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CYCLIC NUCLEOTIDE PHOSPHODIESTERASE FROM A PARTICULATE FRACTION OF RAT HEART. SOLUBILIZATION AND CHARACTERIZATION OF A SINGLE ENZYMATIC FORM.

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SUMMARY : Approximatively 2-8% of the cyclic nucleotide phosphodiesterase activity of a crude 1000 g supernatant from rat heart was associated with the washed 105,000 g pellet fraction. This activity exhibited biphasic Lineweaver-Burk plots over a large range of cyclic nucleotides concentrations. Concave-downward plots were obtained with cyclic AMP as the assay substrate, while cyclic GMP gave rise to concave-upward plots. Treatment of this particulate fraction by freezing and thawing and then with 2% Lubrol PX released the major part of phosphodiesterase activity into the supernatant (70 and 90% for cyclic AMP and cyclic GMP phosphodiesterase activities respectively). Isoelectric focusing of the solubilized enzyme revealed a single peak of phosphodiesterase activity. While the Lineweaver-Burk plots of cyclic AMP phosphodiesterase activity were not markedly modified by detergent treatment kinetic plots of cyclic GMP phosphodiesterase activity underwent a drastic transformation during the overall solubilization procedure. The substantial increase in the cyclic GMP rate of hydrolysis observed at low substrate level might explain the difference in the apparent yield of solubilization between cyclic AMP and cyclic GMP phosphodiesterase activities.

INTRODUCTION : In most tissues, cyclic nucleotide phosphodiesterase was found to be present in both soluble and particulate fractions (1). In contrast with the great deal of informations related to the cytosolic enzyme (2,3), only few reports concern the membrane-bound cyclic nucleotide phosphodiesterase activity. Biochemical details of these particulate enzymes have only been provided in a few systems such as rabbit (4) and rat kidney (5), bovine (6) and rat thyroid (7), rat liver (8-9) and adipose tissue (10), rat brain (11). Although phosphodiesterase activity in the particulate fraction generally only represents a few percents of the whole homogenate activity, the membrane-bound enzyme probably plays an important role in regulating cyclic nucleotides levels, and specially cyclic AMP level, at the membrane site during hormonal stimulation of adenylate cyclase. As shown by recent theoretical analyses, 68-95% of the cellular cyclic AMP is hydrolyzed by the membrane-associated phosphodiesterase (12). Furthermore, hormonal regulation of phosphodiesterase activity pointed out in some tissues (1,3,7-10) almost exclusively affects the particu-

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late enzyme. Heart cyclic nucleotide phosphodiesterase which contributes to the control of cardiac cyclic nucleotides levels might play an important role in the regulation of myocardial contractility. So, this enzyme is a possible target for several cardioactive drugs (13,14). Most of the work on cardiac phosphodiesterases has been done with tissue homogenates (ammonium sulfate precipitate from 4000 g supernatant) (15), sonicated (16) or cytosolic (17) preparations obtained by differential centrifugation. Subcellular localization studies of the cardiac enzyme led to conflicting results. Although it is clear that cardiac phosphodiesterases exist in particulate as well as in soluble fractions (18), striking discrepancies appeared concerning the soluble to particulate activity ratio. Thus, Kakiuchi et al (19) found more than 70% of the total cyclic AMP phosphodiesterase activity in the 105,000 g pellet of rat heart whereas St Louis and Sulakhe (20) reported that about 20% of the total phosphodiesterase activities of guinea pig ventricles are associated with the particulate fraction. Biochemical properties of this particulate enzyme have not been examined thoroughly . The following paper reports the biochemical characterization of the phosphodiesterase activity from a particulate fraction of rat heart and its solubilization with a non-ionic detergent. Isoelectric focusing of the solubilized enzyme reveals the presence of a single peak for both cyclic AMP and cyclic GMP phosphodiesterase activities.

MATERIALS AND METHODS : Male Sprague Dawley rats (200-230 g) were killed by decapitation. The hearts were excised and perfused with 0.32 M sucrose in 10 mM Tris HCl buffer pH 7.5. Fresh muscle freed of connective and vascular tissue was homogenized in 3 volumes (v/w) of the above-mentioned buffer in a glass-glass homogenizer and then in a glass-teflon homogenizer (10 strokes at maximal speed). The homogenate was centrifuged at 1000 g for 10 minutes and the resultant supernatant was centrifuged for 1h at 105,000 g. The resultant pellet was resuspended and rehomogenized in 3-fold the original volume of hypotonic 10 mM Tris-HCl buffer pH 7.5 and subjected to this centrifugation - resuspension procedure two additional times. The final 105,000 g pellet was either freshly used for cyclic nucleotide phosphodiesterase determination or frozen at -20°C as mentioned in the text.

Thawed 105,000 g washed pellet was homogenized in 10 mM Tris-HCl buffer pH 7.5 containing 2% Lubrol PX (Approx. 15 mg proteines/3 ml Lubrol-Tris buffer). After 1 h incubation at 4°C, centrifugation of the detergent treated homogenate was carried out at 105,000 g for 1 hour. The above procedure was repeated for the pellet, and the combined supernatants used as solubilized enzyme preparation were immediatly submitted to isoelectric focusing.

Thin layer isoelectric focusing was performed using extensively washed Sephadex G 75 (5g) suspended in distilled water containing 5 ml of Ampholine carrier ampholytes and layered on a 12.5 x 26 cm plate (5 ml Ampholine pH 3.5-10 gave a final pH range of 4-9 ; 2.5 ml ampholine pH 5-7 plus 2.5 ml Ampholine pH 7-9 mixture gave a final pH range of 4.6 to 8.4).

After formation of the pH gradient, 8-10 mg of solubilized proteins were applied in the pH 7.9-8 zone and the migration effected at 4°C overnight. At the

end of the experiment, the gel was sliced into 7.5 mm wide bands. The pH of each band was determined and the proteins were eluted out of the gel by 2 ml of 160 mM Tris-HCl buffer (pH 8) containing 0.1 mM $CaCl_2$, 5 mM $MgCl_2$ and 1 mg/ml of bovine serum albumin.

Cyclic nucleotide phosphodiesterase activity was assayed by a two step radioisotopic procedure based on that described by Thompson et al. (21) as modified by Boudreau and Drummond (22). In preliminary control experiments we made sure of the validity of this procedure in our experimental assay conditions. The acidification of the resin slurry (AG 1X2 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%), slightly better for guanosine (95%). Adenosine and guanosine recoveries were measured in every assay by means of [U-^{14}C] adenosine and guanosine adjunction simultaneously with snake venom. Phosphodiesterase activities were systematically corrected for these recoveries. In control experiments, with phosphodiesterase of crude 105,000 g rat heart pellet and 0.25 μM cyclic AMP, a good agreement was observed between results obtained by means of acidic resin batch method and with PEI-cellulose-thin layer chromatography of assay medium performed according to Böhme and Schultz (23). In usual experiments, no more than 15% of substrate was hydrolyzed in an assay.

Proteins were determined according to Lowry et al.(24) using bovine serum albumin as standard.

Computer-aided curve-fitting procedure : the experimental enzyme kinetic data were fitted to different models (michaelian model, sum of two michaelian enzymes, Hill model) by a nonlinear least-squares computer program, according to the method of Marquardt (25). Statistical analysis of the results was achieved according to Draper and Smith (26). In all the cases, the best fitting was obtained with Hill model.

RESULTS : In the rat heart homogenates (crude 1000 g supernatants), only 2-4% of the cyclic GMP phosphodiesterase activity and 4-7% of the cyclic AMP phosphodiesterase activity, determined at low substrate level, were found to be associated with the particulate cell fractions through repeated washings (Table 1). The partition of phosphodiesterase between soluble and particulate fractions was found very similar, when measured with both cyclic nucleotides at high substrate concentration (25 μM) : 6-8% of the total cyclic AMP and cyclic GMP hydrolyzing activities remained bound to the particulate cell fractions (not shown).

The pH optimum range of the particulate enzyme was rather broad but it exhibited a maximum at pH 8-8.2 for cyclic AMP phosphodiesterase activity and at pH 7.7-7.9 for cyclic GMP phosphodiesterase activity (data not shown). The ommission of Mg^{++} ions in the assay medium led to a drastic (70-80%) inhibition of both cyclic nucleotides hydrolyzing activities. An optimal Mg^{++} concentration of 5 to 10 mM was required to obtain a maximal phosphodiesterase activity. Kinetic analysis of the cyclic nucleotide phosphodiesterase activity associated with freshly prepared washed membrane material showed that this enzyme hydrolyzed about twice more cyclic GMP than cyclic AMP at 25 μM substra-

TABLE 1 : Distribution of cyclic nucleotide phosphodiesterase activities in rat heart fractions

FRACTION	CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ACTIVITY				TOTAL ACTIVITY RATIO CYCLIC GMP/ CYCLIC AMP
	0.25 µM cyclic AMP		0.25 µM cyclic GMP		
	Total activity pmol/min ± S.D.	Specific activity pmol/min/mg ± S.D.	Total activity pmol/min ± S.D.	Specific activity pmol/min/mg± S.D.	
1000 g supernatant	9380 ± 300	60.1 ± 2	13600 ± 420	87.1 ± 3	1.45
First 105,000 g supernatant	9000 ± 240	90.3 ± 2.5	11850 ± 540	118.8 ± 5	1.32
First 105,000 g pellet	980 ± 26	19.7 ± 0.5	930 ± 98	18.7 ± 2	0.95
Second 105,000 g supernatant	480 ± 13	96 ± 2.6	530 ± 45	106 ± 5	1.10
Second 105,000 g pellet	540 ± 24	15.8 ± 0.7	470 ± 60	13.8 ± 1.8	0.87
Third 105,000 g supernatant	58 ± 4	46.4 ± 3	<10		
Third 105,000 g pellet	620 ± 25	19.3 ± 0.8	490 ± 27	15.2 ± 0.8	0.79

The first 105,000 g supernatant and pellet are from the 1000 g supernatant.
The second and third supernatants and pellets are subsequent washings of the first 105,000 g pellet.
Activities were determined in triplicate.

te while an opposite ratio was observed at low substrate level (Fig.1A). Cyclic AMP phosphodiesterase activity, measured with substrate concentrations ranging from 0.25 to 100 µM, clearly gave non michaelian concave downward Lineweaver-Burk plots. Apparent Km values of 1 and 70 µM were obtained by extrapolation from linear portions of the graphs. Such biphasic concave downward kinetics have already been reported for particulate bovine heart (18), dog heart (27) and guinea pig ventricles (20) cyclic AMP phosphodiesterase activity, with apparent Kms values in the same range of magnitude as we observed with the rat heart. In contrast, cyclic GMP phosphodiesterase activity showed anomalous concave upward Lineweaver-Burk plots over a wide range of substrate concentration, giving an extrapolated Km value of about 25 µM. Kinetic parameters calculated by computer curve-fitting to Hill model or graphically determined by Hill plots method are given in Table 2.

The washed 105,000 g pellet submitted to a freezing and thawing process and then treated with 2% Lubrol PX released the major part of phosphodiesterase activity into the 105,000 g supernatant (Fig.2). At high substrate level (25 µM), about 80% of the total cyclic AMP and cyclic GMP phosphodiesterase activities were found in the Lubrol supernatant. Solubilization did not exceed 18% when freezing and thawing were used alone. When examined at low substrate level, the solubilization patterns of the two phosphodiesterase activities seemed to be rather different. Especially, 30-37% of the final cyclic AMP phosphodiesterase activity remained tightly bound to the particulate frac-

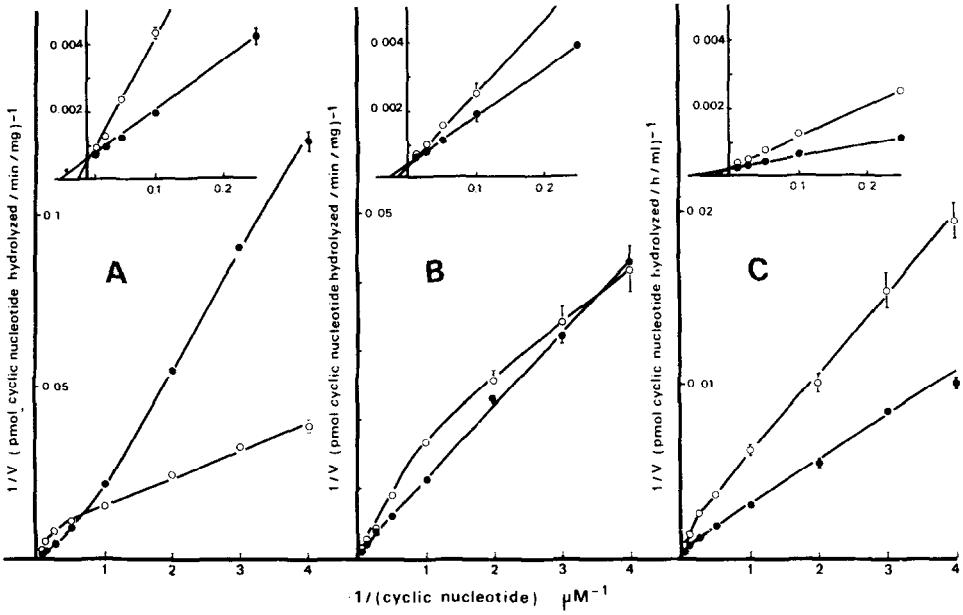

FIG.1 : Kinetic analysis of cyclic nucleotide phosphodiesterase associated with washed membranes from rat heart.
Panel A : freshly prepared 105,000 g pellet. Panel B : frozen thawed pellet. Panel C : Lubrol-solubilized isoelectrofocused fraction.
●—● cyclic GMP and o—o cyclic AMP phosphodiesterase activities were measured in triplicate, with substrate concentration ranging from 0.25 to 100 µM. S.D. are indicated when they are superior to the symbol size.
The inset plots show the same data at an expended scale.

tion in spite of the detergent treatment, whereas 85-90% of the cyclic GMP phosphodiesterase activity was released into the Lubrol supernatant. This cyclic GMP-hydrolyzing activity was also released in a non negligible amount (26%) by freezing and thawing alone. It is noteworthy that freezing and thawing induced a large increase in cyclic GMP phosphodiesterase activity determined at low substrate level (+ 197% with 0.25 µM cyclic GMP) while no important variation was observed with 25 µM cyclic GMP. In addition , cyclic AMP phosphodiesterase activity was only slightly or not modified by this procedure (fig.2). Thus, freezing and thawing of fresh membrane material might unmask enzymatic sites inacessible to cyclic GMP substrate in intact freshly prepared membranes, or only affect kinetic constants of the particulate enzyme. Lubrol treatment of the thawed membranes did not bring further increase in total phosphodiesterase activity. When the detergent-solubilized enzyme fraction was subjected to isoelectric focusing on flat gel bed in a pH range of 4-9, a single peak of activity was detected at pI 5.65. Cyclic AMP and cyclic GMP phosphodiesterase activities were totally superimposable either

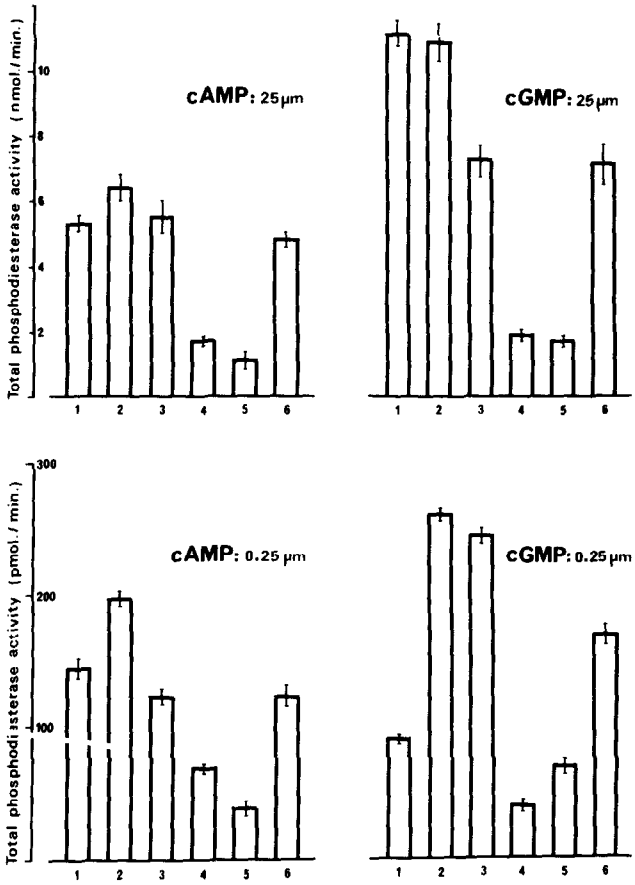

FIG.2 : Solubilization patterns of washed 105,000 g pellet fraction from rat heart. Cyclic nucleotide phosphodiesterase activity was measured with either cyclic AMP or cyclic GMP (0.25 and 25 µM) as substrate : (1) total phosphodiesterase activity of freshly prepared washed 105,000 g pellets ; total protein content : 14.83 mg. (2) total phosphodiesterase activity of the same fraction after freezing and thawing. (3) total phosphodiesterase activity of 105,000 g supernatant fraction of the thawed, Lubrol-solubilized preparation (7.77 mg protein). (4) Total phosphodiesterase activity of 105,000 g pellet fraction of the thawed, Lubrol-solubilized preparation (7.72 mg protein). (5) total phosphodiesterase activity of 105,000 g supernatant fraction of the thawed preparation (2 mg protein). (6) total phosphodiesterase activity of 105,000 g pellet fraction of the thawed preparation (12.84 mg proteins).

at low or at high substrate level. Narrowing the pH range from 4-9 to 5-8, in order to improve the isoelectric focusing resolution did not modify either the position or the shape of the peak (Fig.3). Thus, the isoelectric focusing profile of the detergent-solubilized enzyme clearly indicated the presence of one single enzymatic form of cyclic nucleotide phosphodiesterase.

Kinetic properties of cyclic AMP phosphodiesterase activity in the thawed membrane preparation (Fig.1B) as well as in the solubilized and isoelectro-

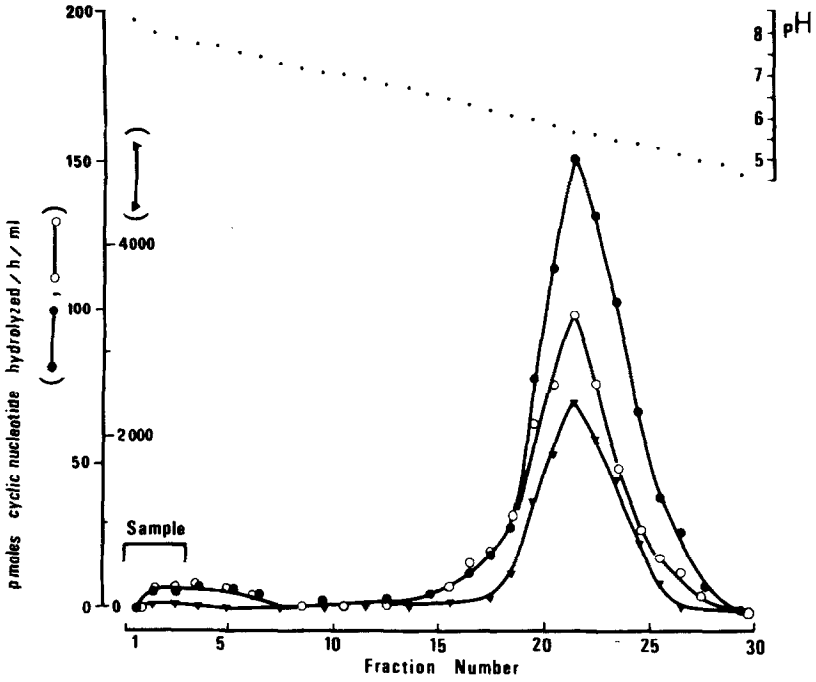

FIG.3 : Isoelectric profile of detergent-solubilized phosphodiesterase. The activity was measured in the presence of 0.25 μM cyclic GMP (●—●), 0.25 μM cyclic AMP(o—o), 25 μM cyclic AMP (▼—▼).

focused fraction (Fig.1C) were found to be rather similar to that of the fresh pellet fraction (Fig.1A and Table 2). Apparent Km values extrapolated from linear portions of Lineweaver-Burk plots were not notably changed. During the overall solubilization procedure, the apparent negative cooperativity of the enzyme seemed to be preserved as shown by the lack of obvious modification of Hill coefficients (Table 2). On the contrary, cyclic GMP phosphodiesterase kinetic properties of the thawed and detergent-solubilized preparation appeared to be markedly different from that observed with the fresh washed pellet fraction : while the latter gave concave upward Lineweaver-Burk plots (Fig.1A), the thawed and detergent-solubilized enzyme showed nearly michaelian kinetic plots (Fig.1B and 1C) which was traduced by the decrease of Hill coefficients (Table 2). However, the modification of kinetic plots induced by these treatments didn't markedly affect either graphically extrapolated or calculated Km values. This agrees with the large increase in total cyclic GMP phosphodiesterase activity observed after the overall solubilization procedure, when measured at 0.25 μM substrate concentration and the lack of variation in enzyme activity at 25 μM (Fig.2).

TABLE 2 : Kinetic parameters of the cyclic nucleotide phosphodiesterase activity of washed rat heart membranes, determined by fitting to Hill model.

ENZYME PREPARATION	SUBSTRATE : cyclic AMP			SUBSTRATE : cyclic GMP		
	V_{max} (a)	Km (μM)	n (b)	V_{max} (a)	Km (μM)	n (b)
fresh membranes		(c)		1370±42	29.0±1.1	1.27±0.012
frozen-thawed membranes	2350±380	32±5	0.85±0.020	2120±120	23.8±1.6	0.94±0.015
Lubrol-solubilized fraction	7300±1500	46±12	0.77±0.017	6745±342	18.1±1.2	0.99±0.018

Substrate range was 0.25 μM to 100 μM. The no. of observations was 30 in each case.
In all the cases except one (fresh membranes assayed with cyclic AMP as substrate), computer-aided curve-fitting of cyclic nucleotide hydrolysis velocity data (the same as in Fig.1) to Hill model was satisfactory, and allowed an accurate determination of kinetic parameters ± S.D.

(a) V_{max} was expressed in pmol hydrolyzed/min/mg protein for fresh and thawed preparations, in pmol hydrolyzed/min/ml for solubilized and isoelectrofocused enzyme.
(b) Hill coefficient
(c) In this case, the fitting to the various models tested didn't allow a precise calculation of parameters. V_{max} was then estimated to be 1680 pmol/min/mg by extrapolation of Lineweaver-Burk plots (Fig.1) and graphic Hill representation was carried out. The data closely followed Hill model with n = 0.62, Km = 26 μM, with cyclic AMP ranging between 0.25 and 10 μM. For higher substrate concentrations, n was near 1.

DISCUSSION : As it was emphasized by Van Inwegen et al. (5), the particulate preparations of cyclic nucleotide phosphodiesterase required extensive washing in order to measure the membrane-bound activity without interference from the soluble enzyme. Indeed, 35-40% of the total phosphodiesterase activity of the first 105,000 g pellet is of cytosolic origin since it is eliminated by further washings. Fractionation and subsequent washings of the particulate material occurs with an overall recovery in phosphodiesterase activity of about 100% as compared with the crude 1000 g supernatant activity, and a notable decrease of the cyclic GMP/cyclic AMP phosphodiesterase ratio from 1.45 in the crude 1000 g supernatant to 0.79 in the final washed pellet. Nevertheless, cyclic GMP phosphodiesterase activity which has been referred to as a soluble enzyme for a long time (1-3) appears to be actually membrane-bound in this heart preparation since the last wash of the 105,000 g pellet does not remove any additional cyclic GMP phosphodiesterase activity. Our results concerning the soluble to particulate phosphodiesterase ratio in the rat heart are quite similar to those of Beavo et al. (18) although we do not

take into account the non-negligible phosphodiesterase activity present in the 1000 g pellet(not shown). Enzymatic activity of this crude fraction containing heterogeneous material, and particularly unbroken cells, is likely strongly contaminated by cytosolic activity. In contrast with Beavo's results and ours, Thompson et al. (28) did not find any phosphodiesterase activity associated with particulate material in 1000 g supernatant from rat heart or skeletal muscle.

The different responses of particulate cyclic AMP and cyclic GMP phosphodiesterase activities to freezing and thawing and solubilization procedures, the complexity of their kinetic patterns in each state of the membrane preparation, might suggest the presence of several membrane-bound cardiac isoenzymes, as found by Marchmont and Houslay in hepatocyte membrane (9). This contrasts with the observation of a clearly unique peak hydrolyzing both cyclic AMP and cyclic GMP after submitting Lubrol-solubilized preparation to isoelectric focusing, a high resolution separation procedure. A minor enzymatic form might however remain bound to Lubrol pellet or be completely labile in isoelectrofocusing conditions. The detergent-solubilized enzyme still exhibits anomalous biphasic Lineweaver-Burk plots. So, we hypothesize that a unique complex enzyme is responsible for hydrolysis of both cyclic AMP and cyclic GMP in this cardiac membrane preparation. Solubilization process would induce the loss of an eventual regulatory subunit, or a modification of the spatial configuration of the protein, or a perturbation in the interactions between eventual subunits, on account of the suppression of the lipidic environment. This would result in a modified enzyme state, behaving rather similarly in the presence of cyclic AMP and cyclic GMP.

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