Adenylate Cyclase, Guanylate Cyclase and Cyclic Nucleotide Phosphodiesterases of Guinea-Pig Cardiac Sarcolemma

By PATRICK J. ST. LOUIS and PRAKASH V. SULAKHE
Department of Physiology, College of Medicine, University of Saskatchewan, Saskatoon, Canada S7N 0W0

(Received 12 April 1976)

1. The activities of the enzymes involved in the metabolism of cyclic nucleotides were studied in sarcolemma prepared from guinea-pig heart ventricle; the enzyme activities reported here were linear under the assay conditions. 2. Adenylate cyclase was maximally activated by 3mm-NaF; NaF increased the \( K_m \) for ATP (from 0.042 to 0.19mm) but decreased the \( K_m \) for Mg\(^{2+}\) (from 2.33 to 0.9mm). In the presence of saturating Mg\(^{2+}\) (15mm), Mn\(^{2+}\) enhanced adenylate cyclase, whereas Ca\(^{2+}\) was inhibitory. \( \beta \)-Adrenergic amines (10–50μM) stimulated adenylate cyclase (38±2%). When added to the assay mixture, guanylyl nucleotides (GTP and its analogue, guanylyl imidophosphate) stimulated basal enzyme activity and enhanced the stimulation by isoproterenol. By contrast, pre-incubation of sarcolemma with guanylyl imidophosphate stimulated the formation of an "activated" form of the enzyme, which did not reveal increased hormonal sensitivity. 3. The guanylate cyclase present in the membranes as well as in the Triton X-100-solubilized extract of membranes exhibited a \( K_m \) for Mn\(^{2+}\) of 0.3mm; Mn\(^{2+}\) in excess of GTP was required for maximal activity. Solubilized guanylate cyclase was activated by Mg\(^{2+}\) only in the presence of low Mn\(^{2+}\) concentrations; Ca\(^{2+}\) was inhibitory both in the absence and presence of low Mn\(^{2+}\). Acetylcholine as well as carbamoylcholine stimulated membrane-bound guanylate cyclase. 4. Cyclic nucleotide phosphodiesterase activities of sarcolemma exhibited both high- and low-\( K_m \) forms with cyclic AMP and with cyclic GMP as substrate. Ca\(^{2+}\) ions increased the \( V_{max} \) of the cyclic GMP-dependent enzyme.

We described previously a method for the isolation of cardiac sarcolemma in a high degree of purity (P. V. Sulakhe et al., 1976a). The sarcolemmal preparation (fraction \( F_{sc} \)) contained significant amounts of adenylate cyclase (EC 4.6.1.1), guanylate cyclase (EC 4.6.1.2) and cyclic nucleotide phosphodiesterase (EC 3.1.4.17) activities. Studies of these enzymes in a highly purified heart sarcolemma have not been reported in the literature. The present observations show some interesting differences in the properties of sarcolemmal adenylate cyclase as well as of cyclic nucleotide phosphodiesterases when compared with those reported for these enzymes present in either washed particulate fractions or soluble fractions from cardiac tissue (Beavo et al., 1970; Drummond et al., 1971; Thompson & Appel, 1971; Sulakhe & Dhall, 1973; Tada et al., 1975; Kakiuichi et al., 1975; Sulakhe, 1975). Additionally, cardiac plasma-membrane guanylate cyclase showed stimulation by cholinergic agents.

Methods

Isolation of cardiac sarcolemma

Sarcolemmal fractions were isolated from guinea-pig heart as described previously (Sulakhe, P. V. et al., 1976a). Sarcolemmal preparations were observed to contain negligible activities of putative marker enzymes for mitochondria, lysosomes and the microsomal fraction (P. V. Sulakhe et al., 1976a); succinate dehydrogenase (EC 1.3.99.1) and cytochrome c oxidase (EC 1.9.3.1) were used as mitochondrial markers, acid phosphatase (EC 3.1.3.2) as a lysosomal marker and rotenone-insensitive NADPH-cytochrome c oxidoreductase (EC 1.6.2.4) as a microsomal marker. By contrast, adenylate cyclase, guanylate cyclase and ouabain-sensitive (Na\(^{+},K\(^{-}\))ATPase [(Na\(^{+}+K\(^{-}\))]-dependent adenosine triphosphatase, EC 3.6.1.3] were enriched five- to ten-fold, compared with homogenate, in the sarcolemmal membranes (St. Louis, 1975; Sulakhe, P. V., et al., 1976a). When examined by electron microscopy, sarcolemmal preparations appeared as empty vesicles of various sizes; occasionally mitochondrial contaminants and some partially extracted fibres were also evident. Sarcolemmal preparations were used within 1h after isolation for determination of the various enzyme activities. Under the assay conditions used, reaction velocities were proportional to time.
Enzyme assays

Adenylate and guanylate cyclase activities were determined as previously described (P. V. Sulakhe et al., 1976b). Cyclic nucleotide phosphodiesterases were determined by the procedure described by Thompson & Appleman (1971). The assay system (final vol. 0.4ml) contained 20–75μg of enzyme protein, 40mM-Tris/HCl (pH8.0), 5mM-EGTA [ethanedioxybis(ethylamine)tetra-acetic acid], 3.75mM-mercaptoethanol, 5mM-MgCl₂ and various concentrations of [³H]-labelled cyclic nucleotides (48–66c.p.m./pmol). Snake venom (Ophiophagus hannah) was used as the source of nucleotidase for the secondary reaction. Appropriate controls using heat-treated (exposure at 100°C for 30min) fractions, devoid of phosphodiesterase activity were included. Radioactivity was counted in an Isocap 300A (Nuclear–Chicago) liquid-scintillation spectrometer; the counting efficiency for ³H was about 35%. Protein was determined by the method of Lowry et al. (1951).

Results and Discussion

Adenylate cyclase

Effect of NaF. When adenylate cyclase activity was determined with various concentrations of ATP in the absence and presence of NaF, a change in the affinity for ATP, due to NaF, was observed (Kᵢ increased from 0.042 to 0.19mm). The observed change in the affinity due to NaF is a novel finding for the cardiac enzyme, since earlier work failed to reveal such an effect of NaF (Drummond et al., 1971; Sulakhe & Dhalla, 1973; McNamara et al., 1974). However, NaF increased the Vₘₐₓ of the sarcolemmal enzyme (Fig. 1) and the extent of this increase depended on the concentration of Mg²⁺ in the assay; at 2mM-Mg²⁺, NaF increased the velocity from 0.214 to 1.07nmol of cyclic AMP/min per mg, whereas at 15mM-Mg²⁺, Vₘₐₓ increased from 0.438 to 0.937nmol/min per mg. The optimal NaF concentration for the sarcolemmal enzyme was found to be 3mm (in the presence of 15mM-Mg²⁺ and 0.75mm-ATP) and, additionally, there was inhibition of the enzyme in the presence of 15mM-NaF (basal activity, 500pmol of cyclic AMP/min per mg; with NaF, 450pmol/min per mg). Previously, inhibition of the cardiac enzyme by NaF (up to almost 40mm in the assay) was not observed (Drummond & Duncan, 1970; Sulakhe & Dhalla, 1973; Tada et al., 1975). The nature of this inhibitory effect was not examined further in the present study. However, an inhibitory effect of NaF has been documented for the enzyme from brain (Johnson & Sutherland, 1973) and adipose tissue (Harwood et al., 1973).

Effect of Mg²⁺, Mn²⁺ and Co²⁺. The sarcolemmal enzyme can be saturated with Mg²⁺ and increasing Mg²⁺ increased the Vₘₐₓ of the enzyme (185pmol/min per mg at 2mm-Mg²⁺, 403pmol/min per mg at 15mm-Mg²⁺). As opposed to its effect on Kᵢ (ATP), NaF caused a decrease in the Kᵢ for Mg²⁺ (2.33mm, F⁻ absent; 0.9mm, F⁻ present) at 0.15mm and 0.74mm-ATP (Fig. 2). Birnbaumer et al. (1969) have noted an increase in the affinity for Mg²⁺ (i.e. decreased Kᵢ) due to the action of NaF on the adenylate cyclase from adipose tissue. When NaF was present in the assay, an S-shaped plot was observed. Drummond & Duncan (1970) previously reported that when the NaF
The stimulation due to β-adrenergic agonists (isoproterenol, epinephrine and norepinephrine) was further enhanced in the presence of GTP or its phosphohydrolase-resistant analogue, guanylylimidodiphosphate. In a typical experiment, the stimulation due to isoproterenol (50 μM) was increased from 25% to 65% and to 100% in the presence of 2 μM-GTP and -guanylylimidodiphosphate respectively. This was consistently observed in many experiments. Additionally, the stimulatory effect of guanylylnucleotides was dependent on the concentration of these nucleotides in the assay mixtures; the maximal stimulatory effect was observed at 50 μM-GTP (twofold) and 100 μM-guanylylimidodiphosphate (fivefold).

When sarcolemma were pre-incubated in the presence of various concentrations of guanylylimidodiphosphate (either in the absence or presence of isoproterenol) and thoroughly washed by centrifugation, a remarkable activation of the enzyme was observed (Fig. 4). Similar findings were noted with GTP (results not shown). The activation of the enzyme was greater in the presence of isoproterenol concentration was greater than that of Mg2+, myocardial adenylate cyclase was partially inhibited.

It is well documented that either Mn2+ or Co2+ can satisfy the bivalent-cation requirement of cardiac adenylate cyclase (Drummond et al., 1971; Perkins, 1973). The results shown in Fig. 3 clearly reveal that in the presence of saturating Mg2+ concentrations (15 mM), Mn2+ ions further increased the activity of the sarcolemmal enzyme; the increase in the activity in the absence of NaF was less dramatic than that observed in the presence of NaF, and was optimal at 1.0 mM- and 3 mM-Mn2+ respectively. On the other hand, under these conditions (i.e. 15 mM-Mg2+ present) Co2+ ions inhibited the enzyme both in the absence and presence of NaF. Further, the extent of adenylate cyclase stimulation by NaF in the presence of Mg2+ plus Mn2+ was greater than when only saturating amounts of Mg2+ were present.

Effect of isoproterenol and guanylylnucleotides. It has been shown that cardiac adenylate cyclase is stimulated by β-adrenergic amines and also that the nature of this stimulation suggests the coupling of the enzyme to a typical β-receptor (Kaumann & Birnbaumer, 1974; Lefkowitz, 1975). Sarcolemmal adenylate cyclase showed modest but consistent stimulation (38 ± 2%, mean ± s.e. of five paired experiments) with β-adrenergic amines (10 μM–50 μM). A similar degree of stimulation of adenylate cyclase has been reported for canine (McNamara et al., 1974) and guinea-pig (Tada et al., 1975) cardiac sarcolemmal preparations.
Fig. 4. Preincubation of sarcolemma with isoproterenol and guanylyl imidodiphosphate

Sarcolemma (2 mg of protein in 1.5 ml of 0.25 M sucrose/10 mM Tris/HCl, pH 7.5) was incubated with various concentrations of guanylyl imidodiphosphate for 7 min at 30°C in the absence (●) or presence (○) of 10 μM isoproterenol. Inset:—sarcolemma was incubated as above with various concentrations of isoproterenol in the absence (▲) or presence (△) of 1 μM guanylyl imidodiphosphate. After incubation, all samples were immediately chilled on ice, centrifuged for 15 min at 8000 g, the supernatants discarded and the pellets washed by resuspension in 3.0 ml of 0.25 M sucrose/10 mM Tris/HCl, pH 7.4, and centrifuging (15 min at 8000 g). The final washed pellets were suspended in 1.0 ml of buffer and assayed for adenylate cyclase by using standard assay conditions (see Fig. 1). Assay tubes contained 20–25 μg of enzyme protein, 15 mM Mg²⁺ and 0.8 mM [α-³²P]ATP (18.18 c.p.m./pmol).

plus guanylyl imidodiphosphate in the preincubation mixture and was time- and temperature-dependent. When sarcolemma was preincubated at 10°C, the maximal activation was observed between 15 and 20 min, whereas at 30°C, it was reached between 5 and 10 min in the presence of guanylyl imidodiphosphate and isoproterenol. In the absence of isoproterenol, longer preincubation times were required for the maximal activation, which was considerably less than that reached in the presence of guanylyl imidodiphosphate and isoproterenol. Further, isoproterenol was found to increase the rate of activation of the enzyme by guanylyl imidodiphosphate (results not shown). It is evident from these data that exposure to guanylyl imidodiphosphate somehow catalyses the transformation of the enzyme to an 'activated' state and that β-adrenergic agonists (such as isoproterenol) influences the speed and the degree of this 'activation' process. However, such 'preactivated' preparations did not exhibit increased sensitivity to isoproterenol when added in the assay. Similar activation of adenylate cyclase present in other membranes has been described (Cuatrecasas et al., 1975; Lefkowitz & Caron, 1975).
Sarcolemmal fraction (6.2 mg) was treated with Triton X-100 (1% final concn.) for 40 min at 4°C; the preparation was centrifuged for 45 min at 40000g, and the supernatant fraction used as the source of enzyme (35 μg of protein/assay). Standard incubation mixtures contained 50 mM-Tris/HCl, pH 7.5, 2 mM-dithiothreitol, 10 mM-theophylline, 1 mM-cyclic GMP, 0.1% bovine serum albumin, 50 μg of creatine kinase, 20 mM-creatine phosphate and 0.62 mM-[γ-32P]GTP (14.23 c.p.m./pmol) in a total volume of 0.15 ml. Incubations were carried out at 30°C for 10 min. (a) Effects of various concentrations of Mn2+ (○), Mg2+ (△) and Ca2+ (□); (b) effects of various concentrations of Mg2+ (△) or Ca2+ (□) in the presence of 0.2 mM-Mn2+.3

**Guanylate cyclase**

Effect of Mn2+, Mg2+ and Ca2+. Sarcolemmal enzyme, which was solubilized by Triton X-100, was remarkably stimulated by Mn2+ (Fig. 5a). We showed previously that Triton X-100 solubilizes membrane-bound guanylate cyclase (P. V. Sulakhe et al., 1976a). Triton X-100, however, is a powerful inhibitor of adenylate cyclase; about 80–90% inhibition was observed in the presence of 1% Triton X-100. The Ks for Mn2+ was about 0.3 mM, which is similar to that observed earlier for the particulate or membrane-bound enzyme (Sulakhe, S. J. et al., 1975, 1976). A Mn2+ concentration greater than that of GTP was required for maximal activity, and the plot for activity against [Mn2+] was sigmoidal. Mg2+ ions were rather poor activators of the enzyme, whereas in the presence of Ca2+ the enzyme activity was inhibited (Fig. 5a, inset). However, in the presence of low Mn2+ concentrations ([GTP] greater than [Mn2+]) the enzyme was activated by Mg2+, whereas Ca2+ ions were inhibitory (Fig. 5b). A stimulatory effect of Ca2+ on the enzyme, either solubilized or membrane-bound, was not observed under any conditions. We described previously (S. J. Sulakhe et al., 1976) that the soluble guanylate cyclase from heart, present in the cytosol, was indeed stimulated by Ca2+ in the presence of low concentrations of Mn2+. These results therefore support the view that the particulate guanylate cyclase exists as a metallo-enzyme with Mn2+ as the bound metal, and that Mg2+ increases the catalytic reactivity; further, fluxes of Ca2+ across these membranes could modulate the activity of this enzyme.

**Effect of cholinergic agents.** Fig. 6 shows that sarcolemmal guanylate cyclase was stimulated by both acetylcholine and carbamoylcholine; the maximal stimulation (30 ± 5.5%, mean ± S.E. of four paired experiments) was achieved at 1 mM-acetylcholine or 100 mM-carbamoylcholine. Atropine, a muscarinic blocker, partially decreased the stimulation due to carbamoylcholine. In other studies with [3H]atropine, muscarinic receptors were detected in these membranes, which exhibited specificity (bound [3H]atropine displaced by unlabelled atropine, carbamoylcholine and acetylcholine, but not by nicotine, tubocurarine and β-adrenergic agonists) and saturability (binding constant was 2 nm) (S. K. Ma &

Vol. 158
P. J. St. Louis and P. V. Sulakhe

Fig. 6. Effects of cholinergic agents on sarcolemmal guanylate cyclase

Guanylate cyclase was assayed as described in Fig. 5 (standard incubation mixture), with guinea-pig cardiac sarcolemma (38 µg of protein/assay), 2 mM-MnCl₂ and 0.57 mM-[α-3²P]GTP (27.8 c.p.m./pmol). Acetylcholine (A) and carbamoylcholine (O) were added to the assay medium as shown. Atropine, when present (-) with carbamoylcholine, was 10 µM.

P. V. Sulakhe, unpublished results). Limbird & Lefkowitz (1975) have failed to observe effects of cholinergic agents in vitro on cardiac guanylate cyclase. Our findings agree with the stimulatory effect of cholinergic agonists on the intracellular concentrations of cardiac cyclic GMP (George et al., 1973). We would like to suggest that changes in situ in the concentration of this cyclic nucleotide in heart, due to the action of cholinergic agonists, results from the interactions of these agents with a muscarinic receptor–guanylate cyclase system of sarcolemma.

Cyclic nucleotide phosphodiesterase activities. About 5% of the homogenate phosphodiesterase activities (with either cyclic AMP or cyclic GMP as substrate) were recovered in the isolated sarcolemmal membranes. In guinea-pig ventricles, about 20% of the total phosphodiesterase activities are associated with the particulate fractions (B. L. Russell, S. J. Sulakhe & P. V. Sulakhe, unpublished work). Hence about 25% of the particulate enzyme was recovered in the sarcolemma. As shown in Fig. 7(a), this membrane diesterase exhibited S-shape kinetic curves when titrated with either cyclic AMP or cyclic GMP, both in the

Fig. 7. Cyclic nucleotide phosphodiesterase activities of sarcolemma; titration with cyclic AMP or cyclic GMP

(a) Assays were performed as described in the Methods section, with 61 µg of protein/assay and [³²H]cyclic AMP (A, Δ) (63.08 c.p.m./pmol) or cyclic [³²H]GMP (48.57 c.p.m./pmol) (■, □). When included (-----), Ca²⁺ was present at 5.125 mM (5 mM-EGTA present). Inset: this is an enlargement of the lower left-hand portion of (a); v is the phosphodiesterase activity expressed as nmol of cyclic nucleotide hydrolysed/min per mg of protein. (b) shows the reciprocal of the velocity (v) plotted against the reciprocal of the cyclic nucleotide concentration, [S]. □, Cyclic GMP (−Ca²⁺); ■, cyclic GMP (+Ca²⁺); Δ, cyclic AMP (−Ca²⁺); A, cyclic AMP (+Ca²⁺).

1976
absence and presence of Ca\textsuperscript{2+}. Significant stimulation of the diesterase activity by Ca\textsuperscript{2+} was only observed with cyclic GMP as substrate both at low (Fig. 7a, inset) and high (Fig. 7a) substrate concentrations. Lineweaver–Burk analysis of these data is shown in Fig. 7(b), which clearly shows the upward-bending of the curves. One possible interpretation of these data supports the concept of multiple binding sites on the enzyme, with interaction occurring between these sites (Koshland, 1970).

With cyclic AMP as substrate, both high-\(K_m\) (270 \(\mu\text{M}\)) and low-\(K_m\) (1.13 \(\mu\text{M}\)) forms of diesterase were detected, the respective \(V_{\text{max}}\) being 2.5 nmol/min per mg and 0.022 nmol/min per mg; with cyclic GMP, high-\(K_m\) 166 \(\mu\text{M}\) (\(V_{\text{max}}\), 1.1 nmol/min per mg, \(-\text{Ca}\textsuperscript{2+}\); 1.6 nmol/min per mg, +\text{Ca}\textsuperscript{2+}) and low-\(K_m\) 11.8 \(\mu\text{M}\) (\(V_{\text{max}}\), 0.25 nmol/min per mg, \(-\text{Ca}\textsuperscript{2+};\) 0.33 nmol/min per mg, +\text{Ca}\textsuperscript{2+}) forms were present. The kinetic properties of the sarcolemmal phosphodiesterases are different from those observed for the cytoplasmic enzyme from cardiac muscle (Goren & Rosen, 1972; Appleman & Terasaki, 1975) and from other tissues (Beavo et al., 1970). These membrane-bound phosphodiesterases can be considered to play a critical role in the regulation of the concentrations of cyclic nucleotides in the vicinity of cardiac plasma membranes. Stimulation of the cyclic GMP-dependent activity by Ca\textsuperscript{2+} suggests the possible regulation of this activity during trans-sarcolemmal Ca\textsuperscript{2+} movements.

**Conclusion**

Numerous studies have shown the critical involvement of cyclic nucleotides as well as Ca\textsuperscript{2+} ions in the regulation of myocardial metabolism and function [see Rasmussen et al. (1975) for a review]. Our studies provide evidence that the enzymes involved in the synthesis, degradation and action of cyclic nucleotides are present in the plasma membranes of cardiac muscle. Further, cyclases displayed the anticipated modulation of their activities by the respective neurohormones (Figs. 4 and 6), whereas phosphodiesterase (cyclic GMP) was activated by Ca\textsuperscript{2+} ions (Fig. 7). From such observations it can be suggested that the primary alterations occurring in these membranes may subserve the physiological effects of cholinergic–adrenergic agents on heart. The presence of an active Ca\textsuperscript{2+}-transport system in these membranes, and its modulation via protein kinase-catalyzed phosphorylation (Hui et al., 1975; St. Louis, 1975; P. V. Sulakhe, et al., 1976a) would further support the view that cardiac sarclemma is intimately involved in regulation of heart metabolism and function.

This work was supported by a grant from the Saskatchewan Heart Foundation. P. J. St. L. is Research Fellow of the Canadian Heart Foundation. We are grateful to Professor George Drummond for sending us his manuscript before publication.

**References**