ENZYME CYTOCHEMICAL STUDY OF RAT CARDIAC MUSCLE I. ADENYLATE CYCLASE AND GUANYLATE CYCLASE^{1,2}

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The cytochemical localization of adenylate cyclase and guanylate cyclase in rat cardiac muscle was demonstrated with the lead nitrate method using dimethyl sulfoxide (DMSO) (Fujimoto *et al.*, 1981). Tissues were fixed in a mixture of 2% paraformaldehyde, 0.25% glutaraldehyde and 5% DMSO in 0.1 M cacodylate buffer, pH 7.4, for 30 min, and then washed with 5% DMSO in 0.1 M cacodylate buffer. Vibratome or Microslicer sections were incubated in the following medium for 30 min at 37°C.

Medium for adenylate cyclase (ACLase): 80 mM Tris-maleate buffer, pH 7.4; 4 mM MgSO₄; 10 mM NaF; 2 mM theophylline; 2 mM lead nitrate; 0.25 M sucrose; 5% DMSO; 2.5 mM levamisole; 0.5 mM AMP-PNP. Medium for guanylate cyclase (GCLase): 80 mM Tris-maleate buffer, pH 7.4; 3 mM MnCl₂; 2 mM theophylline; 2 mM lead nitrate; 0.25 M sucrose; 5% DMSO; 2.5 mM levamisole; 0.5 mM GMP-PNP. The most intense reaction of ACLase was localized in the sarcolemma, sarcoplasmic reticulum and gap junction. GCLase activity was also seen in the same regions. In the unfixed isolated gap junctions, the reaction product was seen to interrupt the interspace of the junction, forming bridges between the junctional membranes. ACLase was activated by NaF and isoproterenol. Alloxan was effective as an inhibitor of ACLase. GCLase was stimulated by the *in vitro* acetylcholine treatment.

Heart activity is regulated by action potentials which are relayed from the pacemakers. Each action potential leads to an increase in the intracellular level of calcium which activates contraction. Relaxation follows as this calcium is removed by uptake and extrusion mechanisms. In this constant ebb and flow of calcium, which follows each action potential, there are subtle adjustments of calcium signal

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Abbreviations: ACLase, adenylate cyclase; GCLase, guanylate cyclase; cAMP, cyclic 3' : 5'-adenosine monophosphate; cGMP, cyclic 3' : 5'-guanosine monophosphate; AMP-PNP, adenylyl imidodiphosphate; GMP-PNP, guanylyl imidodiphosphate; DMSO, dimethyl sulfoxide; SR, sarcoplasmic reticulum.

during each contraction-relaxation cycle. The modulation concerns both the rate and quantity of calcium delivered during the initiation of contraction and the rate at which calcium is removed during relaxation. This modulation of the calcium signal is primarily the concern of cyclic 3',5'-adenosine monophosphate (cAMP) and cyclic 3',5'-guanosine monophosphate (cGMP) (3, 10, 26, 33, 34). In order to appreciate how both cAMP and cGMP act, it is necessary to recognize the localizations of adenylate cyclase (EC 4.6.1.1, ACLase) and guanylate cyclase (EC 4.6.1.2, GCLase) within cardiac muscle. Attempts to elucidate the ACLase and GCLase activities have, thus far, been based to some extent on the biochemical study of the subcellular fraction (7, 16, 33). However, because of a rigid intracellular organization and the extensive invagination of the sarcolemma, it has not been possible to obtain a homogenous subcellular fraction of cardiac muscle. Thus, the subcellular localization of ACLase and GCLase activities has not been clearly determined; it is necessary to use cytochemical methods for demonstrating the subcellular localization of the both enzymes in cardiac muscle. In 1970, Reik *et al.* (23) developed a method for the cytochemical localization of ACLase activity, using ATP as a substrate and lead nitrate as a capture agent. However, the use of ATP as a substrate was strongly criticized since it also serves as the substrate for ATPase, and subsequently it was impossible to distinguish the reaction products based on ACLase from that of ATPase. Thus, Howell and Whitfield (12) and Wagner *et al.* (36) used adenylyl imidodiphosphate (AMP-PNP), an analog of ATP, instead of ATP in the cytochemical medium, because AMP-PNP is converted into cAMP and imidodiphosphate by ACLase, but not hydrolyzed by ATPase (27). Since then, Slezak and Geller (31) and Revis (25) reported the cytochemical demonstration of ACLase in cardiac muscle using a method of Howell and Whitfield. However, Lemay and Jarett (18) questioned the usefulness of AMP-PNP as a substrate and lead nitrate as a capture agent, and claimed that this method of Howell and Whitfield can not be reliably used. GCLase activity can also be demonstrated by the lead method used as in the case of ACLase. GTP is substituted with guanylyl imidodiphosphate (GMP-PNP) (38) in the cytochemical methods (28, 35) to eliminate a non-specific reaction with other GTPase. However, there are similar problems on the appropriateness of the use of lead as a capture agent as in the case of a lead method for ACLase. To the best of our knowledge, cytochemical demonstration of GCLase in cardiac muscle has not been reported before.

Recently, we assessed in a previous study (9) that: 1) ACLase and GCLase activities were increased by dimethyl sulfoxide (DMSO) added to the fixatives as well as to the incubation medium for the cytochemical demonstrations of the both cyclases, 2) 20–40% of enzyme activity of fixed tissues was retained at 2 mM lead nitrate, 3) lead nitrate did not cause the nonenzymic hydrolysis of AMP-PNP or GMP-PNP, and developed an alternative lead capture procedure for the electron microscopic demonstrations of ACLase and GCLase activities.

In this study, we demonstrated the localizations of ACLase and GCLase in the rat cardiac muscle by using this new cytochemical procedure. Furthermore, the present investigation extended the previous study (9), indicating the presence of cyclase activity in gap junctions of granulosa cells, by using this procedure to obtain the more detailed subcellular localizations of both cyclases in the isolated gap junctions from the rat cardiac muscle.

MATERIAL AND METHODS

Male Wistar rats, weighing 180–250 g, were sacrificed by decapitation, and the cardiac muscles were removed and cut slices less than 1 mm thick. Tissues were then fixed in a mixture of 2% paraformaldehyde and 0.25% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, with 5% DMSO for 30 min at 4°C. Fixed tissues were washed in 0.1 M cacodylate buffer, pH 7.4, with 5% DMSO and 0.25 M sucrose for 30–60 min. Then tissues were cut on a Vibratome (Oxford, California) or a Microslicer (Dosaka EM Co. Ltd., Kyoto) at 30 μ m in thickness. Vibratome or Microslicer sections were incubated in the following reaction medium for 30 min at 37°C.

Medium for ACLase: 80 mM Tris-maleate buffer, pH 7.4; 0.5 mM adenylyl imidodiphosphate (Boehringer Mannheim GmbH., West Germany); 10 mM NaF; 4 mM MgSO₄; 2 mM theophylline; 2 mM lead nitrate; 0.25 M sucrose; 5% dimethyl sulfoxide; 2.5 mM levamisole (Sigma Chem. Co. Ltd., St. Louis).

Medium for GCLase: 80 mM Tris-maleate buffer, pH 7.4; 0.5 mM guanylyl imidodiphosphate (Boehringer Mannheim GmbH., West Germany); 3 mM MnCl₂ or MgSO₄; 2 mM theophylline; 2 mM lead nitrate; 0.25 M sucrose; 5% dimethyl sulfoxide; 2.5 mM levamisole.

Some reaction medium for GCLase also contained 5 mM sodium azide (NaN₃), which is known to increase GCLase activity (15).

Incubated sections were then postfixed in 1% OsO₄ in 0.1 M cacodylate buffer, pH 7.4, for 5–10 min. In some cases postosmifixation was omitted. Serial dehydration in graded alcohols was followed by embedding in Spurr's medium (32). Ultrathin sections were prepared with an LKB ultratome, unstained or stained with uranyl acetate, and observed under a JEM-100 CX electron microscopy.

Procedure for the cytochemical localization of cyclase activity in the isolated gap junctions from cardiac muscles

Gap junctions were isolated from the hearts of male Wistar rats weighing 180–250 g, by a modification of the procedure of Goodenough (11). Incubation was carried out in centrifuge tubes. 1 ml of unfixed gap junction fraction and 2 ml of concentrated (1.5 $\times$) incubation medium were added to each tube. They were allowed to equilibrate for 5 min at 37°C followed by 10 min of incubation at 37°C in a mechanical shaker bath. The reaction was terminated by filling the tubes with cold 1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. The tubes were kept on ice for 30 min to fix and then spun at 20,000 g max for 30 min. The ensuing pellets were cut into suitably sized pieces and then processed for electron microscopy as previously described.

Control experiments

As a cytochemical control for demonstrating the ACLase activity, specimens were incubated in a substrate-free medium, a medium excluding NaF by replacing 1 mM DL-isoproterenol (Sigma Chem. Co. Ltd., St. Louis) and a medium containing 10 mM alloxan (Sigma Chem. Co. Ltd., St. Louis) as an inhibitor of ACLase activity (22). As controls for demonstrating the GCLase, specimens were incubated in a substrate-free medium and a medium containing 1 mM acetylcholine (Sigma Chem. Co. Ltd., St. Louis).

RESULTS

Preservation of both cyclase activity and ultrastructure was enhanced by adding DMSO to the incubation medium as well as to fixatives. When fixation was performed with over than 0.5% glutaraldehyde, cyclase activity was dramatically diminished. Thus, the ultracytochemical observation was mainly carried out by using immersion fixation for 30 min with a mixture of 2% paraformaldehyde, 0.25% glutaraldehyde and 5% DMSO as fixatives.

Cytochemical localization of adenylate cyclase activity

Basal activity of ACLase was demonstrated in the sarcolemma, subsarcolemmal cisterna (Fig. 1), junctional sarcoplasmic reticulum (j-SR) adjacent to T-tubules,

FIG. 1. Rat cardiac muscle prefixed with 2% paraformaldehyde, 0.25% glutaraldehyde and 5% DMSO in cacodylate buffer, and incubated with the standard ACLase medium. Reaction product is localized in the sarcolemma and the subsarcolemmal cisternae(SC). $\times 52,000$

FIG. 2. Precipitate, demonstrating ACLase activity, is seen in the junctional sarcoplasmic reticulum (arrows) surrounding the T-tubule (T) and the longitudinal sarcoplasmic reticulum (arrowheads). $\times 12,000$

longitudinal sarcoplasmic reticulum (l-SR) (Fig. 2) and gap junction of the intercalated disc (Fig. 3). At high magnification, the reaction products were found to be associated with the cytoplasmic side of the sarcolemmae and the gap junctions (Fig. 4). However, in the subsarcolemmal cisternae, j-SR and l-SR, the reaction products were most prominent in the luminal spaces of these structures.

Isoproterenol stimulated ACLase activity in the absence of, or at low concentrations of NaF (5 mM), although in the presence of 5–20 mM NaF isoproterenol had no further effect on the intensity of the reaction (Fig. 5). The isoproterenol-stimulated reactions could be found in the SR and subsarcolemmal cisterna as well as the sarcolemma and gap junction.

Control experiments in which AMP-PNP was omitted, as well as boiled controls, did not show any specific precipitate (Fig. 6). The preincubation in 10 mM alloxan followed by the incubation in the reaction medium containing 10 mM alloxan partly reduced the reaction (Fig. 7).

Cytochemical localization of guanylate cyclase activity

The most intense reactions of GCLase occurred in the SR and subsarcolemmal cisterna as well as at the sarcolemma and gap junction (Figs. 8, 9). The precipitates

FIG. 3. The gap junction of intercalated disc shows deposition of reaction product. $\times 11,000$

FIG. 4. At high magnification, the reaction product is associated with the cytoplasmic side of the gap junction. $\times 135,000$

FIG. 5. Rat cardiac muscle incubated in the presence of 1 mM isoproterenol and 10 mM NaF. Isoproterenol seems to have no further effect on the intensity of the reaction. SR, sarcoplasmic reticulum; T, T-tubule. $\times 34,000$

were most prominent in the luminal spaces of the subsarcolemmal cisterna, j-SR and l-SR, while at the sarcolemma and gap junction the reaction products tended to concentrate on the cytoplasmic side of membranes.

In the experiment of acetylcholine, more intense reactions resulting from the incubation with 1 mM acetylcholine were obtained within 15 min of incubation at 37°C than that of the incubation without acetylcholine. However, no difference from the standard incubation was observed in the localization of the reaction.

GCLase activity of the rat cardiac muscle was not cytochemically affected by adding sodium azide to the incubation medium.

Control experiments in which GMP-PNP was omitted, as well as boiled controls, did not show any specific precipitate.

Cyclase activity in the isolated gap junctions

In thin sections, the isolated preparation appeared highly enriched for gap junctions of variable length (Fig. 10). At high magnifications (Fig. 11), the reaction products of cyclase could be seen to interrupt the interspace of the junction, forming bridges (at apposed arrows) between the junctional membranes. The crossing reaction products between the junctional membranes were periodically separated by a distance of about 9–10 nm.

DISCUSSION

Lemay and Jarett (18) and Kempen *et al.* (13), claimed the validity of using lead nitrate as a capture agent to demonstrate ACLase activity. However, other investigators have demonstrated its usefulness (4, 6). In the previous study (9),

FIG. 6. Control cardiac muscle incubated in the absence of AMP-PNP. No reaction product is present. $\times 27,000$

FIG. 7. Cardiac muscle incubated in the presence of alloxan. Alloxan partly reduced the reaction in the sarcoplasmic reticulum (SR). $\times 24,000$

we have confirmed that DMSO enhances cyclase activity, and a short period (30 min) of fixation with a mixture of 2% paraformaldehyde, 0.25% glutaraldehyde and 5% DMSO, might provide cytochemical stability for cyclase. In the present study, the incubation and fixation with DMSO resulted in an intense reaction and adequate preservation of the fine structure of the cardiac muscles. Kim and Han (14) and Cutler (6) suggested that omission of prefixation might provide a degree of protection of cyclase against the inhibitory effects of lead. Since then, Revis (25) demonstrated the localization of ACLase with unfixed cardiac muscles, although

FIG. 8. GCLase activity is localized in the sarcolemma and the subsarcolemmal cisternae (arrow). $\times 45,000$

FIG. 9. GCLase activity is seen in the junctional sarcoplasmic reticulum (arrows) and the longitudinal sarcoplasmic reticulum (SR). $\times 32,000$

the morphological preservation of tissues seemed to be insufficient to assess cytochemical data. It is well known that DMSO crosses cell membranes in a reversible manner and does not alter membrane integrity (37). We believe that the effect of DMSO may also contribute to increased permeability of membranes for the fixatives, allowing more effective penetration of the incubation medium. It has been reported that an increase in a concentration of 1.41 M DMSO was shown to produce concentration-dependent stimulation of microsomal Ca^{++} -transport and ACLase activity and inhibition of $\text{Na}^{+}\text{-K}^{+}\text{-ATPase}$ activity in guinea pig with the observed positive inotropy (37). We could demonstrate biochemically and cytochemically enhanced ACLase activity by DMSO, at 5% concentration, using cardiac muscle cells and various cells (9). Whereas it has been also reported that DMSO blocked some membrane receptors, although the effect of DMSO could be reversed by washing, and norepinephrine receptors in cardiac muscle were unaffected (30).

FIG. 10. The isolated gap junction, incubated in the medium for GCLase. Osmification is omitted. GCLase activity is associated with the junctional membranes. $\times 56,000$

FIG. 11. The isolated gap junction, incubated in the medium for ACLase. The reaction products span across the two junctional membranes (arrows). $\times 540,000$

According to the present results, indicating that the residual GCLase activity, after the fixation with DMSO, still responded to acetylcholine, acetylcholine receptor may also be unaffected by DMSO.

In considering the localization of cyclase in cardiac muscle, it is important to assess that the cytochemical reaction is a specific response to various hormones or drugs. NaF has been used, in concentrations less than 20 mM, to stimulate ACLase activity (14). Cutler (6) suggested that NaF protects the activity of ACLase by

forming a weak complex with lead nitrate, and also used NaF concentrations as high as 40 mM. In contrast, Cheng and Farquar (4) showed that concentrations of NaF less than 20 mM may inhibit ACLase activity, whereas they found increased activity above 20 mM. We observed an increased intensity of the reaction product when 10 mM NaF was added to the incubation medium for ACLase, however could not appreciate further significant enhancement by using a higher concentration. In addition, NaF was shown to stimulate ACLase activity in fractionated gap junctions.

Isoproterenol has been widely known as a potent *in vivo* stimulant of ACLase (22). In our results, isoproterenol was added only to the incubation medium and its *in vitro* effect could be demonstrated. It is also known that perfusion with isoproterenol for periods of 15 to 45 sec lead to significant decreases of 50% in tissue cGMP concentrations and marked adrenergic effects on both heart rate and force of contraction (10). However we could not demonstrate altered GCLase activity by isoproterenol.

In the present investigation, ACLase and GCLase activities are seen in sarcolemma, SR and gap junction, and these cytochemical findings confirm the presence of cyclases in some intracellular organelles as well as in plasma membranes (sarcolemma). According to the commonly accepted model for cyclase system based on biochemical studies (21), the enzymes themselves are situated on the inside of the plasma membrane, whereas the receptors are on the outside. In the present investigation, the reaction products are found to be associated with the cytoplasmic sides of the sarcolemmae and gap junctions, and this finding is in agreement with the model for cyclase (21).

Cytochemical localization of ACLase in the sarcolemma and SR is consistent with the findings of Slezak and Geller (31) and supports the role of these structures for Ca^{++} transport and release. One important action of catecholamines is to prolong the slow inward current, and this effect can be mimicked by dibutyryl-cAMP. This ability of cAMP may be due to prolongation of the influx of calcium across the sarcolemma. By increasing calcium entry, cAMP will increase the amount of Ca^{++} entering the sarcoplasmic reticulum. In turn, the mechanism of action of cAMP involves an increase in permeability of specific Ca^{++} -channels of sarcolemma and uptake of Ca^{++} into SR (2). The cytochemical demonstration of ACLase on the sarcolemma and SR observed in the present study directly supports the possibility that ACLase located on these organelles responds to hormonal signals which regulate the calcium metabolism taking place within these organelles. Whereas comparatively little is known of the molecular basis of GCLase and cGMP in cardiac muscles, but several studies exist. George *et al.* (10) reported that the cGMP levels in the perfused heart were elevated by acetylcholine. In addition, dibutyryl-cGMP as well as carbachol, an acetylcholine analog, is known to decrease the rate of rhythmic beating of cultured rat cardiac cells, while epinephrine and dibutyryl-cAMP accelerate this rate. In the present study, localization of GCLase in the same region as ACLase supports the hypothesis that cellular Ca^{++} metabolism may be regulated by interaction between cAMP and cGMP.

It is well known that cell-to-cell movement of cyclic nucleotides may be involved in the coordination of various cell activities (29, 20). Since cyclic nucleotides are smaller than some dye molecules capable of cell-to-cell movement, it is certainly conceivable that these nucleotides may also diffuse between cell interiors. However,

other studies have implicated gap junctions in the control of intracellular cAMP or cGMP levels in a different manner. This evidence argues on the basis of the possibility that gap junctions are themselves aggregates of macromolecular complexes composed in part of hormone receptors and cyclases. Larsen (17) suggests that the opaque deposits formed on the cytoplasmic sides of gap junction membrane are formed when calcium enters the cell during fixation and precipitates with pyrophosphate produced by the breakdown of endogenous ATP by ACLase located at gap junction. In addition, the correlation between the gap junction and the receptor-cyclase complex is based on several other studies (1, 8, 39). In the present investigation, it has been observed that the crossing reaction products between the isolated junctional membranes are periodically separated by a distance of about 9–10 nm. This finding is somewhat surprising in view of the usually reported measurement for the distance of the central axis of each connexon (8–9 nm) determined from stained thin sections (24) and freeze-fractured preparations (19). However, a difference from *in situ* cytochemical findings using sectioned tissues was observed in the distribution of the reaction product. This may be due to the effect of fixation which changes the molecular configuration of the junctional membranes.

In conclusion, the demonstration of ACLase and GCLase in the sarcolemma, subsarcolemmal cisterna, SR and gap junction suggests that the effect of myocardial contractility by hormones known to activate ACLase and GCLase may occur as a result of activation of cyclases localized to these structures.

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