The Inducible Alkaline Phosphatase of Rat Heart

SOME PROPERTIES OF THE ENZYME AND FACTORS INFLUENCING ITS ACTIVITY

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Subcutaneous injection of isoprenaline into rats produces a rapid rise in alkaline phosphatase activity which is largely confined to the right atrium of the heart. The phosphatase activity of extracts of atrial tissue from treated animals is on average four times that of controls. The rise in activity is largely suppressed by previously treating the animals with cycloheximide or actinomycin D. Comparison of the properties of the alkaline phosphatase in extracts of atria from isoprenaline-treated rats with control preparations shows the stimulated enzyme activity to be similar to that present in the resting state. In a number of respects, including substrate specificity, response to activators and inhibitors, rate of loss of activity on heating and electrophoretic mobility of the native and modified enzyme, the atrial phosphatase resembles alkaline phosphatases from other mammalian non-intestinal and non-placental tissues. These results suggest that the effect of isoprenaline is to increase the rate of synthesis of atrial alkaline phosphatase, and the ability of the tissues of the right atrium to respond in this way may be related to the rich sympathetic innervation of this part of the heart.

Alkaline phosphatase activity has been demonstrated in the right atrium of rat heart by histochemical procedures, with β -glycerophosphate, 1-naphthyl phosphate or naphthol AS-TR (4-chloro-3-hydroxy-2-naphtho-*o*-toluidide) phosphate as substrate (Müller & Pearse, 1969). The activity is much more pronounced in the right atrium than in the left, and elsewhere in the heart optical microscopy shows alkaline phosphatase only in capillary walls and endocardium. The phosphatase activity is increased by various stimuli, among the most potent of which are injections of the catecholamines adrenaline and noradrenaline or their synthetic analogue isoprenaline, and is greater in the hearts of animals that have been exercised than in those of resting animals. No increase was observed in similar circumstances in a range of other enzymic activities, including acid phosphatase, $\text{Na}^+ + \text{K}^+$ -dependent adenosine triphosphatase, non-specific esterase, monoamine oxidase, lactate dehydrogenase, succinate dehydrogenase and α -glycerophosphate dehydrogenase. The present paper describes quantitative studies on the atrial alkaline phosphatase of rat heart and factors that influence its activity. Comparisons have also been made between certain properties of the phosphatase in extracts of atria of isoprenaline-treated and control rats. The results suggest that the atrial tissue responds to this stimulus by increased

synthesis of an alkaline phosphatase which is similar in properties to an enzyme present in the resting state.

MATERIALS AND METHODS

Animals. Male Wistar rats (170–300g) were kept at 22°C with free access to water and a standard pellet diet (Dixon's 41B). A single subcutaneous injection (40 mg/kg body wt.) of isoprenaline [1-(3,4-dihydroxyphenyl)-2-isopropylaminoethanol] sulphate [Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.] in pyrogen-free water was chosen as the stimulus to production of alkaline phosphatase in most experiments. Cycloheximide {3-[2-(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]glutaramide; Sigma} and actinomycin D (Merck, Sharpe and Dohme, West Point, Pa., U.S.A.) were administered by subcutaneous injection and intraperitoneal injection respectively, in amounts indicated in the Results section. The animals were killed by exposure to CO_2 24h after injection of isoprenaline except when otherwise stated. The hearts, lungs, kidneys, livers, spleens and portions of the small intestine were removed immediately after death. Those tissues not required for processing at once were stored at -20°C .

Extraction of enzyme. Hearts were removed and washed in water, and then right and left atria were dissected away from the remainder of the heart and separated. Atria from several animals were pooled, weighed and homogenized in a motor-driven Potter-Elvehjem glass homogenizer with the addition of either 4 vol. of water, or 2 vol.

of water and 2 vol. of butan-1-ol, or 4 vol. of 0.32 M-sucrose. The average weight per atrium was 35 mg. The homogenates were left to autolyse at 4°C for 18 h and were then centrifuged at 1500g for 40 min. Re-extraction of the sedimented debris increased the yield of alkaline phosphatase by about 10%.

Assay of enzyme activity. Alkaline orthophosphatase activity was measured with *p*-nitrophenyl phosphate (BDH Chemicals Ltd., Poole, Dorset, U.K.) as substrate at 30°C by the method of Bowers & McComb (1966). The pH optimum at 5 mM-*p*-nitrophenyl phosphate concentration was determined in 0.1 M-Na₂CO₃-NaHCO₃ and 0.1 M-sodium borate buffers and the Michaelis constant at pH 10.0 in 0.1 M-Na₂CO₃-NaHCO₃ buffer. The effects on phosphatase activity of additions of Mg²⁺ ions and L-phenylalanine were also observed in the Na₂CO₃-NaHCO₃ buffer. For comparison of the relative rates of hydrolysis of different substrates, P_i was measured by the method of Delsal & Manhoury (1958) after incubation of each substrate solution (2 mM) with tissue extract (10 µl) at 37°C in Na₂CO₃-NaHCO₃ buffer at pH 9.8 or 8.8, depending on the substrate, for 15 min with and without addition of 10 mM-Mg²⁺. The substrates used were: 1-naphthyl sodium hydrogen phosphate and disodium β-glycerophosphate (Sigma), disodium AMP, trisodium ADP, disodium ATP, and disodium glucose 6-phosphate (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany), tetrasodium pyrophosphate (BDH Chemicals Ltd.) and pyridoxal 5'-phosphate (Sigma).

Horizontal starch-gel electrophoresis at pH 8.6 with subsequent location of alkaline phosphatase zones was carried out as previously described (Moss, 1963).

RESULTS

Alkaline phosphatase activities per mg of atrial tissue were similar in extracts prepared by each of the three methods, but the results reported here were obtained with extracts made by homogenization in sucrose solution. Activities in extracts of right and left atria of isoprenaline-treated rats and controls are shown in Table 1. The mean resting enzyme activity in the left atrium is approx. one-third of that in the right atrium. After treatment with isoprenaline the right-atrial phosphatase activity increases on average nearly fourfold, whereas the left-atrial activity is increased rather more than twofold. The greatest increase in right-atrial alkaline phosphatase after injection of isoprenaline was observed in three rats which, at 300 g body wt., were larger than those of 170–200 g body wt. used in the other experiments and the lowest activities were recorded in two smaller (150 g) animals. After a single injection of isoprenaline the most rapid increase in atrial phosphatase activity occurs between 4 and 8 h, with a slower decline after 24 h (Fig. 1). The alkaline phosphatase activities in right and left atria measured 24 h after injection are strongly dependent on the dose of isoprenaline up to about 40 mg/kg, but a further increase in the amount of isoprenaline injected

Table 1. *Alkaline phosphatase activity in extracts of right and left atria of control and isoprenaline-treated rats*

Extracts were made 24 h after injection of 40 mg of isoprenaline/kg body wt. Figures in parentheses indicate the number of animals from which tissues were pooled to obtain each value.

Alkaline phosphatase activity (nmol of substrate hydrolysed/min per mg of tissue)			
Controls		Isoprenaline-treated rats	
Left atrium	Right atrium	Left atrium	Right atrium
2	7 (3)	7	23 (2)
2	6 (2)	9	26 (2)
3	9 (2)	5	15 (2)
3	7 (10)	5	38 (3)
		6	25 (3)
		7	21 (5)
		6	26 (7)
		9	22 (5)
		8	24 (5)
		5	33 (10)
Weighted mean activities:			
3	7	6	27

Fig. 1. Time-course of alkaline phosphatase activity in extracts of the left (○) and right (●) atria of rats given an injection of isoprenaline (40 mg/kg) at zero time. C, Values from control animals. Tissues from two rats were pooled to obtain each value.

produces a comparatively small additional response in activity (Fig. 2).

Injections of cycloheximide (1 mg/kg body wt.), or actinomycin D (250 µg/kg body wt.) depressed the isoprenaline-stimulated increase in atrial alkaline phosphatase (Fig. 3). These results show that cycloheximide had most effect when administered 60 or 30 min before injection of isoprenaline: simultaneous injection of cycloheximide and isoprenaline or administration of cycloheximide after isoprenaline resulted in some stimulation of enzyme activity. Because of the high toxicity of

Fig. 2. Alkaline phosphatase activity in extracts of left (○) and right (●) atria 24h after injection of different amounts of isoprenaline. Tissues from four animals were pooled to obtain each value, except at the two highest doses of isoprenaline, for each of which six animals were used.

actinomycin D, even at the low doses used, treatment with this drug was continued for a maximum of 2 days only. However, depression of the response to isoprenaline was observed in rats treated with actinomycin D such that the right-atrial phosphatase activity did not reach the lowest value recorded for animals treated with isoprenaline alone. One animal in the actinomycin D-treated group was given a total dose of the drug that was twice that received by the others in the group over a period of 2 days and in this case the atrial phosphatase activities were lower than those in the remainder of the group, but these results have not been represented separately in Fig. 3.

None of the other organs examined showed an increased alkaline phosphatase activity 24h after an injection of isoprenaline (40mg/kg) similar to that observed in atrial tissue. The activity of alkaline phosphatase in extracts of right atrium in the resting state was approximately equal to that in extracts of liver prepared by similar methods, and was about 50 and 20% of the activity in intestinal and kidney extracts respectively. After stimulation with isoprenaline the right-atrial

Fig. 3. Effect of cycloheximide (1 mg/kg) and actinomycin D (250 μ g/kg) on stimulation of atrial alkaline phosphatase by isoprenaline (40 mg/kg). C, Controls; I, isoprenaline-treated; Cx, injected with cycloheximide only. Cx+I, Treated with cycloheximide and isoprenaline: 1, 2, cycloheximide 60 and 30 min before isoprenaline respectively; 3, cycloheximide and isoprenaline simultaneously; 4, 5, cycloheximide 30 and 60 min after isoprenaline respectively. A, Injected with actinomycin D only; A+I, treated with isoprenaline 24h after actinomycin D. Activities were measured 24h after injection of isoprenaline and at equivalent times for control groups. □, Left atrium; ■, right atrium. Tissues from several animals in each group, indicated by numbers in parentheses above columns, were pooled for preparation of extracts.

activity exceeded the liver activity and approximated to that in extracts of kidney. The right atrium of treated animals is thus a phosphatase-rich tissue, but when the activity of the whole heart is considered this is not the case, accounting for the generally accepted view of the heart as an organ poor in this enzyme (Folley & Kay, 1936).

Because of the small amounts of atrial tissue obtained from each animal and the correspondingly limited volume of enzyme solution, purification of the atrial alkaline phosphatase was not attempted in the present work. Instead, some of the properties of the isoprenaline-stimulated alkaline phosphatase have been inferred from comparisons of extracts of the atria of isoprenaline-treated and control rats. The atrial phosphatase activities of both treated and control rats had similar alkaline pH optima at 5mM substrate concentration (Fig. 4). Michaelis constants for the hydrolysis of *p*-nitrophenyl phosphate at pH 10.0 were calculated from the regression of *s/v* on *s*. Average values for extracts of right atrium were 1.79 mM (control) and 2.29 mM

Fig. 4. Dependence on pH of alkaline phosphatase activity of extracts of right atria of isoprenaline-treated rats (●, ▲) and controls (○, △), in borate buffers (▲, △) and $\text{Na}_2\text{CO}_3\text{--NaHCO}_3$ buffers (●, ○). The substrate concentration was 5 mM-*p*-nitrophenyl phosphate.

(isoprenaline-treated), whereas for extracts of left atrium the corresponding values were 1.98 and 1.62 mM respectively. Since individual estimates of K_m ranged from 1.38 to 2.72 mM with overlapping between the control and treated and left- and right-atrium groups, the stimulation of atrial phosphatase activity by isoprenaline does not appear to be accompanied by a significant change in K_m . After dialysis of the atrial tissue extracts overnight at 4°C against a large volume of water, the alkaline phosphatase activity from both isoprenaline-treated and control animals was increased by approx. 40% by addition of Mg^{2+} at a final concentration of 10 mM to the reaction mixture at pH 9.8, compared with dialysed extract assayed without added Mg^{2+} . Addition of 5 mM-L-phenylalanine, a selective inhibitor of alkaline phosphatases of intestinal and placental types (Fishman & Ghosh, 1967), resulted in a negligible inhibition of the atrial phosphatase.

The relative rates of hydrolysis of various phosphate esters by dialysed atrial extracts are shown in Table 2. Comparison of the activities of the extracts from isoprenaline-treated rats with those from control animals shows a twofold increase in hydrolysis of *p*-nitrophenyl phosphate and 1-naphthyl phosphate at pH 9.8, both with and without added Mg^{2+} , after injection of isoprenaline. Adenosine monophosphatase activity at this pH value is increased

Table 2. Release of P_i at 37°C from different substrates by extracts of right atria of control and isoprenaline-treated rats

Concn. of each substrate was 2 mM; Mg^{2+} , when present, was at 10 mM. Action on *p*-nitrophenyl phosphate, 1-naphthyl phosphate, AMP, ADP and ATP was measured at pH 9.8 and on β -glycerophosphate, glucose 6-phosphate, pyridoxal 5'-phosphate and PP_i at pH 8.8. Tissues from 10 animals in each group were pooled in preparing the extracts. Activities are expressed as percentages of the hydrolysis of *p*-nitrophenyl phosphate in the presence of Mg^{2+} by the extract from treated rats.

Substrate	Control		Isoprenaline-treated	
	- Mg^{2+}	+ Mg^{2+}	- Mg^{2+}	+ Mg^{2+}
<i>p</i> -Nitrophenyl phosphate	34	51	73	100
1-Naphthyl phosphate	54	76	72	129
AMP	13	39	24	50
ADP	9	24	10	21
ATP	14	53	9	47
PP_i	7	25	18	22
β -Glycerophosphate	5	15	6	30
Glucose 6-phosphate	4	8	9	11
Pyridoxal 5-phosphate	5	7	9	13

by 85% without added Mg^{2+} and by 30% when these ions are present in the assay. Marked enhancement of activity towards β -glycerophosphate, glucose 6-phosphate and pyridoxal phosphate is also observed in extracts from treated rats when measured at pH 8.8, which is closer to the pH optima for these substrates at 2 mM concentration. The extracts contained significant amounts of Mg^{2+} -stimulated adenosine triphosphatase and adenosine diphosphatase activities at pH 9.8, but these fell slightly in the isoprenaline-treated animals. Inorganic pyrophosphatase activity at pH 8.8 was somewhat lower in isoprenaline-stimulated animals when assayed in the presence of 10 mM- Mg^{2+} , but when these ions were omitted from the assay a 2.7-fold increase in inorganic pyrophosphatase activity was observed.

When phosphatase-containing extracts of right atrium were heated at various temperatures in tris-HCl buffer at pH 8.0, enzymic activity declined at similar rates in extracts from both control and isoprenaline-treated rats (Fig. 5). The rate of loss of activity at 55°C, equivalent to 50% in 4 min, was similar to that observed for other mammalian alkaline phosphatases from non-placental, non-intestinal sources such as bone or kidney (Moss, 1970a).

Zones of atrial alkaline phosphatase activity migrated to a position between intestinal and liver phosphatase zones on starch-gel electrophoresis at

Fig. 5. Decline in alkaline phosphatase activity of extracts of right atria of isoprenaline-treated rats (●, ▲, ■, ▼) and controls (○, △, □, ▽) on heating at different temperatures at pH 8.0 in tris-HCl buffer: ○, ●, 45°C; △, ▲, 50°C; □, ■, 55°C; ▽, ▼, 58°C.

pH 8.6. Only a single band was present in atrial extracts, and there was no difference in mobility between the phosphatase zones of right and left atrium. Extracts from atria of isoprenaline-treated rats showed alkaline phosphatase zones with the same mobilities as those in extracts from control animals, but of greater intensity; no additional phosphatase zones were apparent in extracts from treated rats. Treatment of the right-atrial extract with acetic anhydride (Moss, 1970b) increased the anodal mobility of the phosphatase zone by about 5%, an acceleration similar to that observed with liver phosphatase but smaller than the 20–30% increase in the rate of migration of placental or intestinal phosphatases. Incubation with neuraminidase before electrophoresis (Moss, Eaton, Smith & Whitby, 1966) considerably retarded the migration of the atrial phosphatase zone: the response of the atrial enzyme to this treatment thus resembles that of other mammalian alkaline phosphatases of non-intestinal origin and suggests the presence of terminal sialic acid residues in the enzyme molecule.

DISCUSSION

The present results indicate an increase in activity, in response to injection of isoprenaline, of an alkaline phosphatase in atrial tissue which

resembles in many respects enzymes of this type occurring in tissues such as liver or bone, but which is unlike the alkaline phosphatases associated with small intestine or placenta (Moss, 1970a). Thus the atrial phosphatase is of low stability to heat, is not inhibited by L-phenylalanine, and shows only a small increase in electrophoretic mobility after acetylation. Similarly, the observed changes in rates of hydrolysis of different phosphate substrates after injection of isoprenaline are consistent with the pattern of substrate specificity expected from an alkaline phosphatase of this class: the non-placental, non-intestinal alkaline phosphatases are also inorganic pyrophosphatases at low (but not at high) concentrations of Mg^{2+} (Moss, 1969) and the increase in inorganic pyrophosphatase activity without added Mg^{2+} relative to *p*-nitrophenyl phosphate hydrolysis in extracts prepared from the hearts of isoprenaline-treated rats is of the magnitude associated with these enzymes. Mammalian alkaline phosphatases release P_i from ADP and ATP, but the activity of the non-intestinal, non-placental enzymes on these substrates is relatively small compared with their action on orthophosphate monoesters, particularly in the presence of Mg^{2+} (Eaton & Moss, 1967). The atrial alkaline phosphatase may therefore be expected to contribute to only a small extent to the total adenosine triphosphatase activity of the extracts so that any small increase in breakdown of ATP from this source would not be significant in the presence of a much greater activity of specific Mg^{2+} -activated adenosine triphosphatase. Although it appears that increased activity of a single alkaline phosphatase can account for the changes observed in a range of phosphatase activities, a final decision on the question of whether more than one type of phosphatase is stimulated by isoprenaline must await purification of the atrial alkaline phosphatase.

The increase in alkaline phosphatase activity in the atrium is not accompanied by any marked change either in catalytic properties such as Michaelis constant and pH optimum, or in molecular properties such as electrophoretic mobility and stability to heat. These findings, together with the effects of cycloheximide and actinomycin D in almost completely suppressing the response, suggest that the isoprenaline-stimulated increase in phosphatase activity is due to increased synthesis of an enzyme of a type that already exists within the tissue: however, because the enzyme has not been purified, an increase in specific activity without an increase in total amount of enzyme protein cannot be discounted completely. A number of instances in which alkaline phosphatases in animal cells respond to a variety of stimuli by increased synthesis have been reported. The alkaline phosphatase activity in rat kidney is influenced by the dietary

content of phosphate (Melani, Farnararo & Chiarugi, 1971) and biliary obstruction in the rat increases the activity of liver phosphatase (Kaplan & Righetti, 1970). In both these cases inhibition of the increase in enzyme activity by cycloheximide and actinomycin D has been interpreted as indicating that new synthesis of enzyme is involved, and in both cases also the induced enzyme has similar properties to that present in the basal state. The increased alkaline phosphatase activity in mouse tail epidermis which follows local application of vitamin A (Riley & Spearman, 1968) and the induction of alkaline phosphatase in intestinal brush border by vitamin D in chicks (Haussler, Nagode & Rasmussen, 1970) have also been ascribed to a stimulation of enzyme synthesis. In tissues in which alkaline phosphatase activity is influenced by adrenocorticoids, however, a different mechanism appears to operate. Cortisone and adrenocorticotrophic hormone promote the development of alkaline phosphatase activity in the duodenum in the infant mouse and other species (Moog, Etzler & Grey, 1969) but the transition to adult values for enzymic activity is accompanied by alterations in the relative rates of hydrolysis of different phosphate esters, whereas antibiotics that interfere with protein synthesis mimic the action of glucocorticoids and act additively with them in stimulating alkaline phosphatase development in these tissues. This phenomenon does not therefore seem to be primarily one of protein synthesis *de novo*. A three- to ten-fold increase in activity of alkaline phosphatase is produced by adrenocorticoids in certain HeLa-cell lines cultured from human cervical cancers, an effect which has been attributed to a steroid-induced change in conformation of the enzyme, since the specific activity but not the total amount of enzyme protein was increased (Griffin & Cox, 1966).

If the increase in activity of alkaline phosphatase in the rat atrium reflects increased synthesis of enzyme protein, it presumably accompanies an increased concentration of catecholamines in the tissue, either reaching the heart from extra-cardiac sources by way of the bloodstream or produced *in situ* by stimulation of the sympathetic nervous system, or arising from a combination of these effects. The observation that increased atrial phosphatase activity can accompany physiological stress such as exercise (Müller & Pearse, 1969) does not distinguish between these alternatives; neither

does the effect of injections of isoprenaline, since this substance stimulates the sympathetic system as well as raising the concentration of circulating catecholamine directly. The high degree of localization of the phosphatase activity in the tissues of the right atrium compared with other parts of the heart may be correlated with the rich adrenergic innervation and high noradrenaline concentrations in this region (Jacobowitz, 1967; Muscholl, 1959), which together with the association of phosphatase activity with nerve-endings visible with the electron microscope suggests that local stimulation of sympathetic nerves may be the most important factor. However, only tissues adjacent to sympathetic nerve-endings may be adapted to respond to increased catecholamine concentration by enhanced enzyme production.

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