Differential Effects of Non-Ionic Detergents on Microsomal and Sarcolemmal Adenylate Cyclase in Cardiac Muscle

By PRAKASH V. SULAKHE and NJANOOR NARAYANAN

Department of Physiology, College of Medicine, University of Saskatchewan,
Saskatoon, Canada S7N 0W0

(Received 21 February 1978)

1. About 4 and 23% of the homogenate adenylate cyclase activity was recovered in the microsomal and sarcolemmal fractions isolated from guinea-pig heart ventricles. 2. Cardiac microsomal adenylate cyclase activity [basal as well as p[NH]ppG (guanyl-5'-yl imidodiphosphate)- and NaF-stimulated] was increased over 2-fold in the presence of Lubrol-PX (0.01–0.1%). 3. The sarcolemmal enzyme, however, showed concentration-dependent inhibition caused by the detergent under all assay conditions, except when p[NH]ppG was included in the assay. In the latter case, the detergent (0.01–0.02%) caused a modest increase (30–45%) in enzyme activity. 4. Another non-ionic detergent, Triton X-100, also stimulated the microsomal cyclase and inhibited the sarcolemmal enzyme. 5. With either membrane fraction, Lubrol-PX solubilized the enzyme when the detergent/membrane protein ratio was 2.5 (μmol of detergent/mg of protein). 6. The findings with homogenate and a washed particulate fraction resembled those obtained with sarcolemma, and those with isolated sarcoplasmic reticulum resembled those with microsomal preparations. 7. p[NH]ppG, and to some extent NaF, protected the detergent-induced inactivation of the enzyme observed at higher detergent concentrations (0.5% Lubrol-PX and 0.05–0.5% Triton X-100). 8. In the absence of detergents, p[NH]ppG increased the basal enzyme activity about 2-fold in microsomal fractions, but did not appreciably stimulate the sarcolemmal enzyme. Isoproterenol, on the other hand, increased the sarcolemmal enzyme activity (>2-fold) in the presence of p[NH]ppG and caused only moderate stimulation (31%) of the microsomal enzyme under these conditions. 9. These findings support the view that, although the bulk of adenylate cyclase resides in heart sarcolemma (plasma membrane), the microsomal activity cannot be accounted for solely by contamination of the microsomal fraction with sarcolemma, as has been suggested by others [Besch, Jones & Watanabe (1976) *Circ. Res.* **39**, 586–595; Engelhard, Plut & Storm (1976) *Biochim. Biophys. Acta* **451**, 48–61]. Further, the results of this study show that cardiac sarcoplasmic-reticulum membranes possess this enzyme.

Localization of adenylate cyclase (EC 4.6.1.1) in heart has been a controversial subject. Although earlier studies (Entman *et al.*, 1969; Dhalla *et al.*, 1970; Sulakhe & Dhalla, 1973; Katz *et al.*, 1974) reported the presence of this enzyme activity in microsomal fraction of cardiac muscle, the recovery of the enzyme was low (at best 3–5% of the homogenate activity) (Sulakhe & Dhalla, 1973; Drummond & Dunham, 1978) and therefore it has been suggested that contamination of microsomal fraction by sarcolemma would account for this enzyme activity in microsomal fractions (Besch *et al.*, 1976; Engelhard *et al.*, 1976). This view is strengthened by the observations that the sarcoplasmic-reticulum-enriched

microsomal fraction also contains sarcolemmal membranes (Besch *et al.*, 1976; Jones *et al.*, 1977) and that purified sarcolemma possesses highly active adenylate cyclase activity recovered in good yield (20–40%) (Hui *et al.*, 1976; Sulakhe *et al.*, 1976a; St. Louis & Sulakhe, 1976a). We found that phospholipase A treatment of microsomal fractions enhanced adenylate cyclase activity (Sulakhe & Dhalla, 1973), but recently we noted that such treatment caused inhibition of the sarcolemmal enzyme (Narayanan & Sulakhe, 1977). This observation suggested that microsomal and sarcolemmal enzyme respond differently to membrane-perturbing agents, despite the fact that the catalytic subunit of this enzyme present in these membranes displayed similarities in its kinetic behaviour (for example, affinities towards MgATP^{2-} and Mg^{2+}) (Sulakhe & Dhalla, 1973; St.

Abbreviation used: p[NH]ppG, guanyl-5'-yl imidodiphosphate.

Louis & Sulakhe, 1976b; Drummond & Dunham, 1978). In the present study, we show that non-ionic detergents such as Lubrol-PX and Triton X-100 produce contrasting effects on the enzyme activity, depending on the membrane fraction used. At the same time, with either membrane, Lubrol-PX treatment resulted in solubilization (80–90%) of this membrane-bound enzyme. These and other observations reported in the present paper support the view that adenylate cyclase is a component of sarcoplasmic reticulum membrane, whereas its greater content in isolated sarcolemma suggests that its main locale in heart is the surface membrane.

Experimental

Materials

Triton X-100 was from Sigma Chemical Co., St. Louis, MO, U.S.A., and Lubrol-PX from ICI Products, Montreal, P.Q., Canada. An average mol.wt. of 600 was used for Lubrol-PX; concentrations of this detergent are expressed as % (v/v) or μmol of detergent/mg of membrane protein. A mol.wt. of 646 was used for Triton X-100; its concentrations are presented as % (v/v). All other materials and their sources have been described (Sulakhe *et al.*, 1976b; Narayanan & Sulakhe, 1977).

Methods

Cardiac muscle (ventricles) from female guinea pigs (280–350 g) was used and all of the fractionation procedures described below were carried out at 4°C.

Isolation of washed particles. Washed particulate fraction was isolated as previously described (Sulakhe *et al.*, 1976a; St. Louis & Sulakhe, 1976a). Briefly, 10% (v/w) homogenate (after exposure to 1.25 M-KCl for 10 min) was centrifuged for 10 min at 9000g_{av.}, the pellet was washed twice by suspension in 10 vol. of homogenizing buffer (10 mM-Tris/HCl, pH 7.5, containing 2 mM-dithiothreitol; TD buffer) and centrifuging as above and was finally resuspended in TD buffer containing 10% (w/v) sucrose. This pellet constituted the washed particulate fraction used in the present study.

Isolation of sarcolemma. Sarcolemmal membranes (fraction F_{3c}) from the washed particulate fraction (see above) were isolated as previously described (Sulakhe *et al.*, 1976a; St. Louis & Sulakhe, 1976a). Briefly, the washed particulate fraction was subjected to two successive discontinuous sucrose-density-gradient centrifugations. The particle band (F_{3c}) obtained at the 52.5/55% (w/v) sucrose interface (after the second gradient centrifugation) constituted the sarcolemma-enriched membrane fraction that was used in the present study. The particle band fraction F_{3b} [50/52.5% (w/v) sucrose interface] also contained considerable amounts of sarcolemmal

membranes. However, since the responses of adenylate cyclase activity of fraction F_{3b} to Lubrol-PX and Triton X-100 were identical with those observed with fraction F_{3c} (see the Results section), the results obtained with the fraction F_{3c} only are presented. Also our previous work clearly showed that the fraction F_{3c}, amongst the gradient fractions, represented cardiac sarcolemma isolated in a high degree of purity (see St. Louis & Sulakhe, 1976a). Therefore the values for the recovery of adenylate cyclase in sarcolemma (see Table 1) are those of the fraction F_{3c}. In other experiments, the combined recovery of the cyclase activity in the sarcolemmal subfractions F_{3b} and F_{3c} was $50 \pm 3\%$ (mean $\pm$ S.E.M. for six experiments) with the homogenate activity set to represent 100% (also see St. Louis & Sulakhe, 1976a).

Isolation of microsomal fractions (sucrose-microsomal fractions). For the preparation of microsomal fractions, ventricular tissue was homogenized in 10 vol. (v/w) of buffer (10 mM-Tris/HCl/0.2 mM-dithiothreitol/0.25 M-sucrose, pH 7.0) as previously described (Sulakhe *et al.*, 1976b). The homogenate was centrifuged at 10000g_{av.} for 20 min and the residue discarded. The particulate material obtained by centrifuging the supernatant fluid at 40000g_{av.} for 1 h was used as the microsomal fraction.

Isolation of sarcoplasmic reticulum (water-microsomal fraction). This was essentially done by the method of Harigaya & Schwartz (1969), except that the initial homogenization of the tissue in 10 mM-NaHCO₃ buffer was for 45 s (3 $\times$ 15 s with a 30 s waiting period in between bursts), instead of the 15 s used by Harigaya & Schwartz (1969). This step (longer homogenization) resulted in the increase (about 3-fold) of the recovery of protein in the sarcoplasmic-reticulum fraction without any detrimental effect on its activities (e.g. Ca²⁺ uptake, adenylate cyclase); a similar observation has also been reported by Jones *et al.* (1977). Harigaya & Schwartz (1969) have referred to this fraction as cardiac-relaxing system. Katz *et al.* (1974) showed that the Harigaya-Schwartz (1969) procedure resulted in isolation of the microsomal fraction [which Katz *et al.* (1974) referred to as 'water-microsomes'] that was more enriched in the membrane fragments derived from the sarcoplasmic reticulum compared with other conventional techniques, an observation based on the estimation of the specific activities of Ca²⁺ uptake (and other activities) of isolated microsomal fractions. We have confirmed this observation. However, since the responses of the cyclase activity of the sarcoplasmic reticulum (cardiac-relaxing system or water-microsomal fraction) and the microsomal fraction (sucrose-microsomal fraction) to Lubrol-PX and Triton X-100 were similar (see Tables 2 and 3), the data obtained with the microsomal fraction are

described in more detail in the present paper (also see under 'General discussion'). For the sake of simplicity, the sucrose-microsomal fraction and the water-microsomal fraction are referred to as microsomal and sarcoplasmic-reticulum fractions respectively.

Assay of adenylate cyclase. Adenylate cyclase activity was determined at 30°C by the procedure described (Sulakhe *et al.*, 1976b). The reaction mixture (0.15 ml) consisted of 50 mM-Tris/HCl (pH 7.5), 8 mM-theophylline, 1 mM-cyclic AMP, 50 µg of creatine kinase, 20 mM-phosphocreatine, 10 mM-MgCl₂, 0.4 mM-[α-³²P]ATP (specific radioactivity 15–20 c.p.m./pmol) and 45 µg of membrane protein. The reaction was initiated by the addition of [α-³²P]-ATP after preincubation of the membranes for 5 min with the rest of the assay components. The reaction was terminated 10 min after the addition of substrate. Detergents, p[NH]ppG, isoproterenol and NaF, when indicated, were present during preincubation. The measured cyclase activity was linear with respect to time under all assay conditions.

Solubilization of cardiac adenylate cyclase by Lubrol-PX. Particulate fraction (1.35 mg of protein) was treated with Lubrol-PX (mol.wt. 600) at the ratio of 2.5 µmol of detergent/mg of protein in the reaction mixture (final volume 0.5 ml) containing 10 mM-Tris/HCl, pH 7.5, 4 mM-MgCl₂, 2 mM-dithiothreitol and 10% sucrose (Mix C). In some instances, 10 µM-p[NH]ppG with (Mix A) and without (Mix B) 50 µM-isoproterenol were also added to the reaction mixture. The treatment was carried out for 10 min at 30°C. After this, the tubes were centrifuged for 60 min at 40000g_{av.}, the supernatant fluid was removed and the pellet was resuspended in the buffer (10 mM-Tris/HCl, pH 7.5, 2 mM-dithiothreitol and 10% sucrose) containing Lubrol-PX at a concentration identical with that used for solubilization. Essentially similar results were obtained by centrifuging the tubes at 100000g_{av.} for 60 min, after treatment with Lubrol-PX. The pellet and the supernatant fluid were assayed (in duplicate) for adenylate cyclase activity. The original fractions were also assayed for enzyme activity without and with Lubrol (2.5 µmol of detergent/mg of protein).

Results and Discussion

Recovery of cardiac adenylate cyclase in various membrane fractions

The recoveries of protein and adenylate cyclase activity are presented in Table 1. The washed particulate fraction contained nearly all of the enzyme activity of the starting homogenate; in fact, the recovery of the cyclase was greater than 100%. This is in accord with the previously reported findings (e.g. see Drummond & Duncan, 1970; St. Louis &

Table 1. Recoveries of protein and adenylate cyclase activity in various fractions isolated from guinea-pig heart ventricles

Fraction	Protein		Adenylate cyclase			
	Amount (mg of protein/ g of tissue)	Recovery (%)	Specific activity (pmol/min per mg of protein)		Total activity (nmol/min per g of tissue)	
			Basal	+NaF	Basal	+NaF
Homogenate (12)	126.0 ± 3.00	100	56 ± 4	96 ± 2	7.060 ± 0.29	12.100 ± 0.15
Washed particles (6)	28.3 ± 1.00	22.5 ± 0.50	270 ± 13	468 ± 7	7.640 ± 0.21	13.240 ± 0.11
Sarcolemma (12)	3.5 ± 0.10	2.8 ± 0.06	466 ± 28	940 ± 56	1.630 ± 0.05	3.300 ± 0.12
Microsomal fraction (12)	2.5 ± 0.16	2.0 ± 0.05	126 ± 6	323 ± 26	0.315 ± 0.01	0.808 ± 0.03
Sarcoplasmic reticulum (6)	3.5 ± 0.30	2.8 ± 0.14	76 ± 4	170 ± 11	0.270 ± 0.01	0.595 ± 0.02
					Basal	+NaF
					100	100
					108.21 ± 1.7	109.42 ± 0.5
					23.09 ± 0.4	27.27 ± 0.6
					4.46 ± 0.1	6.68 ± 0.1
					3.82 ± 0.1	4.92 ± 0.1

Sarcolemmal membrane (gradient fraction F_{3c}) and washed (extracted) particles were isolated by the method of Sulakhe *et al.* (1976a), sarcoplasmic reticulum (water-microsomal fraction) by the method of Harigaya & Schwartz (1969) and microsomal fractions (sucrose-microsomal fractions) as described in the Experimental section. Basal and NaF (5 mM)-stimulated adenylate cyclase activities of these fractions and homogenates were determined as described in the Experimental section. Results are expressed as means ± s.e.m. for the numbers of experiments given in parentheses.

Lubrol-PX; marked stimulation in this case occurred at a narrow range (0.02–0.05%) of detergent concentration. With either membrane fraction, p[NH]ppG, with and without isoproterenol, and NaF alone appeared to protect the enzyme against inactivation by higher concentration of the detergent. The effects of Lubrol-PX on adenylate cyclase activities of sarcolemma and washed particles were clearly different from those observed with microsomal or sarcoplasmic-reticulum fractions. The detergent produced concentration-dependent inhibition of cyclase activities in washed particles and sarcolemma when assayed under basal conditions, with NaF, or with p[NH]ppG and isoproterenol. The maximum inhibition (in the presence of 0.5% Lubrol-PX) amounted to 86–93% under basal assay conditions, 78–86% when assayed with p[NH]ppG plus isoproterenol and 70–80% when assayed with NaF. Interestingly, however, when assayed with p[NH]ppG alone (isoproterenol absent) lower concentrations (0.01–0.05%) of the detergent did not inhibit the cyclase activities in sarcolemma and washed particles. Instead, a modest stimulation (14–45%) was observed. Also, p[NH]ppG offered the best protection against Lubrol-induced inactivation of the enzyme. With either membrane fraction, it appeared that isoproterenol antagonized the protective effect of the guanyl nucleotide against detergent-induced inhibition.

Stimulation of adenylate cyclase activities in microsomal and sarcoplasmic-reticulum fractions and inhibition of the cyclase activities in sarcolemma and washed particles were also observed with another non-ionic detergent, Triton X-100 (Table 3). The stimulatory effects of Triton X-100 on microsomal and sarcoplasmic-reticulum fractions were similar to those observed with Lubrol-PX; however, the degree of stimulation, especially of the basal enzyme, was relatively low. The inhibitory effects of Triton X-100 on the cyclase activities of sarcolemma and washed particles were similar to those noted in the presence of Lubrol-PX. Unlike Lubrol-PX, Triton X-100 did not produce any significant stimulation of the cyclase activity in washed particles and sarcolemma when assayed with p[NH]ppG alone (cf. Tables 2 and 3). Nevertheless, the protective effect of the guanyl nucleotide against detergent-induced inhibition was clearly discernible with Triton X-100 as well. Also isoproterenol appeared to counteract the protective effect of the nucleotide.

Comparative effects of Lubrol-PX on adenylate cyclase activities in homogenate and various membrane fractions derived from the homogenate, when assayed under various conditions, are presented in Table 4. In these experiments the concentration of Lubrol (2.5 μ mol of detergent/mg of protein) that optimally stimulated the microsomal enzyme (see

Table 3. Effect of various concentrations of Triton X-100 on adenylate cyclase activities of various membrane fractions

Membranes (48 μ g of protein) were assayed for adenylate cyclase activities in the absence and presence of various concentrations of Triton X-100 (% v/v) under different assay conditions (B, G, G+I, F; see Table 2). Abbreviations: B, basal; G, 10 μ M-p[NH]ppG; G+I, 10 μ M-p[NH]ppG plus 50 μ M-isoproterenol; F, 5 mM-NaF. The cyclase activities determined in the absence of detergent were set to represent 100% and were similar to those described in Table 2. The cyclase activities are expressed as ratios ($\times 100$) of activities determined with and without Triton $\times 100$ under each assay condition. Results represent means $\pm$ S.E.M. for six experiments.

Concentration of Triton X-100 (%)	Assay conditions	Adenylate cyclase activity																
		Sarcolemma				Washed particles				Microsomal fraction				Sarcoplasmic reticulum				
		...	B	G	G+I	F	B	G	G+I	F	B	G	G+I	F	B	G	G+I	F
0.005		91 ± 2	106 ± 9	108 ± 2	102 ± 2	89 ± 2	89 ± 1	96 ± 1	96 ± 1	101 ± 4	110 ± 5	102 ± 2	100 ± 2	105 ± 1	91 ± 2	96 ± 2	102 ± 1	
0.01		78 ± 2	115 ± 5	94 ± 3	92 ± 2	85 ± 5	87 ± 2	99 ± 2	97 ± 3	116 ± 3	138 ± 4	112 ± 1	112 ± 2	111 ± 2	123 ± 3	104 ± 2	112 ± 1	
0.02		48 ± 3	107 ± 5	60 ± 1	79 ± 4	80 ± 1	93 ± 6	94 ± 1	92 ± 2	130 ± 8	142 ± 1	125 ± 5	131 ± 5	120 ± 2	116 ± 3	112 ± 1	121 ± 3	
0.05		23 ± 2	92 ± 2	38 ± 2	45 ± 2	62 ± 3	90 ± 1	82 ± 2	81 ± 1	77 ± 3	153 ± 2	138 ± 3	156 ± 4	112 ± 3	164 ± 4	150 ± 1	158 ± 2	
0.10		15 ± 2	94 ± 3	41 ± 1	43 ± 3	37 ± 1	73 ± 1	41 ± 1	47 ± 1	64 ± 4	229 ± 7	142 ± 1	152 ± 4	76 ± 4	182 ± 3	161 ± 4	137 ± 2	
0.50		4 ± 1	53 ± 3	22 ± 1	24 ± 2	4 ± 1	49 ± 3	22 ± 1	20 ± 1	20 ± 7	229 ± 6	155 ± 3	132 ± 5	16 ± 2	171 ± 3	129 ± 2	98 ± 2	

Table 2) was chosen. The homogenate cyclase activity was inhibited 50–70% by Lubrol under all conditions, except when assayed in the presence of p[NH]ppG alone. In the presence of p[NH]ppG alone (isoproterenol absent), Lubrol caused only minimal inhibition (30%) of the homogenate cyclase activity, apparently owing to the protective effect of the guanyl nucleotide against detergent-mediated inactivation. In conformity with the results described (see Table 2) Lubrol markedly stimulated the cyclase activities of microsomal and sarcoplasmic-reticulum fractions, but inhibited the cyclase activities of washed particles and sarcolemma. It can be suggested that the sarcolemmal enzyme contributes to most of the activity in the homogenate, since the response of the cyclase to detergents (inhibition) was similar in homogenate, washed particles and sarcolemma (Tables 2, 3 and 4). In addition, the relatively small amount (4–7%) of total homogenate cyclase activity recovered in microsomal or sarcoplasmic-reticulum fraction (see Table 1) does not appear to be due to contamination of these fractions by sarcolemmal membranes, since the detergents produced conspicuously diverse effects: inhibition of the enzyme in sarcolemma and stimulation of the enzyme in microsomal fractions and sarcoplasmic reticulum.

Relationship between the sidedness of isolated membranes and the observed effects on the enzyme activity

Since the membrane fractions studied are vesicular (Harigaya & Schwartz, 1969; St. Louis & Sulakhe, 1976a; Besch *et al.*, 1976), the cyclase activity measured without detergent could be due to limited accessibility of the ligands (such as Mg^{2+} and $MgATP^{2-}$) and other effectors (NaF, p[NH]ppG, isoproterenol) to their respective sites, either because of the permeability properties of the membranes or because of the sidedness of the membrane fractions. It is believed that catalytic site for $MgATP^{2+}$, metal (Mg^{2+})-binding site, and site(s) for guanyl nucleotides such as GTP and p[NH]ppG and for NaF in the

adenylate cyclase complex are exposed to the cytoplasmic side, whereas the hormone-receptor site is exposed to the extracellular side of the plasma membrane. This would imply that inside-out vesicles in which the ligand and effector sites are exposed to the assay medium would contribute to the activity (measured in the absence of detergent), whereas right-side-out vesicles do not, since the catalytic and effector sites are not readily accessible to the ligands. The situation after exposure to detergent would be different. Since the detergent would destroy the permeability barriers (and therefore the sidedness), the ligands can now freely interact with these sites and the measured activity would represent contributions of both right-side-out and inside-out vesicles.

The sidedness of the microsomal and sarcolemmal membranes was evaluated with the ouabain-sensitive ($Na^+ + K^+$)-dependent adenosine triphosphatase system, since the vectorial properties of this enzyme are extensively studied and further, their analysis has been used to assess the sidedness of a variety of isolated membranes [see Schwartz *et al.* (1975) for a detailed review]. With isolated sarcolemma, $48 \pm 6\%$ (mean $\pm$ S.E.M. of three experiments) of membrane vesicles were inside-out (St. Louis & Sulakhe, 1978), whereas the content of inside-out vesicles in microsomal fractions was $66 \pm 3\%$ (N. Narayanan & P. V. Sulakhe, unpublished work). The latter estimation is in excellent agreement with the reported value of 60–70% (Besch *et al.*, 1976). Such analysis would predict that, if detergents were to increase enzyme activity solely by increasing accessibility of the ligands, the increase should have amounted to 50% in microsomal fractions. However, the present data (Tables 2, 3 and 4) indicated much greater stimulation (above 2-fold), which suggests that the detergent effect cannot be accounted for entirely by destruction of the sidedness of vesicles resulting in greater accessibility towards the ligands. The fold stimulations of basal enzyme activity by NaF (5mM) in all fractions studied were similar in the absence ($2.01 \pm$

Table 4. *Effect of Lubrol-PX on adenylate cyclase activities of various fractions*

Adenylate cyclase activity was measured in the absence and presence of Lubrol-PX ($2.5 \mu\text{mol}$ of detergent per mg of protein). Abbreviations (B, G, G+I, F) represent assay conditions as described in Table 3. The numbers in parentheses indicate the numbers of experiments carried out with each fraction. Results are expressed as means $\pm$ S.E.M.

Fraction assayed	Assay conditions ...	Adenylate cyclase activity ($\frac{\text{activity with Lubrol}}{\text{activity without Lubrol}} \times 100$)			
		B	G	G+I	F
Homogenate (8)		33 ± 3	70 ± 3	40 ± 2	53 ± 3
Washed particles (5)		60 ± 4	123 ± 9	40 ± 3	52 ± 2
Sarcolemma (8)		75 ± 5	125 ± 3	49 ± 2	64 ± 4
Microsomal fractions (10)		179 ± 7	222 ± 7	196 ± 8	182 ± 6
Sarcoplasmic reticulum (8)		176 ± 3	196 ± 5	183 ± 6	175 ± 7

Table 5. *Solubilization of cardiac adenylate cyclase by Lubrol-PX*

Washed particles, microsomal fractions and sarcolemmal membranes were isolated as described under 'Methods'. Protein (1.35 mg) of each fraction was exposed to Lubrol-PX (2.5 μ mol of detergent/mg of protein) for 10 min at 30°C in the reaction mixture (0.5 ml) containing 10 mM-Tris/HCl, pH 7.5, 4 mM-MgCl₂, 2 mM-dithiothreitol and 10% (w/v) sucrose in the absence (Mix C) and in the presence (Mix A) of 10 μ M-p[NH]ppG plus 50 μ M-isoproterenol. Soluble (supernatant) and insoluble (pellet) fractions were obtained by centrifugation (see under 'Methods') and assayed for the cyclase activity under basal assay conditions. Results are means $\pm$ S.E.M. of three experiments assayed in duplicate. Specific activities of the starting material assayed under identical conditions were used to estimate the percentage recovery of the enzyme. The numbers in parentheses indicate the percentage distribution of the recovered enzyme activity and protein (pellet+supernatant = 100%). In two experiments, 10 μ M-p[NH]ppG (without isoproterenol) was present during the treatment of the fractions with Lubrol-PX. Results of these experiments were similar to those shown for Mix A and hence are not shown in this Table. Under each condition of detergent treatment (Mix A, Mix B or Mix C) the amount of protein solubilized remained the same; this was true for all the membrane fractions used.

	Adenylate cyclase activity							
	Protein		Mix A			Mix C		
	(mg/fraction)	(%)	(Specific activity)	(Total activity/fraction)	(%)	(Specific activity)	(Total activity/fraction)	(%)
Sarcolemma	1.35	100	205 $\pm$ 16	277 $\pm$ 12	100	135 $\pm$ 10	182 $\pm$ 8	100
Pellet (P)	0.95 $\pm$ 0.09	70 $\pm$ 4 (76)	30 $\pm$ 9	28 $\pm$ 4	10 $\pm$ 1 (7)	15 $\pm$ 3	14 $\pm$ 2	8 $\pm$ 1 (18)
Supernatant (S)	0.30 $\pm$ 0.02	22 $\pm$ 1 (24)	1237 $\pm$ 163	368 $\pm$ 25	133 $\pm$ 5 (93)	215 $\pm$ 17	64 $\pm$ 3	35 $\pm$ 1 (82)
Washed particles	1.35	100	115 $\pm$ 9	155 $\pm$ 6	100	105 $\pm$ 8	142 $\pm$ 6	100
Pellet (P)	0.82 $\pm$ 0.01	60 $\pm$ 1 (67)	72 $\pm$ 2	59 $\pm$ 1	38 $\pm$ 1 (12)	14 $\pm$ 1	11 $\pm$ 1	8 $\pm$ 1 (15)
Supernatant (S)	0.41 $\pm$ 0.01	30 $\pm$ 1 (33)	1085 $\pm$ 2	439 $\pm$ 1	283 $\pm$ 1 (88)	158 $\pm$ 1	64 $\pm$ 1	45 $\pm$ 1 (85)
Microsomal fraction	1.35	100	192 $\pm$ 15	259 $\pm$ 11	100	68 $\pm$ 5	92 $\pm$ 4	100
Pellet (P)	0.65 $\pm$ 0.05	48 $\pm$ 2 (54)	207 $\pm$ 22	134 $\pm$ 8	52 $\pm$ 2 (24)	15 $\pm$ 2	10 $\pm$ 1	11 $\pm$ 1 (16)
Supernatant (S)	0.55 $\pm$ 0.02	41 $\pm$ 1 (46)	777 $\pm$ 58	428 $\pm$ 15	165 $\pm$ 3 (76)	93 $\pm$ 8	51 $\pm$ 1	55 $\pm$ 1 (84)

Table 6. *Stimulation of Lubrol-solubilized adenylate cyclase activity by p[NH]ppG and NaF*

Membranes (microsomal and sarcolemmal) were treated with Lubrol-PX (2.5 μ mol of detergent/mg of protein) in Mix C (see Table 5) for 10 min at 30°C. After treatment the tubes were centrifuged, soluble (supernatant) and insoluble (pellet) fractions removed and assayed for adenylate cyclase activity. Abbreviations (B, G, G+I, F) represent assay conditions as described in Table 3. Expt. 1, microsomal fraction; Expt. 2, sarcolemma. The results are means $\pm$ S.E.M. for three determinations.

Fraction	Adenylate cyclase activity (pmol of cyclic AMP/min per mg of protein)			
	B	G	G+I	F
Expt. 1				
Supernatant	63 $\pm$ 3	232 $\pm$ 3	214 $\pm$ 4	316 $\pm$ 5
Pellet	10 $\pm$ 2	11 $\pm$ 1	9 $\pm$ 1	35 $\pm$ 2
Expt. 2				
Supernatant	160 $\pm$ 3	322 $\pm$ 8	312 $\pm$ 4	291 $\pm$ 6
Pellet	12 $\pm$ 1	9 $\pm$ 1	7 $\pm$ 1	10 $\pm$ 2

0.16) and presence (2.16 $\pm$ 0.17) of Lubrol-PX (2.5 μ mol of detergent/mg of protein). Since NaF is considered to act directly on the catalytic subunit of the cyclase complex (see Perkins, 1973), these results

also are not compatible with the view that the sidedness of the membrane fractions accounts for the observed responses to both detergents. Instead, activation of the enzyme by lower concentration of detergent seems a likely explanation.

With sarcolemmal membranes, increase in the cyclase activity was not observed, but rather only inhibitory effect was detected under all assay conditions, except with p[NH]ppG (see Tables 2, 3 and 4). In the latter case, Lubrol, but not Triton, produced a moderate increase (up to 45%), which is much lower than the expected value of 200%, based on consideration of the sidedness of sarcolemma. Therefore the action of Lubrol-PX on sarcolemmal enzyme activity appears unrelated to the sidedness of the sarcolemmal vesicles.

Solubilization of adenylate cyclase

It was conceivable that detergents solubilized this membrane-bound enzyme under all assay conditions. Data on the solubilization of adenylate cyclase and membrane protein are in Table 5. In these experiments, solubilization was carried out in the presence of 2.5 μ mol of Lubrol-PX/mg of protein. Treatment of the membrane fractions with the detergent was performed under basal conditions (without p[NH]ppG and isoproterenol) and in the presence of p[NH]ppG with and without isoproterenol. Under

each condition, approx. 80–90% of the recovered enzyme activity from each membrane fraction was found in the supernatant fluid. This clearly meant that the ability of Lubrol to solubilize this enzyme (Table 5) and the action (stimulation or inhibition) of this detergent (Tables 2 and 4) on enzyme activity under these conditions are not related to each other. Although 80–90% of the recovered enzyme was present in the supernatant fluids, the recovery of the cyclase (compared with starting material) was 43–53% for sarcolemma and washed particles, and 66% for microsomal fractions when detergent treatment was carried out under basal conditions, i.e. Mix C (Table 5). This suggested that Lubrol caused considerable inactivation of the enzyme under these conditions. Interestingly, however, when the detergent treatment was carried out in the presence of p[NH]ppG and isoproterenol (Mix A) the recovery of enzyme was above 100% (143% with sarcolemma, 321% with washed particles and 217% with microsomal fractions), indicating that the enzyme was not inactivated and at the same time was also solubilized. The increase in specific activity of the supernatant enzyme was 4–9-fold after exposure to Lubrol-PX in the presence of activators (p[NH]ppG and isoproterenol, Mix A, Table 5), whereas in their absence (Mix C, Table 5) the increase was only 36–60%. Results similar to those seen with Mix A were observed when the detergent treatment was carried out in the presence of p[NH]ppG alone (Mix B) (results not shown).

Stimulation of the solubilized enzyme by p[NH]ppG and NaF

When the solubilized enzyme (Mix C) was assayed in the presence of activators, p[NH]ppG and NaF increased its activity (Table 6); isoproterenol failed

to enhance the activity obtained with p[NH]ppG in both microsomal and sarcolemmal extract. The insoluble residues of either membrane contained very low amounts of enzyme activity, which did not respond to p[NH]ppG; the sarcolemmal residue, but not the microsomal residue, showed enhanced activity with NaF. The stimulatory effects of p[NH]ppG and NaF observed with the solubilized enzyme agree with other published reports (Levey, 1970; Swislocki *et al.*, 1976).

Effects of p[NH]ppG on adenylate cyclase activity of various membrane fractions with and without isoproterenol

As shown in Table 7, in the absence of detergent p[NH]ppG failed to enhance the basal enzyme activity in sarcolemma and washed particles, whereas it caused stimulation (50–80%) of enzyme activity in microsomal and sarcoplasmic-reticulum fractions. We have reported that p[NH]ppG stimulated basal enzyme activity of heart sarcolemma (detergent absent) when assays were carried out at 37°C (St. Louis & Sulakhe, 1976*a,b*). More recently, we found that the stimulatory effect of p[NH]ppG is temperature-dependent, such that at temperatures below 30°C the nucleotide did not significantly stimulate basal cyclase activity (Narayanan & Sulakhe, 1977). In the present study, enzyme was assayed at 30°C to minimize inactivation caused by the non-ionic detergents, observed at the higher assay temperature (37°C) (Drummond & Dunham, 1978; N. Narayanan & P. V. Sulakhe, unpublished work). In the presence of Lubrol, p[NH]ppG caused stimulation of basal cyclase activity in all membrane fractions, although the stimulation was much greater in microsomal fractions (246%) and sarcoplasmic reticulum (175%) compared with sarcolemma (147%) and washed

Table 7. *Effect of p[NH]ppG on basal and effect of isoproterenol on p[NH]ppG-activated adenylate cyclase activity in the absence and presence of Lubrol-PX*

Adenylate cyclase activity was determined in the absence and in the presence of 10 μ M-p[NH]ppG (G) $\pm$ 50 μ M-isoproterenol (I) and with and without Lubrol-PX (2.5 μ mol of detergent/mg of protein). Incubations were carried out for 10 min at 30°C. The results are expressed as means $\pm$ S.E.M. The numbers in parentheses indicate the numbers of experiments carried out with each fraction.

Membrane fraction used	Lubrol in assay	Adenylate cyclase activity	
		$\left(\frac{+G}{-G} \times 100\right)$	$\left(\frac{G+I}{+G} \times 100\right)$
Sarcolemma (10) (6)	Absent	99 $\pm$ 4	235 $\pm$ 8
	Present	147 $\pm$ 8	97 $\pm$ 4
Microsomal (13) (10)	Absent	178 $\pm$ 15	131 $\pm$ 4
	Present	246 $\pm$ 19	111 $\pm$ 2
Washed particles (6) (4)	Absent	93 $\pm$ 5	246 $\pm$ 15
	Present	130 $\pm$ 5	81 $\pm$ 3
Sarcoplasmic (7) reticulum (4)	Absent	153 $\pm$ 12	133 $\pm$ 3
	Present	175 $\pm$ 2	119 $\pm$ 6

particles (130%). On the other hand, isoproterenol greatly increased the p[NH]ppG-dependent cyclase activity in sarcolemma (235%) and washed particles (246%) but only modestly in microsomal fractions (131%) and sarcoplasmic reticulum (133%) assayed in the absence of Lubrol. With Lubrol present, isoproterenol failed to stimulate p[NH]ppG-dependent cyclase activity of washed particles and sarcolemma; stimulation was barely detectable in microsomal fractions and sarcoplasmic reticulum.

General discussion

Even if one were to presume that 50% of the microsomal enzyme activity is due to the contaminating sarcolemmal enzyme (which is a generous estimation), then this should have been reflected in much greater specific activity (basal assay condition) of the microsomal fraction (such as about 200–300 pmol/min per mg). Also, in that case, the stimulatory effect of the detergents with microsomal fractions would not be observed, since the inhibitory effect on the sarcolemmal enzyme would neutralize the stimulatory effect observed at the lower concentration of detergents. Therefore the simplest explanation for the observed responses would be that its responsiveness to detergents depends on the micro-environment surrounding this enzyme. Cholesterol/phospholipid ratios of microsomal fractions and sarcolemma differ greatly (0.1–0.2 versus 0.6–0.8), as do the polypeptide profiles of these membranes by sodium dodecyl sulphate/polyacrylamide-gel electrophoretic analysis (St. Louis & Sulakhe, 1976a). Such observations tend to support the view that isolated microsomal and sarcolemma fractions are derived from separate membranes of the heart muscle and that these differ from each other in the content of cyclase and other enzymes [such as ($Mg^{2+} + Ca^{2+}$)-dependent adenosine triphosphatase] as well as in their chemical composition. The latter appears to regulate responsiveness of the cyclase to membrane-perturbing agents such as non-ionic detergents (present results) and phospholipase A (Sulakhe & Dhalla, 1973; Narayanan & Sulakhe, 1977).

Besides the differences in the responsiveness of the cyclase to the detergents, the catalytic unit of the cyclase present in these membranes exhibits other noteworthy features. Sarcolemmal enzyme is inhibited by high concentration (50 mM) of NaF, whereas microsomal enzyme is not (Sulakhe & Dhalla, 1973; St. Louis & Sulakhe, 1976b). Also NaF (5 mM) changed the affinities of the sarcolemmal enzyme for Mg^{2+} and $MgATP^{2-}$, whereas it was devoid of this action on the microsomal enzyme (Sulakhe & Dhalla, 1973; St. Louis & Sulakhe, 1976b). Such observations also support the claim that microsomal enzyme activity cannot be due to the sarcolemmal enzyme.

Besides consideration of the role of the micro-environment in the observed responses to the detergent, it seems possible that membranes contain a factor(s) that determines the detergent action. In other words, detergent may remove an inhibitory effect of a factor(s) that is present in microsomal fractions and that such a factor(s) is (are) absent from isolated sarcolemma. This could in part explain an increase in microsomal enzyme activity. The sarcolemmal response (i.e. decrease in enzyme activity) may then represent a true response of the enzyme to the detergent in the absence of the cyclase activators (such as p[NH]ppG). Further analysis is therefore needed to know which one of these postulates [chemical composition or presence of inhibitor(s)] accounts for the observed responses to non-ionic detergents. It is worth noting here that microsomal Mg^{2+} -dependent adenosine triphosphatase activity is inhibited (50%), whereas sarcolemmal enzyme is stimulated (30%) in the presence of sodium deoxycholate (P. V. Sulakhe, N. Narayanan & P. J. St. Louis, unpublished work) under identical assay conditions. This is one additional example of the differential response of another enzyme present in these two membrane fractions. Also the antagonistic effect of isoproterenol (on the protective action of p[NH]ppG against the detergent inactivation of the cyclase) was observed with sarcolemma or washed particles and not with microsomal fractions or sarcoplasmic reticulum (see Tables 2, 3 and 4).

The findings noted with microsomal fractions were also obtained with isolated sarcoplasmic reticulum (Tables 2, 3 and 4). Also we found that the microsomal fraction exhibited many activities typical of sarcoplasmic reticulum (such as highly active Ca^{2+} -transport capacity and ($Mg^{2+} + Ca^{2+}$)-dependent adenosine triphosphatase), and, further, polypeptide profiles (sodium dodecyl sulphate/polyacrylamide-gel electrophoresis) of microsomal fractions and sarcoplasmic reticulum were similar (results not shown). Therefore it is likely that sarcoplasmic reticulum fragments comprised the majority of microsomal vesicles. Earlier, we demonstrated that sarcolemmal membranes isolated by our procedure were not contaminated with sarcoplasmic reticulum (or microsomal particles) (Sulakhe *et al.*, 1976a; St. Louis & Sulakhe, 1976a,b,c). In view of these and other observations described here, it appears that adenylate cyclase is a component of sarcoplasmic reticulum (microsomal particles), although the content of this enzyme is much greater in heart sarcolemma.

Drummond & Dunham (1978) reported that Lubrol-PX and Triton X-100 stimulated adenylate cyclase activity of 100000g particles (microsomal fraction) and inhibited the enzyme present in washed particles isolated from rabbit heart ventricles. In their

work washed particulate fraction was enriched in the sarcolemmal membranes. These investigators also concluded from their study that the microsomal enzyme activity is due to the presence of fragments of sarcoplasmic reticulum in the microsomal fraction and that the sarcolemmal contamination could not account for the observed responses of the microsomal enzyme to these detergents. Our results support these findings.

The physiological significance of the dual localization of adenylate cyclase remains unclear at present. It is conceivable that cyclic AMP modulates Ca^{2+} fluxes across both sarcoplasmic reticulum and sarcolemma, since studies *in vitro* support this role of cyclic AMP in membrane Ca^{2+} transport (for a review see Sulakhe & St. Louis, 1976). Recent studies in our laboratory showed the presence of β -adrenergic receptors in sarcolemma as well as sarcoplasmic reticulum of heart (N. Narayanan, J. W. Wei & P. V. Sulakhe, unpublished work). Presence of adenylate cyclase in sarcoplasmic reticulum (as shown by the present study) therefore supports the concept of an internal β -receptor-linked adenylate cyclase system in heart, as is proposed by Entman *et al.* (1976). Finally, the presence of adenylate cyclase in the intracellular organelles (such as the nucleus) of other tissues is already reported by many investigators (Soifer & Hechter, 1971; Liao *et al.*, 1971; Braun *et al.*, 1977; Wedner & Parker, 1977). Whether or not intracellular organelles besides sarcoplasmic reticulum in heart possess this enzyme activity remains to be investigated.

N. N. is a research fellow of the Canadian Heart Foundation. This work was supported by a grant from the Saskatchewan Heart Foundation. We are grateful to Professor George I. Drummond, University of Calgary, for sending us a copy of his paper before its publication.

References

- Besch, H. R., Jones, L. R. & Watanabe, A. M. (1976) *Circ. Res.* **39**, 586–595
- Braun, T., Frank, H., Dods, R. & Seprenwol, S. (1977) *Biochim. Biophys. Acta* **481**, 227–235
- Dhalla, N. S., Sulakhe, P. V., Khandelwal, R. L. & Hamilton, I. R. (1970) *Life Sci.* **9**, 625–632
- Drummond, G. I. & Duncan, L. (1970) *J. Biol. Chem.* **245**, 976–983
- Drummond, G. I. & Dunham, J. (1978) *J. Mol. Cell. Cardiol.* in the press
- Engelhard, V. H., Plut, D. A. & Storm, D. (1976) *Biochim. Biophys. Acta* **451**, 48–61
- Entman, M. L., Levey, G. S. & Epstein, S. E. (1969) *Biochem. Biophys. Res. Commun.* **35**, 728–733
- Entman, M. L., Goldstein, M. A. & Schwartz, A. (1976) *Life Sci.* **19**, 1623–1630
- Harigaya, S. & Schwartz, A. (1969) *Circ. Res.* **25**, 781–794
- Hui, E. C., Drummond, M. & Drummond, G. I. (1976) *Arch. Biochem. Biophys.* **173**, 415–427
- Jones, L. R., Besch, H. R. & Watanabe, A. M. (1977) *J. Biol. Chem.* **252**, 3315–3323
- Katz, A. M., Tada, M., Repke, D. L., Iorio, J. & Kirshberger, M. A. (1974) *J. Mol. Cell. Cardiol.* **6**, 73–78
- Levey, G. S. (1970) *Biochem. Biophys. Res. Commun.* **38**, 86–92
- Liao, S., Lim, A. H. & Tymoczko, J. L. (1971) *Biochim. Biophys. Acta* **230**, 535–538
- Narayanan, N. & Sulakhe, P. V. (1977) *Mol. Pharmacol.* **13**, 1033–1047
- Narayanan, N. & Sulakhe, P. V. (1978) *Arch. Biochem. Biophys.* **185**, 72–81
- Perkins, J. P. (1973) *Adv. Cyclic Nucleotide Res.* **3**, 1–64
- Schwartz, A., Lindenmayer, G. E. & Allen, J. C. (1975) *Pharmacol. Rev.* **27**, 3–134
- Soifer, D. & Hechter, O. (1971) *Biochim. Biophys. Acta* **230**, 539–542
- St. Louis, P. J. & Sulakhe, P. V. (1976a) *Int. J. Biochem.* **7**, 547–558
- St. Louis, P. J. & Sulakhe, P. V. (1976b) *Biochem. J.* **158**, 535–541
- St. Louis, P. J. & Sulakhe, P. V. (1976c) *Can. J. Biochem.* **54**, 946–956
- St. Louis, P. J. & Sulakhe, P. V. (1978) *Biochemistry* in the press
- Sulakhe, P. V. & Dhalla, N. S. (1973) *Biochim. Biophys. Acta* **293**, 379–396
- Sulakhe, P. V. & St. Louis, P. J. (1976) *Gen. Pharmacol.* **7**, 313–319
- Sulakhe, P. V., Leung, N. L. & St. Louis, P. J. (1976a) *Can. J. Biochem.* **54**, 438–445
- Sulakhe, P. V., Sulakhe, S. J., Leung, N. L., St. Louis, P. J. & Hickie, R. A. (1976b) *Biochem. J.* **157**, 705–712
- Swislocki, N. I., Tierney, J. & Essner, E. D. (1976) *Arch. Biochem. Biophys.* **174**, 291–297
- Wedner, J. & Parker, C. W. (1977) *Biochem. J.* **162**, 483–491