosine/adenosine ≥ 0.05), did not cause any underestimate of phosphodiesterase activity. In our usual experiments, no more than 15% of the substrate was hydrolysed in an assay. (iii) With the crude rat heart supernatant, as well as with the separated enzymic forms, a good linearity of phosphodiesterase activity versus time was observed throughout the duration of the assay.

A heated 100 000 g supernatant from rat cerebral cortex was used as calmodulin source (Egrie *et al.*, 1977). The stimulating effect of this preparation was evaluated on a rat brain phosphodiesterase (100 000 g supernatant), with 25 μM -cyclic AMP and 0.1 mM exogenous Ca^{2+} . A dose-dependent stimulation was found over a range of 2–20 μg of protein/assay, with a plateau of 170% stimulation from 20 to 160 μg of protein/assay, whereas EGTA (1 mM) gave 45% inhibition. Protein was assayed by the Lowry method, with bovine serum albumin as standard.

Computer-aided curve-fitting procedures

Estimations of K_m and V_{max} for the various enzyme forms were performed by an iterative curve-fitting procedure, based on the method of Marquardt (1963). The experimental data were fitted to the following models: Michaelian, sum of two Michaelian enzymes, one Michaelian enzyme plus one component proportional to substrate concentra-

tion, and Hill model, by a non-linear least-squares computer program. Statistical analysis of the results was performed as described by Draper & Smith (1966).

Results

Cyclic nucleotide phosphodiesterase of the rat heart cytosolic fraction was at first investigated

by isoelectrofocusing on a polyacrylamide plate, within the pH range 3–9. All the activity was detected in the pH 4.5–6.5 region (results not shown). The experiments were then performed again in the pH 4–7 range. With several heart phosphodiesterase preparations, a reproducible focusing pattern was obtained on a polyacrylamide gel bed as well as on a Sephadex gel bed (Fig. 1).

Fig. 1. Typical isoelectric-focusing profile of cytosolic cyclic nucleotide phosphodiesterase from rat heart, obtained on a Sephadex flat gel bed

Activity was measured with 0.25 μM (a) or 25 μM (b) cyclic nucleotide (○, cyclic AMP; ●, cyclic GMP). Experiments performed with five different enzyme preparations gave similar results, on Sephadex G-75 or polyacrylamide supporting media. The phosphodiesterase activity recoveries were 50–60 and 40–50% with substrate cyclic AMP and cyclic GMP respectively. ...., pH.

When measured at low cyclic AMP concentration ($0.25\mu\text{M}$), phosphodiesterase activity was found between pH 5.2 and 6.7. The most basic peak appearing in Fig. 1 was identified as a non-migrating fraction of activity: its position strictly depended on the position of sample application. It most likely represented a precipitate of a cyclic AMP-specific form; precipitation of phosphodiesterase in pH gradients has previously been reported (Gardner *et al.*, 1978). The amount of non-focused material was greatly decreased by applying the enzyme in a neutral zone, after formation of the pH gradient. Several overlapping peaks hydrolysing specifically cyclic AMP were closely focused at pH 5.55, 5.7 and 6. By contrast, most of the cyclic GMP phosphodiesterase activity measured at low substrate concentration was found between pH 4.8 and 5.6. One of the two major cyclic GMP-hydrolysing peaks migrated to pH 5.45. This peak also hydrolysed cyclic AMP in a non-negligible fashion. Another major peak and a minor one appearing as a shoulder were detected at pH 4.9 and 5.2 respectively. These latter peaks were almost completely devoid of cyclic AMP-hydrolysing activity. At high substrate concentration ($25\mu\text{M}$), the phosphodiesterase pattern proved very different: most of the cyclic AMP- and cyclic GMP-hydrolysing activity was found in the pI-5.45 peak. The pI-4.9 peak was far less important, and also less specific towards cyclic GMP as substrate than it was at low substrate concentration.

The sensitivity of phosphodiesterase to exogenous 'calmodulin' plus Ca^{2+} , or to the calcium chelator EGTA, was investigated in the original heart muscle soluble fraction and in the separated forms (Table 1). The unseparated phosphodiesterase preparation was found to be nearly insensitive to exogenous 'calmodulin' plus Ca^{2+} under every substrate condition used. By contrast, the effect of the calcium chelator EGTA depended on the nature and concentration of the substrate used. At low substrate concentration ($0.25\mu\text{M}$), cyclic AMP hydrolysis was not modified by EGTA, whereas cyclic GMP hydrolysis was inhibited by about 50%. When measured at $25\mu\text{M}$ substrate, hydrolyses of cyclic AMP and cyclic GMP were inhibited by about 20%. The sensitivity of the different isoelectric forms was variable: cyclic AMP-specific peaks were not responsive to either 'calmodulin' plus Ca^{2+} , or EGTA. The less substrate-specific peak with pI 5.45 was only slightly sensitive to EGTA inhibition (20%). The activities of minor (pI 5.2), and major (pI 4.9) cyclic GMP-specific peaks were markedly inhibited by EGTA (64 and 63% respectively), but not sensitive to addition of 'calmodulin' plus Ca^{2+} .

Effects of exogenous 'calmodulin' plus Ca^{2+} and of EGTA were also assayed in the presence of 100 mM-KCl, which has been reported to enhance greatly Ca^{2+} stimulation of a Ca^{2+} -activatable cerebral phosphodiesterase by selectively inhibiting its basal activity (Davis & Daly, 1978). With

Table 1. Ca^{2+} -dependence of phosphodiesterase in the heart muscle soluble fraction and in the forms obtained by isoelectrofocusing

The original 100 000 g supernatant fraction was obtained and fractionated as described in the Experimental section. Phosphodiesterase was assayed in the presence of bovine serum albumin (0.2mg/ml) to minimize non-specific activation of the enzyme by proteins contained in the calmodulin preparation (Bergstrand & Lundquist, 1976; Ho *et al.*, 1977), and with various additions of effectors. Control specific activity (without any effector) of the original preparation was: with $0.25\mu\text{M}$ -cyclic AMP, 52; with $0.25\mu\text{M}$ -cyclic GMP, 60; with $25\mu\text{M}$ -cyclic AMP, 1425; with $25\mu\text{M}$ -cyclic GMP, 1404 (in pmol hydrolysed/min per mg of protein). 'Calmodulin' ($40\mu\text{g}$ of protein/assay) was added simultaneously with 0.1mM exogenous CaCl_2 ; EGTA (solution in 40mM -Tris, pH adjusted to 8) was 1mM , and KCl was 0.1M .

		Phosphodiesterase activity [% of control (enzyme without addition)]					
		Calmodulin + Ca^{2+}			Calmodulin + Ca^{2+} + K^+		
		EGTA	Ratio*		EGTA + K^+	Ratio*	
Substrate							
Original cytosol	Cyclic AMP ($0.25\mu\text{M}$)	101	93.5	1.07	—	—	—
	Cyclic AMP ($25\mu\text{M}$)	103	76	1.36	—	—	—
	Cyclic GMP ($0.25\mu\text{M}$)	114	52	2.19	108	43.5	2.48
	Cyclic GMP ($25\mu\text{M}$)	114	86	1.33	—	—	—
pI 6 peak	Cyclic AMP ($0.25\mu\text{M}$)	117	115	1.02	—	—	—
pI 5.7 peak	Cyclic AMP ($0.25\mu\text{M}$)	104	98	1.06	—	—	—
pI 5.55 peak	Cyclic AMP ($0.25\mu\text{M}$)	117	101	1.16	—	—	—
pI 5.45 peak	Cyclic GMP ($0.25\mu\text{M}$)	99.5	80	1.24	94.5	72	1.31
pI 5.2 peak	Cyclic GMP ($0.25\mu\text{M}$)	109	36	3.03	96	31	3.11
pI 4.9 peak	Cyclic GMP ($0.25\mu\text{M}$)	103	37	2.78	88	23	3.80

* Activity with calmodulin + Ca^{2+} /activity with EGTA.

Table 2. Kinetic parameters of the different isoelectric forms of soluble heart muscle phosphodiesterase

	Cyclic GMP substrate				Cyclic AMP substrate			
	Concentration range (μM)	K_m (μM)	V_{max} (pmol/h per ml)	No. of observations	Concentration range (μM)	K_m (μM)	V_{max} (pmol/h per ml)	No. of observations
pI-4.9 form	0.25–10	2.0 ± 0.1	268.5 ± 5.5	18	33–200	43 ± 14	569 ± 66	15
pI-5.45 form	5–100	8.4 ± 1.5	2901 ± 143	18	5–100	45.5 ± 2.9	5051 ± 171	18
pI-5.5–5.8 form	1–1000	23.1 ± 1.5	435.5 ± 10	22	0.25–40	$0.86 \pm 0.06^*$	$124 \pm 5.7^*$	29
						86	602	

	V_{max} cyclic AMP	V_{max} cyclic GMP
	0.47	0.47
	0.57	0.57
	†	†

* For this enzyme form, the best computer curve-fitting was obtained for the following models: two Michaelian enzyme species, and one Michaelian enzyme plus one component proportional to substrate concentration (which derives from the first model, with 'high K_m ' $\gg$ substrate concentration). This analysis of our data allowed a precise determination of the 'high-affinity component' parameters, but not of the 'low-affinity component' ones. However, the $V_{\max}/K_m$ ratio for this component was obtained with good precision (7.0 ± 0.36). 'High $V_{\max}$ ' was then estimated by measuring the hydrolysis rate at millimolar concentrations of cyclic AMP and the 'high- K_m ' calculated as $V_{\max}/7.0$. The Hill coefficient calculated by curve-fitting to the Hill model was 0.52 ± 0.03 .

† The ratio of cyclic GMP/cyclic AMP hydrolysis was 0.14 at 0.25 μ M substrate and 0.6 at 1 mM.

Fig. 2. Lineweaver-Burk plot of cyclic AMP phosphodiesterase activity of the refocused *pI*-5.5-5.8 region
Bars indicate S.D. of the values ($n = 3$). The inset shows the same data, on an expanded scale. For assay conditions, see the Experimental section.

heart enzyme, the effect of K^+ was not so drastic (Table 1). Stimulated phosphodiesterase activity was not markedly affected, and the stimulation ratio (activity in the presence of 'calmodulin' plus Ca^{2+} /activity in the presence of EGTA) was somewhat increased in the pI-4.9 peak (2.78 without K^+ , versus 3.80 with K^+).

Each separated major peak (i.e. the two predominant cyclic GMP-specific peaks, and the pH 5.5–5.8 portion of the cyclic AMP-specific region) was submitted to a second isoelectric focusing, without notable change in isoelectric points or sensitivity to EGTA of the resulting peaks (results not shown).

Kinetic parameters of each refocused enzyme form were studied by using the graphic Lineweaver-Burk method and computer-aided curve-fitting procedures. Results are presented in Table 2 and Fig. 2. The effect of cyclic GMP on cyclic AMP hydrolysis by the less-substrate-specific peak (pI 5.45) was also assayed: cyclic GMP at 0.05–10 μM failed to enhance significantly cyclic AMP phosphodiesterase activity at pH 7.4.

Kinetic characteristics of the secondary cyclic AMP-specific peaks (i.e. phosphodiesterase activity remaining at the sample application point and pI6 peak) were indistinguishable from that of the twice-focused pI5.5–5.8 region, which constitutes the predominant part of the cyclic AMP-specific activity (results not shown).

Discussion

Isoelectric focusing on a flat gel bed provides a convenient and efficient separation of several forms

of cyclic nucleotide phosphodiesterase from rat heart. The three major forms obtained seem not to be interconvertible in the conditions of isoelectric fractionation, as shown by refocusing of separated peaks at the same pI values. A striking point is the variation in the relative contribution of each form to the total phosphodiesterase activity when substrate concentration is changed. At low concentration, cyclic AMP is hydrolysed 80–90% by the pI-5.55–6 peaks, and cyclic GMP with nearly equal efficiencies by the pI 5.45 and pI 4.9 peaks. At high substrate concentration, cyclic AMP and cyclic GMP are principally hydrolysed by the pI-5.45 peak. The weak contribution of the specific cyclic AMP phosphodiesterase to cyclic AMP hydrolysis at high substrate concentration could explain the absence of such a form from bovine heart tissue claimed by some authors (Ho *et al.*, 1976).

Peaks with pI 4.9, 5.45 and 5.55–6 present some of the characteristics of the DEAE-cellulose phosphodiesterase fractions (DI, DII, DIII respectively) obtained by Appleman & Terasaki (1975) with a sonicated rat heart preparation. However, some particularities of isoelectric fractionation are observed. A first difference is that the cyclic AMP-specific enzyme appears to be heterogeneous on isoelectric fractionation, whereas it gave a sole broad peak (DIII) on DEAE-cellulose chromatography and gel filtration (Appleman & Terasaki, 1975; Russel *et al.*, 1972), which could indicate a better resolution capacity of the former technique. All the cyclic AMP-specific forms obtained after isoelectric focusing display apparently identical non-Michaelian kinetics, as observed with fraction DIII (Appleman & Terasaki, 1975). The existence of several isoelectric forms with identical kinetic properties could be due to the attachment of different amounts of lipids to the enzyme, as proposed by Minneman (1976) for cerebral enzyme, or to the occurrence of enzyme in different phosphorylation states. Oxidation of thiol groups seems not to be involved, since this property is unchanged when the enzyme is prepared and fractionated in the presence of dithiothreitol (G. Némóz, A. F. Prigent & H. Pacheco, unpublished work). A discrepancy also appears between the DII form and the pI-5.45 isoelectric form: the less substrate-specific form was found to have non-Michaelian kinetics when obtained by DEAE-cellulose chromatography. It was also characterized by the ability of cyclic GMP to stimulate the cyclic AMP-hydrolysing activity (Appleman & Terasaki, 1975; Terasaki & Appleman, 1975). After isoelectric fractionation, the pI-5.45 form exhibited Michaelian kinetics and was refractory to cyclic GMP-activation, under pH and substrate conditions described as allowing the observation of positive co-operativity (Wells & Hardman, 1977). But such behaviour could be

related to the omission of thiols in our preparation, which could favour the fully activated state of the enzyme (Terasaki & Appleman, 1975). This hypothesis requires investigation.

Rat heart phosphodiesterase in crude preparation is wholly saturated by endogenous calmodulin (Table 1) (Lugnier & Stoclet, 1979). By contrast with anion-exchange chromatography (Van Inwegen *et al.*, 1976), isoelectric focusing does not dissociate enzyme-activator complexes, as suggested by the results of Table 1, and as also observed with brain enzyme (Pledger *et al.*, 1975). It appears therefore to be a mild fractionation technique. Calmodulin is an acidic protein with pI 4.7 (Pledger *et al.*, 1975). The calmodulin-complexed state of the pI-4.9 form, and perhaps of the pI-5.45 form, of the enzyme could explain why these peaks display the most acid pI values, whereas the likely corresponding forms in DEAE-cellulose chromatography (DI and DII) are the first eluted (i.e. the least acid): fixation of calmodulin on the enzyme would shift its pI to a greater acidity. The affinity of calmodulin plus Ca^{2+} for the pI-5.45 form is also supported by the weak but significant inhibition of this form by EGTA (Table 1). This inhibition seems not to be related to contamination by the pI-5.2 peak, since it remains unchanged in the twice-focused pI-5.45 peak. The DII form was not reported to be Ca^{2+} -dependent by Appleman & Terasaki (1975) and Terasaki & Appleman (1975), but was slightly activatable by calmodulin plus Ca^{2+} according to Donnelly (1978).

The minor peak observed at pI 5.2 and sharing some common properties with the pI 4.9 peak probably represents a different thiol-oxidation state of the latter peak (G. Némóz, A. F. Prigent & H. Pacheco, unpublished work): the addition of millimolar concentrations of dithiothreitol to homogenization and separation media markedly increases the pI-5.2 peak and decreases the pI-4.9 peak, whereas other peaks are unchanged.

Thus the separation pattern of rat heart phosphodiesterase proves to be somewhat more complex than was suggested by DEAE-cellulose chromatography profiles.

Isoelectric focusing on a flat gel bed appears to be a useful technique for study of cyclic nucleotide phosphodiesterase. The advantages of the method are: the absence of an elution-concentration step, sometimes damaging to phosphodiesterase activity (Thompson *et al.*, 1979b), the short separation time (a few hours with analytical plates, overnight with preparative ones), and the possibility of improving resolution by narrowing the pH range chosen. Furthermore, in contrast with polyacrylamide-gel electrophoresis, which affects the properties of phosphodiesterase in an irreversible manner (Uzunov & Weiss, 1972; Pledger *et al.*, 1974), isoelectric focusing on a gel bed preserves non-

Michaelian kinetics and high affinity of the enzyme. On a preparative scale, it may be employed as an efficient step for separation and purification of iso-enzymes, and associated with classical chromatography techniques or with affinity chromatography (Prigent *et al.*, 1980). As an analytical tool, it may be employed routinely for the comparison of normal and diseased tissues or for the investigation of the consequence of a drug treatment on metabolism of cyclic nucleotides.

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