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ADENYLATE CYCLASE OF HEART SARCOTUBULAR MEMBRANES*

PRAKASH V. SULAKHE** AND NARANJAN S. DHALLA
Department of Physiology, Faculty of Medicine, University of Manitoba, Winnipeg, R3E OW3 (Canada)

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SUMMARY

Subcellular distribution of dog and rabbit heart homogenates revealed highest specific activity of adenylate cyclase (ATP:AMP phosphotransferase, EC 2.7.4.3) in the sarcotubular membranes. Further fractionation of the sarcotubular membranes by sucrose density gradient revealed that the fraction possessing the highest calcium accumulating ability also showed the highest adenylate cyclase activity. The enzyme located in these membranes was activated by NaF and catecholamines. Ca^{2+} was found to inhibit adenylate cyclase activity of the sarcotubular membranes in the absence or presence of NaF and catecholamines. Neither norepinephrine nor NaF changed the affinity of the enzyme for ATP (K_m = 0.13 mM), however, the V (150 pmoles/mg protein per min) was increased 2- and 8-fold by these agents, respectively. Saturation of the enzyme was observed at 9 mM Mg^{2+}. Extraction of these membranes by deoxycholate as well as treatment with phospholipase C (EC 3.1.4.3) or trypsin (EC 3.4.4.4) markedly reduced the adenylate cyclase activity in the absence or presence of NaF and norepinephrine. Addition of sonicated lipids did not restore the reduced activity due to the phospholipase C treatment. Treatment with phospholipase A (EC 3.1.1.4) selectively increased the NaF stimulated activity without any significant effect on the basal activity. Pretreatment of sarcotubular membranes with NaF or norepinephrine and subsequent incubation with and without these activators indicated that F^- activation was partially irreversible and norepinephrine activation reversible. It is speculated that the adenylate cyclase present in the sarcotubular system may be intimately involved in the regulation of heart metabolism during the contraction–relaxation cycle.

Abbreviations: EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid; PCMB, *p*-chloromercuribenzoic acid.

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** Present address: Department of Pharmacology, University of British Columbia, Faculty of Medicine, Vancouver 8, Canada.

INTRODUCTION

It is now well established that adenylate cyclase (ATP:AMP phosphotransferase EC 2.7.4.3) and cyclic AMP are involved in the activation of cardiac metabolism by various hormones including catecholamines[1–3]. A great deal of work has been carried out to show the changes in cyclic AMP levels in the myocardium under the influence of different interventions[3–5]. Some information is also available concerning the properties of adenylate cyclase of the heart and for this purpose most of the investigators have either employed heart homogenate or washed cell particles for their studies describing the activation of this enzyme by NaF and by hormones such as catecholamines, glucagon and thyroxine[6–12].

It is generally believed that adenylate cyclase is located in the cell membrane[1]. However, Entman *et al.*[13] have claimed the presence of adenylate cyclase in cardiac sarcoplasmic reticulum. This observation was confirmed in this laboratory[14]. In addition, Wollenberger *et al.*[15] by employing specific cytochemical techniques for localization of adenylate cyclase have observed that the cardiac sarcoplasmic reticulum, especially the subsarcolemmal cisternae and lateral sacs, was more heavily stained with the reaction product than the myocardial cell membrane. Although all these investigators[13–15] have shown that adenylate cyclase in the sarcoplasmic reticulum is hormone sensitive, no report concerning its properties has yet appeared in the literature. Adequate information concerning the distribution of adenylate cyclase in myocardium is also not available. Therefore in the present paper we wish to describe some observations on the subcellular distribution and properties of adenylate cyclase present in the sarcotubular membranes of the dog and rabbit hearts. In addition, an attempt was made to gain some information concerning the role of membrane structure in the activity of adenylate cyclase as well as the mechanisms of its activation by norepinephrine and NaF.

METHODS

Dog or rabbit heart ventricles, after washing thoroughly to remove blood, were freed of connective tissue and fat. The cardiac tissue was minced, washed in ice-cold 0.25 M sucrose, 10 mM Tris–HCl (pH 7.4) and homogenized with 6–8 vol. of the same buffer in a Waring blendor or Sorval Omnimixer for 20 s (10 s × 2) in the cold room. The homogenate, after filtration through several layers of gauze, was centrifuged at 1000 × *g* for 15 min. The residue was washed, suspended in the buffer and centrifuged at 1000 × *g* for 10 min. This step was repeated twice and the washed residue was suspended in the same buffer at a concentration of 5–10 mg/ml. The combined supernatants were then centrifuged at 10 000 × *g* for 20 min to obtain the mitochondrial fraction which was washed once in the sucrose buffer, suspended and centrifuged at 1000 × *g* for 10 min, the residue discarded and the supernatant centrifuged at 8000 × *g* for 15 min to obtain the mitochondrial sediment which was then resuspended at a protein concentration of 3–5 mg/ml in the buffer sucrose. The post mitochondrial supernatant was then further centrifuged at 40 000 × *g* for 1 h to obtain heavy microsomal (sarcotubules or sarcoplasmic reticulum) fraction. This was suspended in 0.6 M KCl–10 mM Tris–HCl (pH 7.0), centrifuged at 40 000 × *g* for 45 min and suspended thoroughly in the sucrose buffer at a protein concentration of 2–5 mg/ml.

The post 40 000 × *g* supernatant and the above fractions were kept at 0 °C.

In some experiments, the heavy microsomal fraction of the rabbit heart was layered onto a sucrose gradient consisting of 10 ml of 40% sucrose and 15 ml of 20% sucrose. The tubes were centrifuged at 20 000 rev./min for 2 h in a Beckman ultracentrifuge (Model L). The protein fractions were removed by a Pasteur pipet, diluted 2–3-fold with 10 mM Tris–HCl (pH 7.5) and centrifuged as above. The sediments were assayed for calcium uptake by the method described earlier[16,17] and for adenylate cyclase by the following methods.

Various fractions, including the original homogenate, were assayed for adenylate cyclase activity within 15 min of their isolation. The adenylate cyclase activity was assayed at 37 °C in a total volume of 0.1 ml containing 25–30 mM Tris–HCl (pH 7.45), 4 mM $MgCl_2$, 2 mM [8-^{14}C]ATP, 8 mM theophylline, 20 mM phosphoenol pyruvate, about 10 μg of pyruvate kinase (EC 2.7.1.40), 5–10 mM KCl and 0.5 mg albumin/ml (System A). The reaction was started by addition of either fractions or ATP and stopped by boiling the tubes for 4 min with prior addition of unlabelled cyclic AMP (final concentration 2 mM). The tubes were centrifuged and the 50 μl of the clear supernatant spotted on Whatman No. 3 MM paper for ascending or descending chromatography, using 1 M ammonium acetate–95% ethanol (13:35, v/v) as a solvent. The chromatograms were developed for 18 h at room temperature (24–25 °C) and, after drying, the cyclic AMP area was visualized by ultraviolet light, cut out and counted in 20 ml of Bray's solution in a Packard liquid scintillation spectrometer. The chromatograms also had marker spots for ATP, ADP, AMP, and cyclic AMP. The nonenzymatic formation of cyclic AMP was estimated for every fraction by denaturing the particulate material and then following the above procedure. The final activity was then calculated after correcting for nonenzymatic cyclic AMP formation as well as for quenching. The above method is essentially similar to that described by Drummond and Duncan[6]. The adenylate cyclase activity was also determined under the assay conditions (System B) described by Drummond *et al.*[12]. In some experiments the adenylate cyclase assay was carried out in the above medium while the separation and estimation of cyclic AMP was followed according to the method (System C) described by Krishna *et al.*[18]. Results obtained by these procedures are essentially similar. Other conditions and any changes employed are described under the respective tables and figures. The protein concentration was determined according to Lowry *et al.*[19]. Cytochrome *c* oxidase (EC 1.9.3.1), AMP nucleosidase (EC 3.2.2.4) and glucose-6-phosphatase (EC 3.1.3.9) activities were determined according to methods described elsewhere[20–22]

Treatment of sarcotubular membranes with phospholipase C (EC 3.1.4.3)

The sarcotubular membranes (2.0 mg/ml) were treated with different concentrations of phospholipase C for 10 min in medium containing 50 mM Tris–HCl (pH 7.5), 20 mM KCl and 0.2 mM $CaCl_2$. At the end of incubation, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA) was added (2 mM final concentration) and the membranes were separated by centrifugation at 40 000 × *g* for 45 min. The sediment was suspended in 50 mM KCl–20 mM Tris–HCl (pH 7.0) and centrifuged at 40 000 × *g* for 45 min. The membranes thus obtained were resuspended in buffered sucrose solution at a protein concentration of 1.0 mg/ml and used for the adenylate cyclase activity measurement.

Treatment of sarcotubular membranes with phospholipase A (EC 3.1.1.4)

The membranes were incubated with different concentrations of heat-treated (70 °C for 15 min) snake venom *(Naja Naja)* in medium containing 50 mM Tris–HCl (pH 7.5), 100 mM KCl, 2 mM $CaCl_2$ at room temperature for 10 min. At the end of incubation, EGTA was added to a final concentration of 5 mM and the washed particles were assayed for adenylate cyclase activity in the absence or presence of albumin (1%).

Treatment of sarcotubular membranes with trypsin (EC 3.4.4.4)

The vesicles (2.5 mg/ml) were incubated in medium containing 50 mM Tris–HCl (pH 7.6), 20 mM KCl and various concentrations of trypsin for 10 min at room temperature. At the end of incubation, the reaction was stopped by the addition of 2–3-fold excess of trypsin inhibitor and the membranes were separated by centrifugation at 40 000 × *g* for 45 min. The sediment was washed by suspension in buffered sucrose solution followed by centrifugation. The pellet was resuspended in 50 mM KCl–20 mM Tris–HCl (pH 7.0) and assayed for adenylate cyclase activity. Control incubation was carried out in the presence of trypsin and trypsin inhibitor mixture.

Pretreatment with NaF or norepinephrine

The membranes were incubated in medium containing 50 mM Tris–HCl (pH 7.5) in addition to other ions as indicated under the respective tables with and without NaF or norepinephrine bitartarate. The tubes were chilled in ice at the end of incubation period and final membrane suspension was obtained by washing and centrifugation at 40 000 × *g* for 30 min. These membranes were then assayed for adenylate cyclase activity in the standard medium in the absence or presence of NaF or norepinephrine.

[8-^{14}C]ATP was purchased from New England Nuclear Corporation. All other nucleotides were obtained from P.L. Biochemicals or Cal-Biochemicals. Epinephrine bitartarate and norepinephrine bitartarate were purchased from Winthrop Chemicals or Cal-Biochemicals. Phospholipase C (from *Clostridium welchii*) was a Sigma product and trypsin was purchased from Cal-Biochemicals. Dowex-50 H^+ form (X8), 100–200 mesh) was obtained from Baker Chemicals or BioRad Laboratory. All reagents were of analytical grade and distilled deionized water was used throughout the procedure. Special enzyme grade sucrose was purchased from Mann Laboratories. Snake venom *(Naja Naja)* and sonicated lipids (from red blood cells) were kindly provided by Dr A. Fessler.

RESULTS

Dog and rabbit heart homogenates were fractioned by differential centrifugation and the adenylate cyclase activity in the absence and presence of NaF (5 mM) was determined in each fraction. The data on subcellular distribution are shown in Tables I and II. The highest specific activity of this enzyme was observed in the 10 000–40 000 × *g* fraction (sarcotubular membranes) with or without NaF in the incubation medium; however, 50–70% of the total activity of the dog heart homogenate and 80–90% of the total activity of the rabbit heart homogenate sedimented in the 1000 × *g* fraction. The relative specific activities of some marker enzymes in

TABLE I

SUBCELLULAR DISTRIBUTION OF ADENYLATE CYCLASE IN DOG HEART HOMOGENATE

Fresh dog heart ventricles were homogenized in 0.25 M sucrose–10 mM Tris–HCl (pH 7.5) for 20 s. The homogenate was spun down at the speeds and times indicated below, the fractions were suspended in the above buffer and assayed according to System A (see Methods) for adenylate cyclase activity at 37 °C for 5 min. The protein concentrations in the incubation medium were 73 μg, 74 μg, 58 μg, 72 μg and 56 μg for homogenate, 1000 × *g*, 10 000 × *g*, 40 000 × *g* and supernatant fractions, respectively. NaF was present at 5 mM concentration. Assay system A was used.

Fraction	*Total protein (mg)*	*Total activity*		*Percent activity*		*Specific activity*	
		Without F⁻	*With F⁻*	*Without F⁻*	*With F⁻*	*Without F⁻*	*With F⁻*
		(nmoles/min)				*(pmoles/mg protein per min)*	
Homogenate	1428	59.8	249.8	100	100	42.0	175.0
1000 × *g* 10 min	925	36.6	132.2	61	53	39.6	143.0
10 000 × *g* 20 min	112	7.4	11.2	12	4.5	67.0	100.0
40 000 × *g* 60 min	17	1.2	6.4	2	2.6	71.8	379.4
Supernatant	385	3.8	7.0	6.4	2.7	10.0	18.0

TABLE II

SUBCELLULAR DISTRIBUTION OF ADENYLATE CYCLASE IN RABBIT HEART HOMOGENATE

Adenylate cyclase activity was measured according to System B (see Methods). All other conditions are similar to those described under Table I.

Fraction	*Total protein (mg)*	*Total activity (nmoles/min)*		*Percent activity*		*Specific activity (pmoles/mg protein per min)*	
		Without F⁻	*With F⁻*	*Without F⁻*	*With F⁻*	*Without F⁻*	*With F⁻*
Homogenate	955.8	32.8	185.1	100	100	34.4	193.6
1000 × *g* 10 min	571.2	28.6	147.6	86.9	79.7	50.1	258.4
10 000 × *g* 20 min	68.0	3.3	8.6	8.2	4.6	39.8	126.1
40 000 × *g* 60 min	12.1	1.4	4.6	4.2	2.5	113.4	385.6
Sarcoplasmic reticulum*	43.3	4.9	16.7	14.9	9.0	113.4	385.7

* Data calculated after correction for the content of the fragmented sarcoplasmic reticulum/g heart according to the method of Briggs *et al.*[23].

TABLE III

RELATIVE SPECIFIC ACTIVITIES OF MARKER ENZYMES IN THE SUBCELLULAR FRACTIONS OF DOG HEART HOMOGENATE

Various fractions were obtained as described in Table I. Relative specific activity is expressed as the ratio of percent activity in the fraction to the percent protein in the fraction.

Fraction	*Cytochrome c oxidase*	*AMP nucleosidase*	*Glucose-6-phosphatase*	*NaF-stimulated adenylate cyclase*
1000 × *g* 10 min	0.133	0.399	0.299	0.81
10000 × *g* 20 min	7.334	0.550	0.400	0.59
40000 × *g* 60 min	0.600	7.500	4.168	2.18

the dog heart 10 000–40 000 × *g* fraction are shown in Table III for comparison purposes. The lack of inhibitory effect of azide, a well known inhibitor of mitochondrial calcium transport[17], on the "calcium pump" present in the sarcotubular membranes rules out the possibility of contamination by mitochondrial fragments. We have also observed the presence of adenylate cyclase in sarcotubular membranes isolated from rat, guinea pig, hamster and sheep hearts. It may be mentioned that only 2–4% of the total adenylate cyclase activity in the heart was found to be present in the 8000–40 000 × *g* fraction and this is due to the poor yield of this fraction which is isolated mainly for the purpose of demonstrating the presence of a very active "calcium pump". However, on applying the correction factor to obtain the content of these membranous vesicles according to the method described by Briggs *et al.*[23], 9–15% of the total adenylate cyclase activity in the heart was calculated to be associated with these membranes. The content of sarcotubular vesicles in rabbit heart was found to be 5.27 mg/g heart.

In order to obtain evidence that adenylate cyclase resides in the fragmented sarcotubular membranes, the 40 000 × *g* fraction was layered on the sucrose gradient (Fig. 1). The protein fraction exhibiting the highest specific activity of calcium transport (the sarcotubular membrane marker) also showed the highest specific

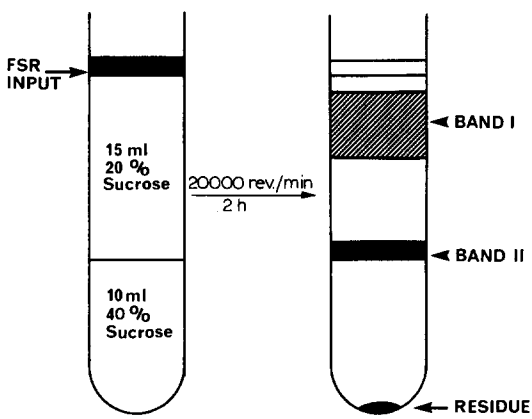

Fig. 1. Sucrose density gradient. The details are described under Methods. FSR, fragmented sarcoplasmic reticulum.

activity of adenylate cyclase (Table IV). If adenylate cyclase in these membranes was present due to contamination by sarcolemmal fragments, then such a distribution pattern of adenylate cyclase and calcium transport activities in the three fractions obtained after gradient centrifugation would not be expected. The protein fraction (Band II) containing the highest specific activity of adenylate cyclase was also used for the kinetic studies described below.

The reaction for the adenylate cyclase activity of the sarcotubular membranes with or without NaF was linear with the time of incubation and the protein concentrations employed in our assay conditions. In the absence of an ATP-regenerating system this linearity was not observed. Since adenylate cyclase activity in sarcotubular membranes was found to decline by 30–40% within 3 h when kept at 0 °C, most of the

TABLE IV

DISTRIBUTION OF PROTEIN, CALCIUM UPTAKE AND ADENYLATE CYCLASE ACTIVITIES IN VARIOUS BANDS OBTAINED BY DENSITY GRADIENT CENTRIFUGATION OF THE RABBIT HEART FRAGMENTED SARCOPLASMIC RETICULUM

See Fig. 1 for sucrose density gradient experiment. Assay system B used.

	Total protein (mg)	*Adenylate cyclase activity*				*Calcium uptake (nmoles/mg)*	
		Total activity (pmoles/min)		*Specific activity (pmoles/min per mg)*			
		Without F^-	*With F^-*	*Without F^-*	*With F^-*	*Without F^-*	*With F^-*
40 000 × *g* fraction	6.90	590	2023	86	293	22	1800
Band I	0.36	73	281	203	782	13	2123
Band II	0.90	191	760	212	844	37	2925
Residue	3.7	114	1124	31	305	31	1675
Recovery (%)	70	64	107	—	—	97	77

experiments were carried out within 15 min of the isolation of these membranes. However, active preparations can be obtained from hearts frozen and stored at −90 °C.

Various concentrations of F^- and norepinephrine were shown to activate adenylate cyclase activity of the sarcotubular system (Fig. 2). The maximal activa-

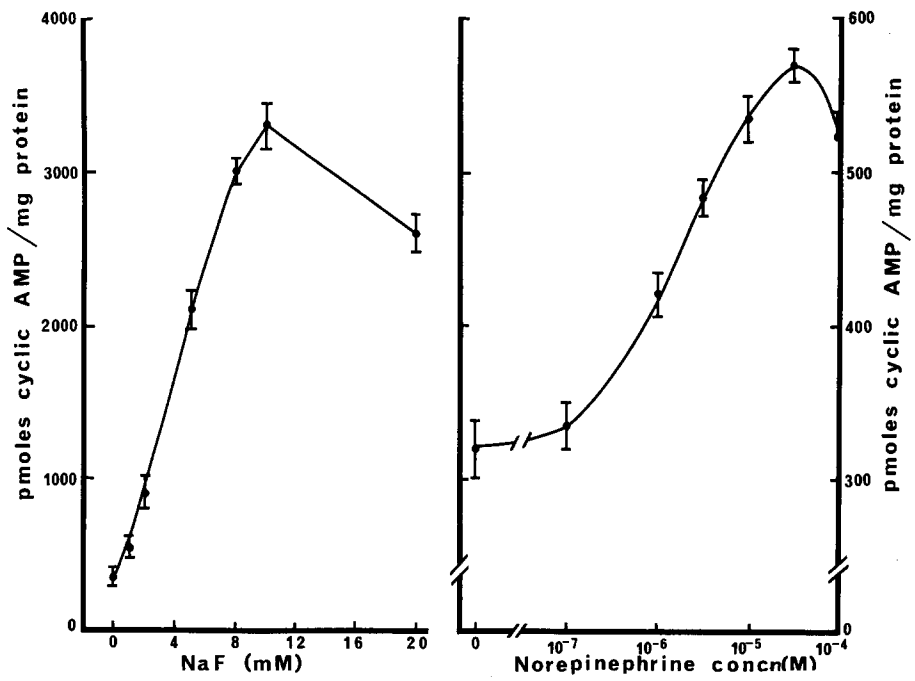

Fig. 2. Activation of dog heart sarcotubular adenylate cyclase by NaF and norepinephrine. F^- experiment, mean ±S.E., $n = 4$. Time of incubation 5 min at 37 °C, protein concentration, 57–85 μg for different preparations. 4 mM $MgCl_2$, 2 mM ATP. Norepinephrine experiment, mean ±S.E., $n = 3$; protein concentration 65–92 μg. Time of incubation 5 min at 37 °C. 4 mM $MgCl_2$, 2 mM ATP. Other conditions as described in the methods. Assay system A used.

tion by NaF was seen at 10 mM concentration whereas higher concentrations of NaF were inhibitory. On the other hand the maximal activation by norepinephrine was obtained at a concentration of $5 \cdot 10^{-5}$ M. Epinephrine exhibited a dose response similar to that of norepinephrine. It may be noted that in contrast to a marked stimulation by NaF, catecholamines showed a modest degree of activation. The adenylate cyclase activity of sarcotubular membranes in the absence or presence of NaF (5 mM) showed a relatively broad pH optimum between 7.2 and 8.0 (Fig. 3).

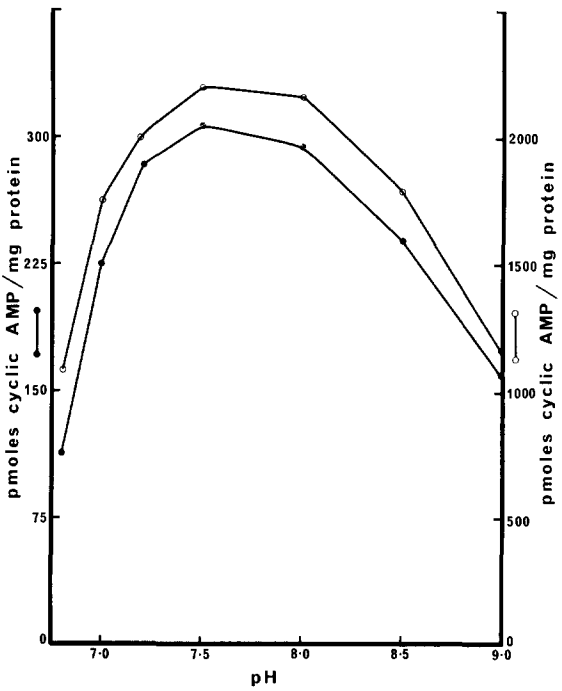

Fig. 3. pH of the incubation medium and dog heart sarcotubular adenylate cyclase activity. The pH of the various solutions to be added in the medium were adjusted to their respective values. 40 mM Tris–HCl of various pH employed, NaF, 5 mM. Protein concentrations were 74 μg and 91 μg for basal and F^--stimulated experiments, respectively. Time of incubation was 5 min at 37 °C. Other conditions as described in Methods. ●—●, basal adenylate cyclase activity; ○—○, F^--stimulated adenylate cyclase activity. Assay system A used.

Adenylate cyclase activity of the sarcotubular membranes depended upon Mg^{2+} concentrations, which were much in excess of ATP. The effect of increasing concentrations of Mg^{2+} on the velocity of the reaction was examined in the absence and presence of norepinephrine or NaF. The results shown in Fig. 4 indicated that saturation was observed at 9–18 mM Mg^{2+}, an apparent K_a being 3–4 mM. Neither norepinephrine nor NaF had any appreciable effect on the apparent K_a for Mg^{2+}; however, at each concentration of Mg^{2+} both norepinephrine and NaF increased the V. The results obtained with washed particles (prepared according to the method of Drummond and Duncan[6]) are also shown in Fig. 4. These data seem to support the conclusion drawn by Drummond and co-workers[6,12] that Mg^{2+} binding at a secondary site results in the selective activation of the V but not in a change in the affinity for Mg^{2+}.

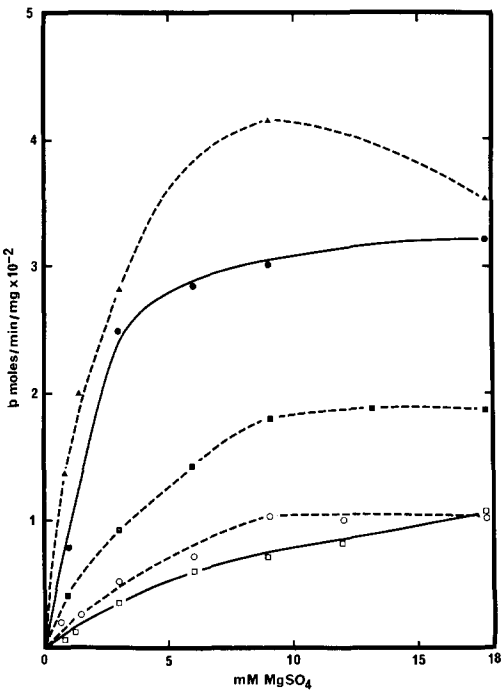

Fig. 4. Effect of Mg^{2+} concentration on the adenylate cyclase. The protein fraction of Band II obtained by sucrose density gradient centrifugation (see Fig. 1) of the rabbit heart sarcotubular membranes was assayed according to System B. The incubation medium contained 40 mM Tris–HCl (pH 7.5), 8 mM theophylline, 2 mM cyclic AMP, 5.5 mM KCl, 20 mM phosphoenolpyruvate, 130 μg per ml of pyruvate kinase, 0.4 mM [α-^{32}P]ATP (20–25 μCi/μmole) and indicated concentrations of $MgSO_4$ at 37 °C. The washed cell particles of rabbit heart prepared according to the method of Drummond and Duncan[6]. Solid lines, washed cell particles (basal, □—□, NaF (9 mM) stimulated, ●—●. Dotted lines, sarcotubular membranes (basal, ○---○; norepinephrine ($5 \cdot 10^{-6}$ M) stimulated, ●---●; NaF (9 mM) stimulated, ▲---▲.

The effect of increasing concentrations of ATP at a fixed concentration of Mg^{2+} on the adenylate cyclase activity is shown in Fig. 5. The apparent K_m for ATP was found to be 0.13 mM. Neither norepinephrine nor NaF had any effect on the K_m; however, these agents increased the velocity of the reaction. Concentrations of ATP in excess of Mg^{2+} were found to be inhibitory.

In view of the remarkable activity of the sarcotubular membranes to transport calcium, the effect of various concentrations of Ca^{2+} on adenylate cyclase was studied. The results shown in Fig. 6 indicate a marked inhibitory effect of Ca^{2+} on the adenylate cyclase activity of heart sarcotubular membranes when studied in the absence or presence of NaF and norepinephrine.

Dog heart sarcotubular preparations were treated with varying amounts of phospholipase C or trypsin and the adenylate cyclase activity was determined with and without NaF or epinephrine. The results in Figs 7 and 8 indicate that the enzyme activity is markedly reduced by these treatments. It was interesting to find that the response to epinephrine was unaltered under conditions of phospholipase C treatment whereas the F^- response was reduced by 50%. Likewise, the epinephrine response was reduced by about 20% when the F^- response was diminished by 80% under condi-

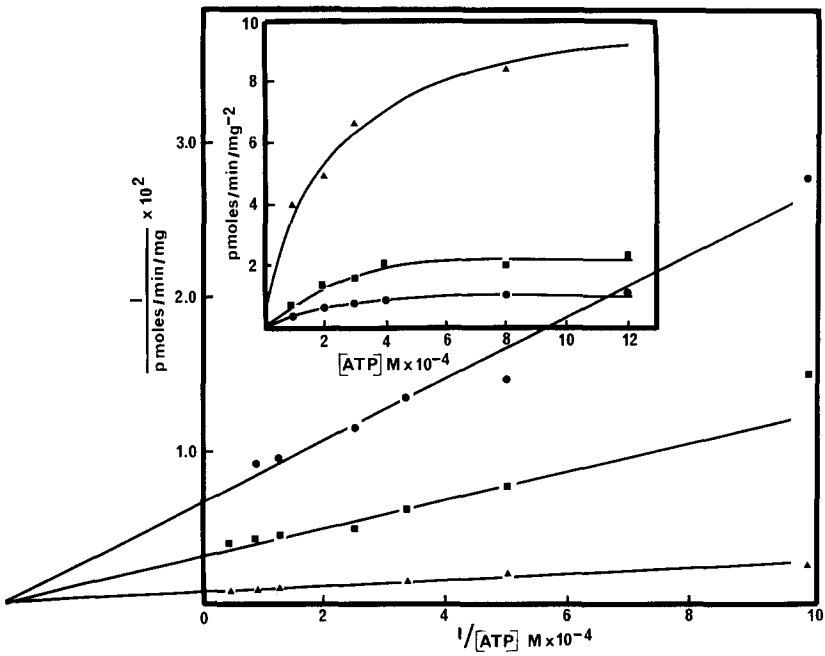

Fig. 5. Effect of ATP concentration on the rabbit heart sarcotubular adenylate cyclase. The protein fraction of Band II obtained by sucrose density gradient centrifugation (see Fig. 1) was assayed according to System B in the presence of different concentrations of [α-^{32}P]ATP (20–25 μCi/μmole). Basal activity, ●—●; norepinephrine ($5 \cdot 10^{-5}$ M) stimulated activity, ■—■; NaF (9 mM) stimulated activity, ▲—▲.

TABLE V

INACTIVATION OF ADENYLATE CYCLASE BY PHOSPHOLIPASE C TREATMENT: EFFECT OF SONICATED PHOSPHOLIPIDS ON RABBIT HEART SARCOTUBULAR MEMBRANES

Rabbit heart sarcotubular membranes were treated with or without phospholipase C as described in the legend to Fig. 7. Equal volumes of these membranes and sonicated phospholipid suspension (2 mg/ml) were incubated for 5 min at room temperature and assayed according to System B.

Phospholipase C concn (μg/ml)	*Treatment time (min)*	*Sonicated lipid*	*Adenylate cyclase activity (pmoles/min per mg)*		
			Basal	*Norepinephrine ($5 \cdot 10^{-5}$ M)*	*NaF (9 mM)*
0	10	—	65.35	100.47	386.07
	10	+	85.82	92.50	418.53
	20	—	35.37	46.00	326.73
	20	+	50.06	58.25	347.92
60	10	—	50.89	85.73	366.40
	10	+	60.12	70.52	369.85
	20	—	30.20	55.12	298.26
	20	+	38.50	45.80	278.70
300	10	—	51.19	87.32	335.29
	10	+	61.40	55.99	367.99
	20	—	21.14	29.78	257.55
	20	+	31.36	23.99	257.92
600	10	—	24.90	11.00	262.80
	10	+	24.20	15.05	219.24
	20	—	0	0	188.43
	20	+	4.84	0	197.00

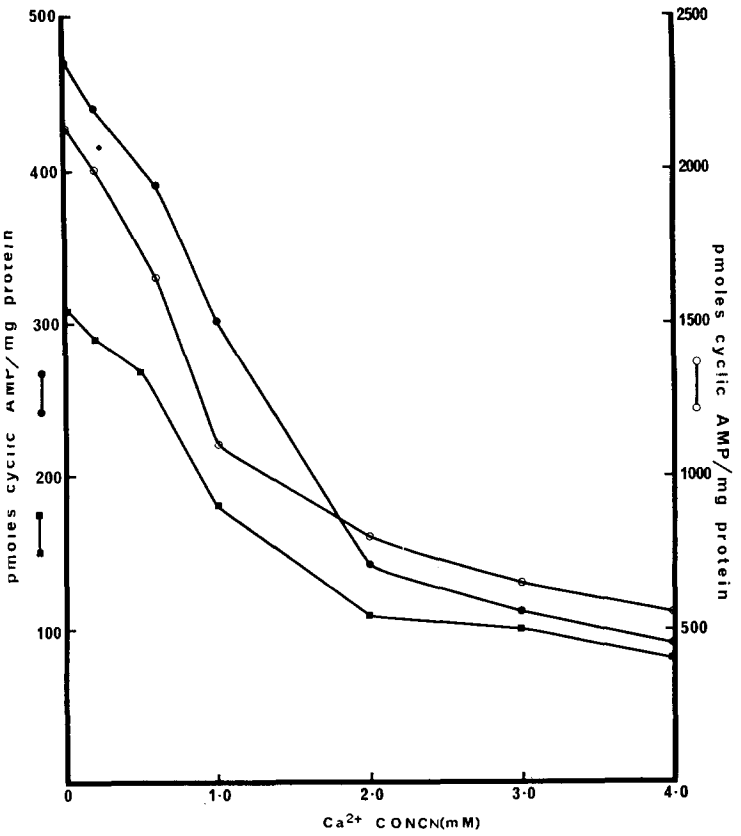

Fig. 6. Inhibition of adenylate cyclase of dog heart sarcotubular membranes by Ca^{2+}. All standard conditions as described under Methods (System A). Time of incubation was 6 min at 37 °C. Basal activity (■—■, protein, 75 μg. Norepinephrine-stimulated activity (●—●), $1 \cdot 10^{-5}$ M; protein, 80 μg. F^--stimulated activity (○—○), 5 mM; protein, 100 μg.

tions of trypsin digestion. Extraction of these membranes with 1% deoxycholate was also found to inactivate the enzyme.

When rabbit heart sarcotubular preparations were treated with phospholipase C, results similar to those described above for dog heart were obtained. Addition of sonicated phospholipids to these phospholipase C treated membranes did not restore the activity of adenylate cyclase when tested in the absence and presence of norepinephrine or NaF. As a matter of fact, high concentrations of lipids were inhibitory (Table V). Further experiments revealed that the response of calcium transport system and adenylate cyclase of the sarcotubular membranes to delipidation differ in that the former is less sensitive than the latter under controlled conditions of phospholipase C digestion followed by reconstitution with sonicated phospholipids (data not shown).

In contrast to phospholipase C, the effect of phospholipase A digestion of sarcotubular membranes is of a different nature. It was of interest to observe that the basal adenylate cyclase activity of the heart sarcotubular membranes was not altered appreciably by phospholipase A treatment (Fig. 9). Similar action of phospholipase A

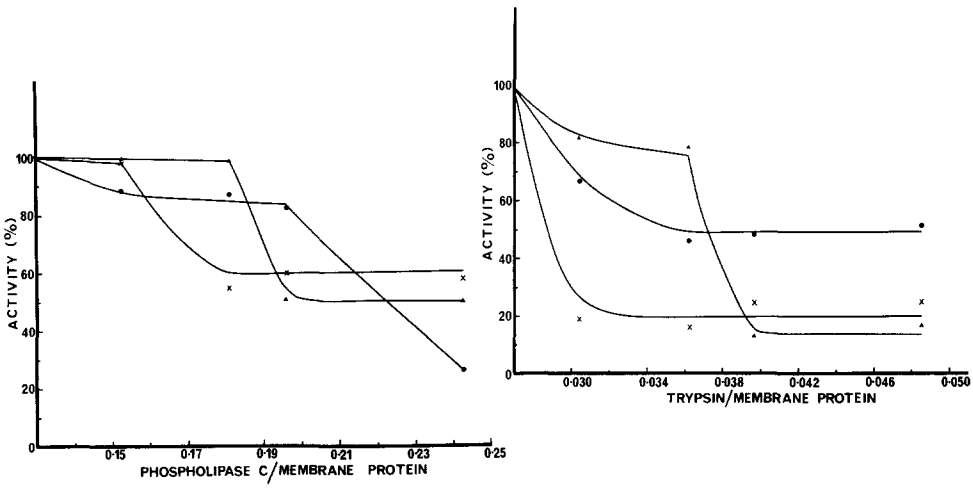

Fig. 7. Effect of phospholipase C treatment on the adenylate cyclase activity. Dog heart sarcotubular membranes were treated with different concentrations of phospholipase C as described under Methods (System C). The membranes obtained after washing and centrifugation were assayed in the absence and presence of NaF (10 mM) or epinephrine ($5 \cdot 10^{-5}$ M) for 10 min at 37 °C under standard assay conditions. ●—●, basal; ×—×, F^-; ▲—▲, epinephrine.

Fig. 8. Effect of trypsin treatment on dog heart sarcotubular adenylate cyclase activity. The trypsin treatment was carried out as described under Methods (System C). The membranes were separated after washing and centrifugation and were assayed immediately for adenylate cyclase activity in the absence or presence of activators, NaF, 10 mM; epinephrine $5 \cdot 10^{-5}$ M. Time of incubation was 10 min at 37 °C. All other conditions were as described under Methods. ●—●, basal; ×—×, F^-; ▲—▲, epinephrine.

on rat liver plasma membranes has also been reported by other investigators[24]. The NaF-stimulated adenylate cyclase activity of these membranes is further enhanced by phospholipase A treatment. The effect of phospholipase A treatment was maximal when fatty acid free albumin was added during the adenylate cyclase assay (Fig. 10). Since the activating effects of phospholipase A digestion were observed in the absence of added albumin, this action can not be attributed to the released free fatty acid but may be due to alteration of the lipoprotein structure of the membrane.

A well known sulfhydryl blocking agent, *p*-chloromercuribenzoic acid (PCMB) (1–10 μM), was found to inhibit adenylate cyclase activity. The addition of cysteine (1 mM) prevented this action but cysteine alone had no effect (Table VI). Similar action of PCMB on both F^-- and norepinephrine-stimulated activities was also observed. A well known adrenergic blocking agent, propranolol ($2.5 \cdot 10^{-4}$ M), was found to inhibit norepinephrine stimulated activity without any influence on the effect of F^- on the sarcotubular adenylate cyclase (Table VII). It was also observed that the responses to NaF and norepinephrine were not additive at the concentration employed in this study.

In another series of experiments, the membranes were treated with NaF or norepinephrine in a medium containing Mg^{2+}, Ca^{2+} or ATP, washed thoroughly and assayed for adenylate cyclase activity. The enzyme activity of NaF-treated particles was higher than for those without F^- (Table VIII). However, Mg^{2+} and ATP when

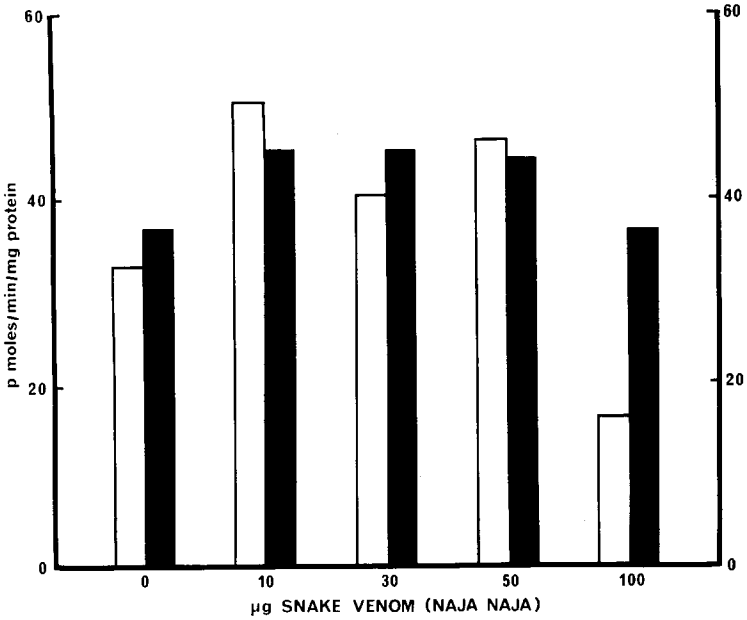

Fig. 9. Effect of snake venom (*Naja Naja*; as a source of phospholipase A) on basal adenylate cyclase of rabbit heart sarcotubular membranes. The membranes were incubated at 23 °C with indicated concentrations of heat treated (70 °C for 15 min to inactivate proteins) snake venom in a medium containing 50 mM Tris–HCl (pH 7.5), 2 mM $CaCl_2$ for 10 min. At the end of the incubation period, EGTA was added to a final concentration of 5 mM and the aliquots were assayed (System B) for adenylate cyclase activity in the absence (open bars) or presence (filled bars) of albumin (1%).

TABLE VI

REQUIREMENT OF –SH GROUPS FOR ADENYLATE CYCLASE ACTIVITY

The membranes were incubated at 37 °C for 3 min with various concentrations of PCMB and/or cysteine (1 mM) in medium otherwise complete and the reaction started by the addition of ATP to give final concentration of 2 mM. NE ($5 \cdot 10^{-5}$ M) and NaF (8 mM) were present before starting the reaction by ATP. The time of incubation was 5 min and the protein concentrations were 85 μg, 92 μg and 78 μg for the basal, norepinephrine and NaF experiments, respectively. Different preparations were used for each experiment. Assay system A was used.

Incubation medium composition	*Adenylate cyclase (pmoles/mg per 5 min) in the presence of*		
	Basal	*Norepinephrine ($5 \cdot 10^{-5}$ M)*	*NaF (8 mM)*
Basal	297	572	2875
1 μM PCMB	202	475	2422
2.5 μM PCMB	159	325	1872
10 μM PCMB	97	139	900
2.5 μM PCMB + 1 mM cysteine	272	550	2753
1 mM cysteine	305	595	3100

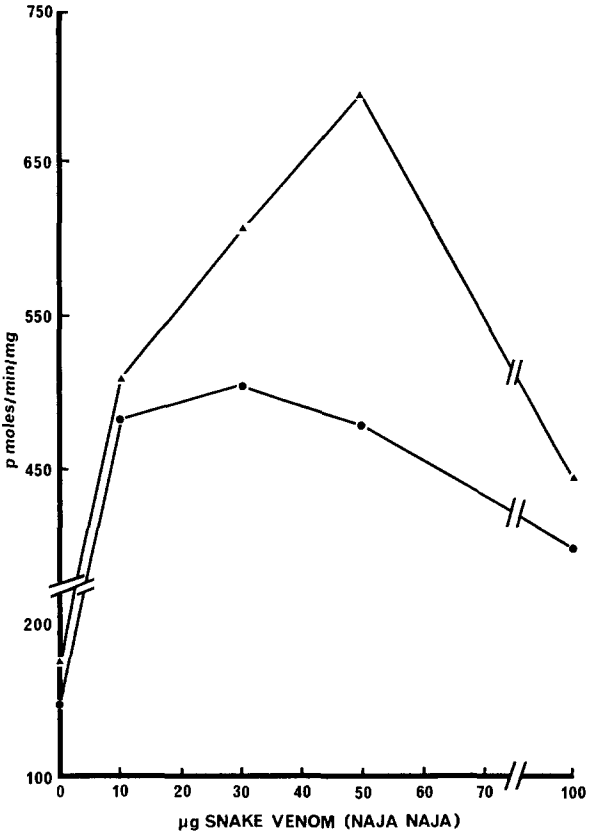

Fig. 10. Effect of snake venom on NaF (9 mM) stimulated adenylate cyclase of rabbit heart sarcotubular membranes. The conditions are exactly similar to those described under Fig. 9. Adenylate cyclase activity was determined in the absence (●—●) or presence (▲—▲) of albumin (1%).

present together with F^- in the pre-incubation medium showed maximum activity when assayed subsequently under basal conditions. Pretreatment with Ca^{2+} reduced the activity while Mg^{2+} or ATP had no appreciable effect. When the adenylate cyclase activity was determined in the presence of F^- (incubation medium) pretreatment of the membranes with NaF had no influence, except that the presence of Ca^{2+} in the pre-incubation medium reduced the activity (Table VIII). These results indicate that F^- is firmly bound to these membranes (or to this enzyme) and is not removed easily by washing. In other words, F^- and hence the activation of adenylate cyclase by F^- is partially reversible. The removal of an inhibitor by NaF is also possible but the present studies do not distinguish between these two separate mechanisms of F^- action. In contrast to F^-, norepinephrine pretreatment did not have an appreciable effect when subsequently assayed in the absence or presence of norepinephrine (Table IX). An inhibitory effect of Ca^{2+}, similar to that reported above, was also seen in these experiments.

TABLE VII

EFFECT OF PROPANOLOL AND Ca^{2+} ON THE STIMULATION OF HEART SARCOTUBULAR ADENYLATE CYCLASE BY NaF AND NOREPINEPHRINE

The composition of the assay medium is similar to that described in Methods. The other additions are as follows, NaF (8 mM), norepinephrine ($5 \cdot 10^{-5}$ M), propranolol ($2.5 \cdot 10^{-4}$ M), Ca^{2+} (2 mM), Mg^{2+} (3 mM), ATP (3 mM) and EGTA (1.0 mM). Temp. 37 °C. Assay system A used.

Composition of incubation medium	*Adenylate cyclase activity (pmoles cyclic AMP/mg protein per 5 min)*
Expt 1	
Basal medium	352
+ norepinephrine	605
+ NaF	2980
+ norepinephrine + NaF	2875
+ propranolol	328
+ norepinephrine + propraholol	393
+ NaF + propranolol	2921
Expt 2	
Basal medium	312
+ norepinephrine	569
+ norepinephrine + Ca^{2+}	310
+ Ca^{2+}	190
+ propranolol + norepinephrine	352
+ propranolol + norepinephrine + Ca^{2+}	258
+ EGTA	387

TABLE VIII

IRREVERSIBILITY OF NaF ACTIVATION OF THE SARCOTUBULAR ADENYLATE CYCLASE

The membranes (1 mg/ml) were first incubated in 50 mM Tris buffer (pH 7.5), containing Mg^{2+} (4 mM), NaF (5 mM), ATP (2 mM) and Ca^{2+} (1.5 mM) either alone or in combination as indicated below for 5 min at 25 °C. The tubes were then chilled in ice and the membranes were separated by washing with sucrose–Tris buffer (pH 7.5) (see Methods) and centrifugation. These particles were suspended in sucrose–Tris medium and used immediately for adenylate cyclase assay in the standard medium with and without 8 mM NaF. Time of incubation was 5 min at 37 °C. Assay system A used. Results are expressed as pmoles cyclic AMP/mg protein per 5 min.

Incubation medium prior to assay	*Activity (pmoles cyclic AMP/mg protein per 5 min)*	
	Without F^-	*With F^- (8 mM)*
Expt 1		
None	293	2785
Mg^{2+}	279	2812
Mg^{2+} + NaF	850	3100
Mg^{2+} + NaF + ATP	1422	3273
Expt 2		
None	328	2972
Ca^{2+}	265	2100
Ca^{2+} + Mg^{2+}	297	2700
Ca^{2+} + Mg^{2+} + ATP	245	2050
Ca^{2+} + NaF	518	2122
Ca^{2+} + ATP + NaF	539	2300
Mg^{2+} + NaF	1020	3200
Mg^{2+} + NaF + ATP	1622	3250
ATP	345	3125
NaF (2 mM)	492	2987

TABLE IX

REVERSIBILITY OF NOREPINEPHRINE STIMULATION OF THE SARCOTUBULAR ADENYLATE CYCLASE

The sarcotubular membranes (2.5 mg/ml) were incubated for 10 min (Expt 1) and for 5 min (Expt 2) at 25 °C in 50 mM Tris–HCl (pH 7.5) containing Mg^{2+} (4 mM), NaF (5 mM), ATP (2 mM), Ca^{2+} (1.0 mM), norepinephrine ($5 \cdot 10^{-5}$ M), and propranolol ($1 \cdot 10^{-4}$ M) either alone or in combination as described below. The tubes were then chilled in ice, the membranes washed in sucrose–Tris buffer (pH 7.5) and centrifuged, and finally suspended in sucrose–Tris buffer (Expt 1) or 50 mM Tris–HCl (pH 7.5) (Expt 2). These particles were assayed immediately for adenylate cyclase activity in the presence or absence of norepinephrine ($5 \cdot 10^{-5}$ M) at 37 °C for 5 min. The protein concentration of the washed membranes suspensions was adjusted to 1.5 mg/ml, so that 75 μg of protein was present during assay. Assay system A used.

Preincubation medium	*Activity (pmoles cyclic AMP/mg protein per 5 min)*	
	Without norepinephrine	*With norepinephrine ($5 \cdot 10^{-5}$ M)*
Expt 1		
None	275	578
Mg^{2+}	283	592
Mg^{2+} + ATP	291	583
Mg^{2+} + ATP + norepinephrine	305	598
Expt 2		
None	318	592
Ca^{2+}	262	489
Ca^{2+} + ATP	295	512
Ca^{2+} + ATP + norepinephrine	278	502
Norepinephrine	305	602
NaF + Mg^{2+} + ATP	1385	1378
Propranolol	257	581

DISCUSSION

The presence of adenylate cyclase activity in dog and rabbit cardiac sarcotubular membranes and its activation by catecholamines of F^- are in agreement with earlier reports[13–15]. We have previously characterized the sarcotubular fraction in terms of its marker enzyme activities and responses to different inhibitors of calcium transport since this fraction is frequently employed for studies concerning the "calcium pump" mechanism in heart muscle[16,17]. These membranes appear as vesicular structures when examined by electron microscopes and are relatively free of other cytoplasmic contaminents as revealed by marker enzyme activities. Furthermore, sucrose gradient experiments revealed that the fraction of the sarcotubular membranes exhibiting highest calcium transport ability also showed highest adenylate cyclase specific activity. This fraction was also found to contain negligible activity of Na^+–K^+-stimulated ouabain-sensitive ATPase (EC 3.6.1.3), an enzyme considered to be mainly localized in the heart sarcolemma[25]. Therefore we believe that the adenylate cyclase in the sarcotubular membranes is not of sarcolemmal origin. Although only 10–15% of the total adenylate cyclase activity of the heart homogenate is present in the sarcotubular membranes, this small fraction may be of some functional importance. Particularly, it is of considerable interest that the presence of cyclic AMP-dependent protein kinase (EC 2.7.1.37) has also been demonstrated in the cardiac sarcotubular membranes[26].

Our finding concerning the presence of adenylate cyclase in the sarcotubular fraction prompted us to examine some of its kinetic properties. The apparent K_a for Mg^{2+} was 3–4 mM and K_m for ATP was about 0.13 mM. Both NaF and norepinephrine increased the velocity of the reaction at all Mg^{2+} concentrations without any effect on the K_m for the substrate (MgATP). These results support the conclusion of Drummond *et al.*[12] that the stimulatory effects of NaF and hormones are due to an increase in V of the enzyme, reflecting increased reactivity of the catalytic site with substrate. On the basis of these data, it is difficult to determine whether or not the adenylate cyclase of the sarcotubular membranes is different from that considered to be present in the heart sarcolemma. It may however be pointed out that since the membrane preparation employed in this study is vesicular, diffusion of substrate may have an important role in the activity of the enzyme. Thus different conditions may alter the accessibility of substrate to the catalytic sites without changing the properties of the enzyme *per se*. Therefore, some caution should be observed in the interpretation of these results.

The sarcotubular adenylate cyclase is dependent upon Mg^{2+} for its activity whereas Ca^{2+} appears to have a modulatory effect. The potent inhibitory action of Ca^{2+} is of special interest since intracellular concentration of Ca^{2+} in the free form is considered to change during cardiac contraction and relaxation[27]. It is therefore likely that Ca^{2+} may play an important role in the regulation of adenylate cyclase activity and formation of cyclic AMP. It is also conceivable that Ca^{2+} may determine the extent of cyclic AMP formation in the heart during sympathetic stimulation where the responses are elicited through the release of norepinephrine from the adrenergic nerve endings. The presence of adenylate cyclase in the sarcotubular membranes and its activation by norepinephrine are of special interest since phosphorylase as well as glycogen in the heart are considered to be mainly localized in the vicinity of the sarcotubular system. The possibility of involvement of the sarcotubular adenylate cyclase in glycogenolysis therefore seems very attractive.

The involvement of a lipoprotein complex in the activity of adenylate cyclase is apparent from the observations reported here concerning the effect of phospholipase C and trypsin pretreatments. The reduction in the basal as well as epinephrine or NaF stimulated activities indicates the importance of the integrity of the membrane in the full expression of adenylate cyclase responses. The treatment of sarcotubular membranes with phospholipase A enhanced the responses to both NaF and norepinephrine. Increased NaF stimulated adenylate cyclase activity of liver plasma membranes was also observed by phospholipase A treatment[24]. It was interesting to note that epinephrine-stimulated activity was decreased only slightly although F^- activation was markedly reduced after treatment with trypsin. Likewise, F^- activation was decreased by 50%, whereas the norepinephrine response was not affected by phospholipase C treatment. These results can be interpreted to suggest differences in the site or mechanism of adenylate cyclase activation by catecholamines and F^-. This is further supported by the fact that propranolol antagonizes the action of norepinephrine but not of F^-. These observations lend support to the suggestion that catecholamines interact with the adrenergic receptor of the membrane and thereby activate adenylate cyclase, possibly through conformational changes, whereas F^- has its binding site on the enzyme molecule itself and thus activates directly[1]. This suggestion is supported by the observation made by Levey[28], that solubilized cardiac adenylate cyclase was activated by NaF but not by catecholamines. Although extensive research concerning

the exact site of the catecholamine action and the mechanism of adenylate cyclase activation is needed, our data on the reversible nature of catecholamine activation of cardiac adenylate cyclase may have some physiological bearing.

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REFERENCES

1 Robison, G. A., Butcher, R. W. and Sutherland, E. W. (1968) *Ann. Rev. Biochem.* 37, 149–174
2 Himms-Hagen, J. (1967) *Pharmacol. Rev.* 19, 367–461
3 Mayer, S. E. (1970) *Fed. Proc.* 29, 1367–1372
4 Robison, G. A., Butcher, R. W., Øye, I., Morgan, H. E. and Sutherland, E. W. (1965) *Mol. Pharmacol.* 1, 168–177
5 Drummond, G. I., Duncan, L. and Hertzman, E. (1966) *J. Biol. Chem.* 241, 5899–5903
6 Drummond, G. I. and Duncan, L. (1970) *J. Biol. Chem.* 245, 976–983
7 Sobel, B. E., Dempsey, P. J. and Cooper, T. (1968) *Biochem. Biophys. Res. Commun.* 33, 758–762
8 Levey, G. S. and Epstein, S. E. (1969) *J. Clin. Invest.* 48, 1663–1669
9 Levey, G. S., Skeleton, C. L. and Epstein, S. E. (1969) *Endocrinology* 85, 1004–1009
10 Sobel, B. E., Dempsey, P. L. and Cooper, T. (1969) *Proc. Soc. Exp. Biol. Med.* 132, 6–9
11 Levey, G. S., Skeleton, C. L. and Epstein, S. E. (1969) *J. Clin. Invest.* 48, 2244–2250
12 Drummond, G. I., Severson, D. L. and Duncan, L. (1971) *J. Biol. Chem.* 246, 4166–4173
13 Entman, M. L., Levey, G. S. and Epstein, S. E. (1969) *Biochem. Biophys. Res. Commun.* 35 728–733
14 Dhalla, N. S., Sulakhe, P. V., Khandelwal, R. L. and Hamilton, I. R. (1970) *Life Sci.* 9, 625–632
15 Wollenberger, A., Krause, E. G., Schulze, W. and Halle, W. (1971) *Abstr. Int. Conf. Physiol. Pharmacol. cyclic AMP, Milan*, p. 59
16 Sulakhe, P. V. and Dhalla, N. S. (1970) *Mol. Pharmacol.* 6, 659–666
17 Sulakhe, P. V. and Dhalla, N. S. (1971) *J. Clin. Invest.* 50, 1019–1027
18 Krishna, G., Weiss, B. and Brodie, B. B. (1968) *J. Pharmacol. Expt. Ther.* 163, 379–385
19 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
20 Nordlie, R. C. and Arion, W. J. (1966) in *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O., eds), Vol. 9, pp. 619–625, Academic Press, New York
21 Smith, L. and Camerino, P. W. (1963) *Biochemistry* 2, 1428–1432
22 Heppel, L. A. and Hilmoe, R. J. (1955) in *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O., eds,) Vol. 2, pp. 546–550, Academic Press, New York
23 Briggs, F. N., Solaro, R. J. and Gertz, E. W. (1971) in *Cardiac Hypertrophy* (Alpert, ,N. R. ed.), pp. 167–175, Academic Press, New York
24 Pohl, S. L., Krans, H. M. J., Kozyreff, V., Birnbaumer, L. and Rodbell, M. (1971) *J. Biol. Chem.* 246, 4447–4454
25 Sulakhe, P. V. and Dhalla, N. S. (1971) *Life Sci.* 10, 185–191
26 LaRaia, P. J. (1972) *Abstr. 5th Ann. Meet. Int. Study Group Res. Cardiac Metab., Winnipeg*, p. 62
27 Sonnenblick, E. H. and Stam, A. C. (1969) *Ann. Rev. Physiol.* 31, 647–674
28 Levey, G. S. (1970) *Biochem. Biophys. Res. Commun.* 38, 86–92