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Membrane-bound Phosphodiesterases in Rat Myocardium

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Abstract

Isoenzyme-specific phosphodiesterase (PDE) inhibitors are potential positive inotropic drugs. For evaluating such drugs in experimental models and to understand the physiological roles of the different isoenzymes, it is necessary to know what isoenzymes are present in the tissues studied. Rat myocardium has been reported to be devoid of the particulate cGMP-inhibited cAMP-PDE (type III isoenzyme). Here we re-evaluate the isoenzyme profile of rat myocardium.

The cAMP-PDE isoenzyme patterns were studied by ion-exchange chromatography using siguazodan and rolipram, specific inhibitors of type III and IV isoenzymes, respectively. In contrast to earlier reports, type III isoenzyme was abundant in the particulate fraction. PDE III-specific antibodies depressed PDE activity and stained bands in Western blot with molecular masses 64 and 71 kDa. Type III isoenzyme of myocardial membranes was found to be unstable at 37°C which may explain why earlier investigators have failed to demonstrate its presence.

The data presented in this paper show that rat heart particulate fraction contains two low K_m PDE isoenzymes, type III and type IV, in equal activities. Thus, in contrast to previous reports, this paper clearly shows the presence of considerable amounts of membrane-bound type III PDE isoenzyme in rat myocardium.

Cyclic AMP plays a pivotal role in the contractile function of the myocardium. Both synthesis and degradation of this second messenger are strictly controlled by complicated regulatory mechanisms. The inactivation of cyclic AMP is accomplished by hydrolysis to AMP by members of families of phosphodiesterase (PDE) isoenzymes. Seven mammalian PDE families have been classified on the basis of their different substrate affinities, responses to specific effectors, sensitivities to specific inhibitors and regulatory control mechanisms (Beavo & Reifsnyder 1990; Thompson 1991; Manganiello et al 1995). Phosphodiesterases can be either soluble or membrane-bound. The membrane-bound particulate enzymes are believed to be most important (Whalin et al 1989). Phosphodiesterase inhibitors such as theophylline and caffeine have long been used in the treatment of asthma and as central nervous system stimulants. However, because of the ubiquitous and diverse functions of cyclic AMP, the usefulness of these drugs is limited by their lack of tissue specificity. In addition, many of the effects of these drugs are not based on phosphodiesterase inhibition but on binding to adenosine receptors (Ohisalo 1987). The elucidation of the diversity of phosphodiesterase isoenzymes and their distinct tissue distribution has now enabled the pharmaceutical industry to develop drugs that are isoenzyme-specific and are not adenosine antagonists. More recently, inhibitors of isoforms III (cGMP-inhibited) and IV (cAMP specific) have emerged as potential positive inotropic drugs. This has raised considerable interest in the distribution and properties of cardiac phosphodiesterases. In most species, the particulate fraction contains type III phosphodiesterase. In some species, including man, also type IV isoenzymes are present in particulate fractions. Unlike the case for other mammalian species studied, particulate type III isoenzyme has been reported to be absent in rat myocardium. In rat heart the particulate phosphodiesterase is believed to be solely of type IV (Beavo & Reifsnyder 1990; Shahid et al 1990). In the course of our studies on hormonal regulation of phosphodiesterase inhibitors it has become obvious that the isoenzyme profile of rat myocardium must be re-evaluated.

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Materials and Methods

Methods

Preparation of particulate fraction. Rat ventricles were homogenized in ice-cold buffer containing 20 mM Tris (pH 7·5), 2 mM EDTA, 10 mM DTT, 2 mM benzamidine, 0·1 mM phenylmethylsulphonyl fluoride and 0·01 mM leupeptin (buffer A). All subsequent procedures were carried out at 4°C. The homogenate was first centrifuged at 1000 *g* for 10 min and the pellet was discarded. The supernatant was centrifuged for 60 min at 100 000 *g*. The pellet was rehomogenized and washed once.

Ion-exchange chromatography. The rehomogenized particulate fractions were incubated overnight with 1% Triton X-100 under continuous stirring at 4°C. The suspension was centrifuged at 100 000 *g* for 60 min. The supernatant was filtered (Sartorius Minisart, mesh size 0·2 m) and applied to a Resource Q column (bed volume 1 mL, Pharmacia) pre-equilibrated with buffer A. After washing with buffer A (flow rate 30 mL h^{-1}) until absorbance at 280 nm was stable, the column was eluted with a linear 40-mL gradient of 0-1·2 M NaCl in buffer A. Fractions of 0·5 mL were collected.

Phosphodiesterase assay. Cyclic AMP phosphodiesterase activity was determined by a modification (Kaasik et al 1994) of the method of Davis & Daly (1979). The assay mixture contained 1 μM cAMP and 10 nM [^{3}H]cAMP in 120 μL 48 mM Tris-6 mM $MgCl_2$ at pH 8. The reaction was initiated by the addition of 30 μL of enzyme preparation and

allowed to proceed for 10 min at 37°C, after which the tubes were boiled for 2 min. The samples were applied to columns of Affi-Gel 601 boronate affinity gel equilibrated with 0·1 M K_2HPO_4/KH_2PO_4–0·1 M NaCl, pH 8·0. The columns were washed with 4 × 5 mL of the same buffer and eluted with 5 mL 0·25 M acetic acid. The [^{3}H]AMP thus eluted was counted in OptiPhase HiSafe 3 (Wallac, Turku, Finland). Total protein was assayed by the method of Lowry et al (1951).

Western blot. Western blots were done essentially as described previously (Ohisalo et al 1989). Chromatographic fractions which showed PDE activity were concentrated and run on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% gel. The proteins were transferred to nitrocellulose electrically at 100 V for 60 min. Type III PDE was detected by using antisera antiT. The second antibody was goat anti-rabbit IgG coupled to horseradish peroxidase, and final staining was done with *o*-dianisidine.

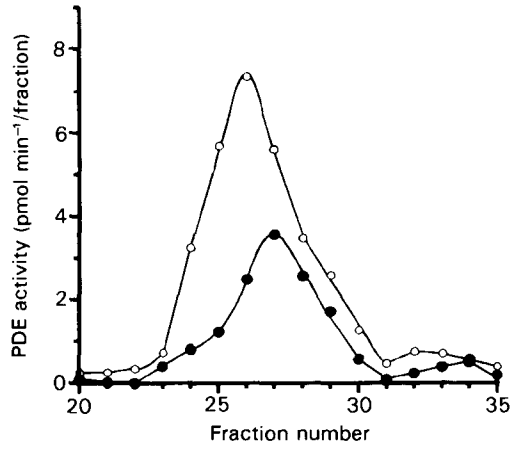

FIG. 1. Chromatographic profiles of type IV (○) and type III (●) cAMP phosphodiesterase activities from solubilized rat heart particulate fraction. Type IV and type III phosphodiesterase activities were calculated by subtracting enzyme activities measured in the presence of 100 μM rolipram or 10 μM siguazodan, respectively from enzyme activities measured in their absence.

Reagents

[2,8-^{3}H]Cyclic AMP (50 Ci mmol^{-1}) was from Amersham, unlabeled cyclic AMP from Sigma, Affigel 601 and goat anti-rabbit IgG coupled to horseradish peroxidase from Biorad. Rolipram (ZK 62711) was generously supplied by Dr B. Fredholm (Karolinska Institutet) and siguazodan (SK&F 94836) by Dr B. H. Warrington from SmithKline Beecham. Antiserum raised against human platelet PDE III and IgG fraction from an antiserum raised against purified bovine adipose PDE III were kindly supplied by Dr. E. Degerman from Lund University.

Results

PDE activity in crude membrane fraction

The isozyme profiles of nonsolublized particulate fractions were directly determined using rolipram which has been shown to be a specific inhibitor of isoenzyme IV (Thompson 1991) or siguazodan a specific phosphodiesterase III inhibitor (Reeves et al 1987). Type IV and III phosphodiesterase activities were calculated by subtracting enzyme activities measured in the presence of 100 μM rolipram or 10 M siguazodan, respectively, from enzyme activities measured in their absence. The activities of type IV and III isoenzymes were 10·4 ± 1·0 and 9·9 ± 0·60 pmol min^{-1} mg^{-1} of particulate protein, respectively and together constituted over 90% of total phosphodiesterase activity. The values shown are means ± s.e.m. of 6 independent determinations using 6 different hearts. The particulate fraction had been washed carefully and the activity of type III isoenzyme was clearly too high to be due to cytoplasmic contamination.

Ion-exchange chromatography

Thirteen milligrams of solubilized particulate protein was applied to a Resource Q column. The column was washed with buffer A and was then eluted with a linear sodium chloride gradient of 0 to 1·2 mol L^{-1} in buffer A. Most of the phosphodiesterase activity was eluted at 0·3–0·5 M NaCl concentration. To identify the isoenzymes in the major peak positively, the fractions were then re-assayed in the presence of isoenzyme-specific inhibitors. Fig. 1 shows that the total activity was divided into two peaks: a rolipram-sensitive and a siguazodan-sensitive peak were separated.

Immunoinhibition

The fractions of the major peak of chromatographic profile were mixed and PDE activity was re-assayed in the presence or absence of 1346 (IgG fraction from an antiserum raised against purified bovine adipose PDE III). Preincubation with 1346 (10 min at 37°C) lowered PDE activity by 56 ± 1% (Table 1). In the presence of 1346, siguazodan had no effect but rolipram decreased PDE activity significantly.

Table 1. PDE activity of peak fraction of PDE chromatographic profile measured in the absence or presence of PDE III-specific antibody 1346. Samples were preincubated for 10 min at 37°C with antibody and assayed in the absence or presence of 100 μM rolipram or 10 μM siguazodan.

	PDE activity (% control)
Control	100 ± 6
1346	44 ± 1
1346 + siguazodan	40 ± 3
1346 + rolipram	6 ± 0
1346 + siguazodan + rolipram	5 ± 6

Western blot

Type III phosphodiesterase in particulate fraction was visualized by using antiserum antiT (raised against human platelet PDE III). In Western blots of the peak fractions of PDE chromatographic profile, antiT recognized two bands with approximate molecular masses 64 and 71 kDa. Neither band was stained by control serum of rabbit (0 serum) to any significant extent and they can be considered as type III PDE.

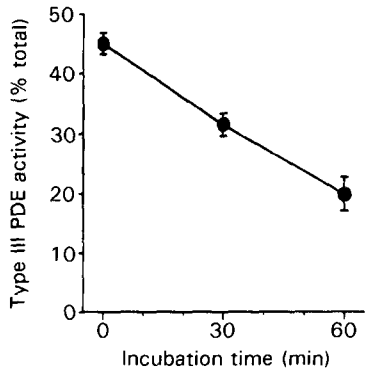

FIG. 2. Relative changes in type III PDE isoenzyme activity in non-solubilized particulate fractions during incubation at 37°C.

Discussion

In contrast to previous reports, this paper clearly shows the presence of considerable amounts of PDE isoenzyme III in rat heart particulate fraction. This conclusion is supported by the following observations.

PDE activity was significantly inhibited by 10 μM siguazodan. Since siguazodan is isoenzyme specific (IC50 for PDE III is 0.6 μM and for other isoforms more than 30 μM), the siguazodan-sensitive part of PDE activity can be considered as type III PDE activity (Murray et al 1990; Torphy et al 1993; Tang et al 1994).

PDE activity was depressed by antibody 1346 raised against purified native PDE III. In the presence of 1346, type III inhibitor did not cause any further inhibition but type IV inhibitor depressed PDE activity almost completely.

Antiserum antiT (raised against human platelet PDE III) stained two bands in Western blot which were not stained by control serum.

Type III PDE is more labile than type IV (Fig. 2) which may be the reason why earlier reports could not demonstrate the presence of particulate type III phosphodiesterase in rat myocardium.

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