Impaired Cardiac Muscarinic Receptor Function in Dogs with Heart Failure

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Abstract

Prior physiological studies have suggested that parasympathetic control is altered in heart failure. The goal of our studies was to investigate the influence of heart failure on the muscarinic receptor, and its coupling to adenylate cyclase. Ligand binding studies using [^{3}H]quinuclidinyl benzilate and enriched left ventricular (LV) sarcolemma, demonstrated that muscarinic receptor density in heart failure declined 36% from a control of 5.6±0.6 pmol/mg, with no change in antagonist affinity. However, agonist competition studies with both carbachol and oxotremorine showed that it was a loss of high affinity agonist binding sites in the sarcolemma from failing LV that accounted for this difference.

The functional efficacy of the muscarinic receptor was also examined. When 1 μM methacholine was added to 0.1 mM GTP and 0.1 mM isoproterenol, adenylate cyclase stimulated activity was inhibited by 15% in normal LV but only 5% in LV sarcolemma from animals with heart failure even when the reduced adenylate cyclase in these heart failure animals was taken into account. Even at 100-fold greater concentrations of methacholine, significantly less inhibition of adenylate cyclase activity was observed in LV failure as compared with normal LV sarcolemma. Levels of the GTP-inhibitory protein known to couple the muscarinic receptor to adenylate cyclase, as measured with pertussis toxin labeling, were not depressed in LV failure. Thus, the inhibitory pathway regulating LV adenylate cyclase activity is defective in heart failure. The decrease in muscarinic receptor density, and in particular the specific loss of the high affinity agonist binding component of this receptor population, appears to be the major factor underlying this abnormality.

Introduction

Several abnormalities in the autonomic control of cardiac function have been identified in heart failure (1–5). While most work has focused on alterations in adrenergic mechanisms, defective parasympathetic regulation of the failing heart has also been surmised from the findings of depressed chronotropic responses to atropine and reduced baroreflex slowing of heart rate in response to an acute elevation in arterial pressure (2–4). Since the mechanism of reflex bradycardia in response to baroreceptor hypertension is primarily vagal (6, 7), this suggests defective parasympathetic control of the failing heart. This defect could be located in the vagus, the central nervous system, at the muscarinic receptor itself, or in its transduction mechanism. Muscarinic receptor activation can affect directly a variety of cellular systems which leads to alterations in cardiac rate and contractility (1). One well-defined mechanism of muscarinic receptor function involves inhibition of adenylate cyclase activity. We have recently observed that in a model of chronic left ventricular (LV)[1] failure in the dog, muscarinic receptor density is reduced (5). The ligand binding studies from that study were conducted using a relatively crude membrane preparation.

To extend these preliminary observations, the present investigation was designed to determine if (*a*) a decrease in muscarinic receptor density was present in an enriched sarcolemmal preparation from failing LV (8), (*b*) abnormalities in agonist binding could also be identified, (*c*) muscarinic inhibition of adenylate cyclase activity, one type of functional measure of muscarinic receptor activity (9), was also depressed, and (*d*) levels of inhibitory guanine nucleotide-binding proteins (10–12) were altered.

Methods

Mongrel puppies of either sex at 7–10 wk were anesthetized with sodium thiamylal (10 mg/kg), and then halothane (1 vol %), and ventilated with a Harvard respirator. A right thoracotomy was performed through the 4th intercostal space using sterile surgical technique. The ascending aorta above the coronary arteries was isolated and dissected free of surrounding tissue. A 1-cm wide Teflon cuff was placed around the aorta, tightened until a thrill was palpable over the aortic arch, and the chest was closed. Nine dogs with chronic pressure overload developed LV failure spontaneously, 9 mo to 2 yr after banding. Animals used in this study were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and the Guide for Care and Use of Laboratory Animals (Department of Health and Human Services publication No. [National Institutes of Health] 85-23, revised 1985).

The data from the group of nine dogs with LV failure were compared with a group of 10 normal dogs, one of which was a sham-operated littermate of the dogs with LV failure. LV and arterial pressures were measured in the conscious dogs using a calibrated Millar micromanometer catheter. The catheter was introduced retrogradely into the LV using a femoral artery approach under local anesthesia with lidocaine 2%. Pressures were recorded on a multichannel oscillograph.

After the dogs were anesthetized with sodium pentobarbital, 30 mg/kg, the hearts were immediately excised and placed into iced saline. All subsequent procedures were carried out at 4°C. Enriched sarcolemma membranes were prepared according to the method of

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1. *Abbreviations used in this paper:* LV, left ventriculars; [^{3}H]QNB, [^{3}H]quinaclidinyl benzilate.

Jones and Besch (8). The sarcolemma were prepared by mincing and homogenizing the tissue in 4 vol of buffer (0.75 M NaCl and 10 mM histidine) for 5 s at low speed with a polytron (Brinkmann Instruments Co., Westbury, NY). The homogenate was centrifuged for 15 min at 14,000 *g* and the resulting pellet was resuspended in the same buffer. This step was repeated twice and the pellet was resuspended in 10 mM $NaHCO_3$ and 5 mM histidine buffer and then the pellet was homogenized for 30 s three times at one-half speed with a polytron. The homogenate was centrifuged at 14,000 *g* for 20 min and the supernatant was saved and centrifuged at 44,000 *g* for 30 min. The pellet was resuspended in distilled water, and an equal volume of 2 M sucrose was added and then layered at the bottom of a sucrose density gradient containing 1.0, 0.6, and 0.25 M sucrose. The yield from this preparation was 7±1 mg enriched sarcolemma protein per 100 g of LV for normal hearts, which was not significantly different from failing hearts (6±2 mg/100 g). A crude membrane preparation was also prepared as previously described (5). The crude membrane preparation was used solely for the agonist binding experiments. The heart was trimmed, minced, homogenized, and centrifuged at 1,000 *g* for 15 min in buffer (0.25 M sucrose, 1 mM $KHCO_3$, 1 mM EDTA). The supernatant was centrifuged at 45,000 *g* for 15 min. The pellet was resuspended in the same buffer and centrifuged two more times. The yield from this preparation was ~ 100 mg protein per 100 g LV.

Na^+,K^+-ATPase activity was determined according to the method of Jones and Besch (8). Na^+,K^+-ATPase activity, a membrane-associated protein that is not involved in ligand binding, was used as a marker for sarcolemma content. The enriched sarcolemma-associated activity of Na^+,K^+ ATPase was 26.8±3.4 μmol inorganic phosphate (P_i)/h per mg in the normal LV and not significantly different (24.0±3.4 μmol P_i/h per mg) in the failing LV.

All studies were performed in the presence of 100 mM Tris, 5 mM $MgCl_2$, and 1 mM EGTA, pH 7.2. Antagonist binding studies were performed using 100 μl [^{3}H]quinuclidinyl benzilate (^{3}H-QNB), 0.05–3 nM, 100 μl atropine (1 μM) or buffer, and 800 μl of membrane protein (2–4 μg/tube). Agonist competitive inhibition binding studies were performed with ^{3}H-QNB, and with increasing concentrations (0.001–100 μM) of carbachol and oxotremorine in the presence and absence of 0.1 mM Gpp(NH)p. Assays were performed in duplicate, incubated at 37°C for 30 min, terminated by rapid filtration on Whatman glass fiber/C filters, and counted in 10 ml Hydrofluor (National Diagnostics, Manville, NJ) for 5 min. Nonspecific binding as determined by 1 μM atropine, comprised 10% of the total binding. Specific binding was determined by subtracting the nonspecific from the total binding. The binding data were analyzed by the "Ligand" program of Munson and Rodbard (13). In the computer analysis, the F test was used to compare the best fit for the ligand binding competition data. The three-site vs. two-site fit was determined by the *P* value for the F test, and by the change in the residual sum of squares for the various fits. One-, two-, and three-site models were tested and the model yielding the least residual sum of squares was used to describe the data. The *P* values and the residual sum of squares for the normal LV indicated that the three-site fit was preferred over the two-site fit, for the inhibition curve using carbachol as the competing ligand.

The adenylate cyclase assays were performed in a total volume of 0.15 ml containing sarcolemma protein (10–20 μg), 100 mM Tris (pH 7.2), 1 mM EGTA, 15 mM $MgCl_2$, 2 μCi [α-^{32}P]ATP, 0.2 mM ATP, 7 mM creatine phosphate, 1 U creatine phosphokinase, 0.3 mM cyclic AMP, [^{3}H]cyclic AMP (10,000 cpm, 40 Ci/mmol), and one or more of the following: 0.1 mM GTP, 0.1 mM or 0.3 μM isoproterenol, 1 μM atropine, and 0.03–100 μM methacholine. Maximal adenylate cyclase activity was assessed by measuring cyclic AMP production in the presence of 0.1 mM isoproterenol plus 0.1 mM GTP. Adenylate cyclase activity was linear, with time of incubation from 1 to 30 min. Increasing concentrations of the muscarinic agonist methacholine (0.03 μM to 0.1 mM) were used to inhibit adenylate cyclase activity that was stimulated by isoproterenol (0.3 μM) plus GTP (0.1 mM). ^{32}P-Cyclic AMP was quantitated according to the method of Salomon et al. (14). The inhibition of adenylate cyclase activity by methacholine was completely blocked with atropine (1 μM). The protein concentrations for each assay were determined by the method of Lowry et al. (15).

The pertussis toxin–catalyzed ADP ribosylation of the sarcolemmal GTP-binding proteins was carried out according to the method of Bokoch et al. (11). Enriched sarcolemma was solubilized in a 2% cholate solution for 60 min immediately before ADP ribosylation. The solubilized membranes (3 mg/ml) were then diluted 1:20 in 20 mM Tris HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, and 0.05% Lubrol (Sigma Chemical Co., St. Louis, MO). The reaction was begun by adding 10 μl of this solution, which contained 1.5 μg of solubilized sarcolemma, to 26 μl of a reaction buffer containing 3 μg/ml pertussis toxin and 1.0 μM [^{32}P]NAD, and then the reaction mixture was incubated at 30°C for the times indicated. The reaction was stopped by the addition of 3 vol of solubilization buffer. The samples were then run on a 17% polyacrylamide gel and autoradiography was carried out overnight. Individual bands representing the inhibitory guanine nucleotide regulatory proteins G_i and G_o were cut out separately, and counted in 10 ml of a toluene-based scintillation mixture. An equivalent area of each lane was cut out for background measurement, and the reported values represented total band counts minus background counts. ADP ribosylation values were reported as picomoles of [^{32}P]ADP-ribose incorporated per milligram of membrane protein added.

Data were expressed as mean value±SEM, and stored in a Digital computer (PDP-11/34). Statistical evaluation was performed by Student's *t* test between groups (16).

Results

Quantitation of receptor density in LV failure. In the 9 dogs with LV failure as compared with 10 normal animals, LV weight, LV free wall weight/body weight, LV systolic, and end-diastolic pressures were elevated, $P < 0.01$ (Table I). The increased LV end-diastolic pressure (33±4.3 vs. 8.1±0.7 mmHg for controls) indicated that LV hypertrophy was no longer compensated. LV failure, reflected by pulmonary vascular congestion, was confirmed in all dogs at autopsy.

Using an enriched sarcolemmal membrane preparation (8), muscarinic receptor density, as assessed with the muscarinic antagonist [^{3}H]QNB, was reduced significantly, $P < 0.05$, in the LV of animals with heart failure (3.6±0.4 pmol/mg, $n = 8$) compared with normal LV sarcolemma (5.6±0.6 pmol/mg, $n = 10$). The affinity for [^{3}H]QNB was similar in normal (0.12±0.02 nM) as compared with failing LV (0.13±0.02 nM) (Fig. 1).

Quantitation of agonist high affinity sites in LV failure. Muscarinic agonist binding was examined by performing competition studies with both carbachol (Fig. 2) and oxotrem-

Table I. Morphology and Hemodynamics

	Normal ($n = 10$)	LV failure ($n = 9$)
Body weight (*kg*)	25±1.3	22±0.8
LV weight (*g*)	81±5.1	157±9.9*
LV weight/body weight (*g/kg*)	3.4±0.1	7.1±0.4*
LV systolic pressure (*mmHg*)	129±3.0	270±28*
LV end-diastolic pressure (*mmHg*)	8.1±0.7	33±4.3*
Mean arterial pressure (*mmHg*)	94±4.0	100±6.7
Heart rate (*beats/minute*)	94±5.0	139±14‡

* $P < 0.01$ from normal values.
‡ $P < 0.05$ from normal values.

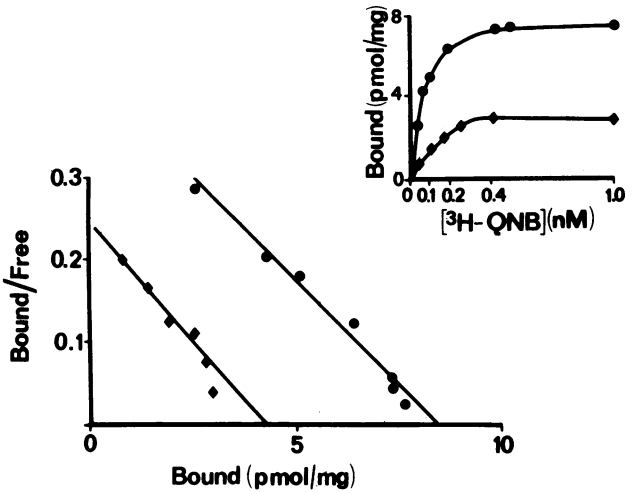

Figure 1. Scatchard analyses of muscarinic receptor binding to LV-enriched sarcolemma are compared in the normal heart (*circles*) and in the heart with LV failure (*diamonds*). The LV failure heart shows a similar affinity and fewer receptors per milligram protein than the normal LV. The inset shows a saturation plot of the same data.

orine (Fig. 3). This was carried out since previous reports have indicated that the number and percentage of interactive high affinity sites detected with these two agonists may be different (17, 18). Using the crude membrane preparation from normal LV, agonist competition binding curves with [^{3}H]QNB and 15 to 22 concentrations (10^{-10}–10^{-4} M) of carbachol, were fitted best to a three-site model (Fig. 4). Sarcolemma from failure LV showed a complete loss of the superhigh affinity site and a 50% reduction in the concentration of the high affinity site (Fig. 4). Using oxotremorine for agonist binding, the data best fit a two-site model for normal LV, i.e., high and low affinity sites, in the membranes from the normal dogs. In LV failure, the number of high affinity sites (56±9 fmol/mg) was significantly reduced, $P < 0.05$, from that observed in normal dogs (89±9

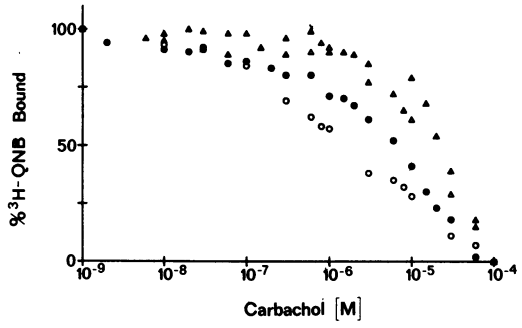

Figure 2. Competitive inhibition binding is shown in sarcolemma membranes from the normal LV of a single animal with increasing concentrations of carbachol. In the normal LV in the absence of Gpp(NH)p (*open circles*), the binding curve has a shallow slope and fits best to three affinity sites. In the presence of Gpp(NH)p (*open triangles*), the curve shifts to the right and steepens to fit two affinity sites. In sarcolemma from the failing LV of a single animal, the absence of Gpp(NH)p (*filled circles*) results in a binding curve that is shifted to the right of the normal LV and fits best to two affinity sites. In the presence of Gpp(NH)p (*filled triangles*), the curve shifts further to the right and fits best to a single affinity site.

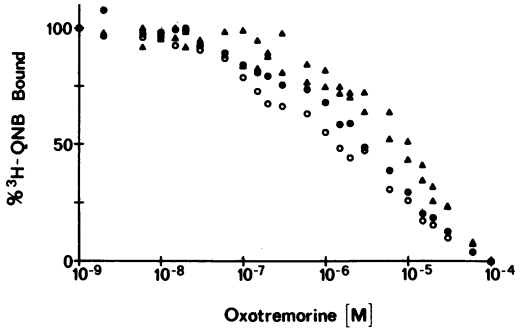

Figure 3. Competitive inhibition binding is shown in sarcolemma membranes from normal LV of a single animal and failing LV of a single animal, with increasing concentrations of oxotremorine. Binding to sarcolemma from normal LV, in the absence of Gpp(NH)p (*open circles*), results in a shallow slope and fits best to two affinity sites. In the presence of Gpp(NH)p (*open triangles*), the curve steepens and shifts to the right. With LV failure, in the absence of Gpp(NH)p (*filled circles*), the binding curve is shifted to the right of the normal LV. In the presence of Gpp(NH)p (*filled triangles*), the curve shifts only slightly further to the right.

fmol/mg) (Table II). In the presence of 0.1 mM Gpp(NH)p, all curves shifted to the right into a low affinity state.

Functional efficacy of muscarinic receptor activation in LV failure. Basal adenylate cyclase activity was lower in LV sarcolemma of animals with failure (385±51 pmol cyclic AMP/mg per min, $n = 5$) as compared with normal animals (804±121 pmol cyclic AMP/mg per min, $n = 6$). Maximal β-adrenergic receptor–mediated stimulation of adenylate cyclase by 0.1 mM isoproterenol and 0.1 mM GTP was also significantly less, $P < 0.05$, in LV failure (815±138 pmol cyclic AMP/mg per min) compared with normal hearts (2,238±427 pmol cyclic AMP/mg per min) as previously reported (5).

When this maximal isoproterenol (0.1 mM) stimulation was inhibited with 1 μM methacholine, adenylate cyclase activity was reduced by 15±2.7% (244±87 pmol cyclic AMP/mg per min) in normal LV and significantly less, $P < 0.01$, by only 5±2.1% (25±9.4 pmol cyclic AMP/mg per min) in the failing LV. The reduced inhibition of adenylate cyclase activity in LV failure was observed both with and without subtracting basal cyclase activity (Fig. 5). After the addition of 1 μM atropine, the inhibition in 0.1 mM isoproterenol-stimulated adenylate

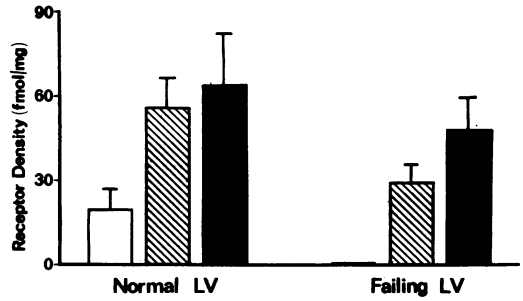

Figure 4. The average±standard error of the mean number of superhigh (*open bar*), high (*hatched bars*), and low (*solid bars*) affinity sites as determined by computer modeling of carbachol competitive inhibition curves with normal ($n = 7$) and failing LV membranes ($n = 7$), shows absence of the superhigh affinity site and a decline in total muscarinic receptors in the failing LV.

Table II. Agonist Binding

	K_{11}	K_{12}	K_{13}	R_1	R_2	R_3	R_1	R_2	R_3
		μM			*fmol/mg*			%	
Carbachol									
Normal LV (n = 7)	0.01±0.01	0.48±0.24	4.68±1.01	20±6	55±10*	64±17	14±5	40±5	46±5
Failing LV (n = 7)	—	1.11±0.96	10.29±4.81	0	29±6	47±11	0	38±6	62±6
Oxotremorine									
Normal LV (n = 9)	—	0.06±0.01	3.53±0.90	0	89±9*	68±12	0	57±3	43±3
Failing LV (n = 9)	—	0.08±0.05	1.66±0.31	0	56±9	65±15	0	46±5	54±5

* High plus superhigh affinity sites greater in normal LV than failing LV, $P < 0.05$.

cyclase activity produced by 0.1 mM methacholine was no longer observed in normal LV (2,328±432 pmol cyclic AMP/mg per min) or in LV failure (786±108 pmol cyclic AMP/mg per min). This reversal by atropine of methacholine inhibition of isoproterenol stimulated adenylate cyclase activity results in adenylate cyclase activity similar to the maximal isoproterenol stimulated activity (2,238±427 pmol cyclic AMP/mg per min in normal hearts and 815±138 pmol cyclic AMP/mg per min in the failing LV). This demonstrates that the methacholine inhibition of adenylate cyclase activity is specific for the muscarinic cholinergic receptor.

Effects of methacholine on submaximal stimulation of adenylate cyclase activity by 0.3 μM isoproterenol. Isoproterenol (0.3 μM) plus GTP (0.1 mM) were used to stimulate adenylate cyclase activity to examine the effects of a lower concentration of isoproterenol, which was near the EC_{50}, and more sensitive to increasing concentrations of methacholine (0.03–100 μM). Fig. 6 shows that in the LV sarcolemma from failing hearts, at any concentration of methacholine, adenylate cyclase was inhibited by only a minor fraction of that observed in the normal LV. Regression analysis was also conducted on these relationships. The slopes for normal and LV failure were tenfold different, $P < 0.001$.

Quantitation of inhibitory GTP-binding proteins in LV failure (19). To use [^{32}P]NAD/pertussis toxin labeling as a method of quantitating the α subunits of G_i and G_0, it was important to show that labeling of this protein was complete. This was demonstrated in two ways. First, saturation of labeling occurred at 60 min as shown in a typical experiment in Figs. 7 and 8. During this time, [^{32}P]NAD concentrations were maintained as demonstrated by polyethyleneimine-cellulose thin-layer chromatography of reaction mixtures and subsequent autoradiography. Secondly, the amount of labeling was linear with respect to amount of sarcolemma protein added, and independent of the [^{32}P]NAD (range, 1 to 4.5 μM) and pertussis toxin (range, 3 to 12 μg/ml) concentrations.

Using this technique, G_i and G_0 were quantitated in sarcolemma from normal and failing LV. There is no significant difference in G_i in normal (23±2.5 pmol/mg protein, n = 5) vs. LV failure (32±6.2 pmol/mg, n = 4) or in G_0 in normal (10±2.1 pmol/mg) vs. LV failure (12±2.1 pmol/mg).

Discussion

Preliminary data from our laboratory using a crude membrane preparation suggested that a decrease in muscarinic receptors occurred in LV failure (5). Compared with the receptor density (0.24 pmol/mg protein) detected in the crude membrane preparation employed in an initial study, a 23-fold enhancement in receptor content (5.6 pmol/mg protein) was obtained in the highly enriched sarcolemma employed in the present investi-

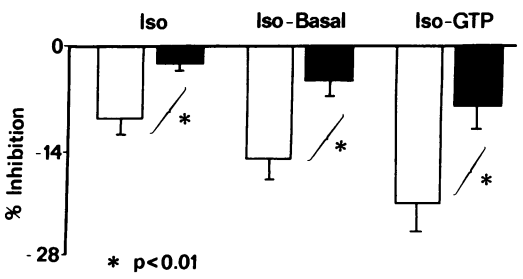

Figure 5. The percent inhibition by 1 μM methacholine of isoproterenol (0.1 mM) stimulated adenylate cyclase activity of cardiac sarcolemma from normal (n = 5) (*open bars*) and failing (n = 4) LV (*solid bars*) is shown. On the left is the percent inhibition of isoproterenol (0.1 mM) plus GTP (0.1 mM)-stimulated cyclase. The middle bars show the same data with basal activity subtracted, and on the right are the same data with the GTP (0.1 mM)-stimulated activity subtracted.

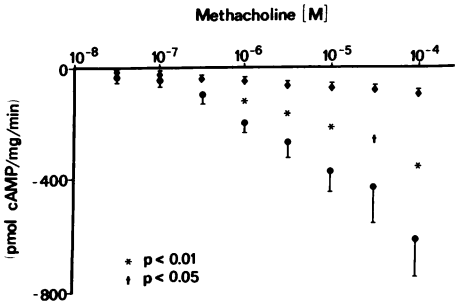

Figure 6. Adenylate cyclase was stimulated with isoproterenol (0.3 μM) plus GTP (0.1 mM) in sarcolemma from normal LV (1,964±393 pmol/mg per min) (n = 6) and from failing LV (631±77 pmol/mg per min) (n = 5), in the presence or absence of increasing concentrations of methacholine (0.03–100 μM) to inhibit the production of cyclic AMP. In comparison to sarcolemma from normal LV (*circles*), there is a marked diminution of methacholine-mediated adenylate cyclase inhibition in sarcolemma from failing LV (*diamonds*).

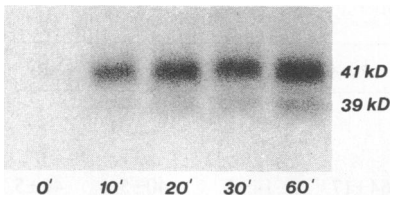

Figure 7. This figure demonstrates the ADP ribosylation of G_i and G_0 in solubilized canine sarcolemma membranes. Each lane contains 1.5 μg protein incubated with 3 μg/ml pertussis toxin and 1.0 μM [^{32}P]NAD at 30°C. The reaction was stopped at the following time points: 0, 10, 20, 30, and 60 s. Note the ability to separate labeled G_i and G_0 (≈ 41,000 and 39,000 D, respectively) as two distinct bands.

gation. The current investigation, using this enriched sarcolemmal preparation (8), confirms the previous finding of decreased muscarinic receptor density in heart failure. While muscarinic receptor density in the sarcolemma of failing hearts is reduced, the absolute number of receptors per cell is not equivalently reduced, since myocyte hypertrophy occurs in this model of heart failure. The decreased density of muscarinic receptors could not be attributed to differences in the yield of membrane protein from each heart, since these values were similar in the normal and failing hearts. However, it is important to note that this reduction in density is not a generalized phenomenon. Firstly, as previously reported from our laboratory, β-adrenergic density is actually increased in the same sarcolemma prepared from the failing LV. Secondly, the sarcolemma marker Na^+,K^+-ATPase activity was similar in the two groups. Thirdly, the content of the inhibitory GTP protein, G_i, and another pertussis toxin substrate, G_0, were not decreased. The content of G_0 was quantitated, since reconstitution studies by Florio and Sternweis (20) suggest that G_0, as well as G_i, may couple to the muscarinic receptor.

Various mechanisms can be suggested that could lead to a reduction in muscarinic receptors as a secondary event. For example, a loss of sympathetic or parasympathetic innervation could be responsible since muscarinic receptors are associated with sympathetic as well as parasympathetic neurons (21, 22). In fact, heart failure is characterized by decreased myocardial

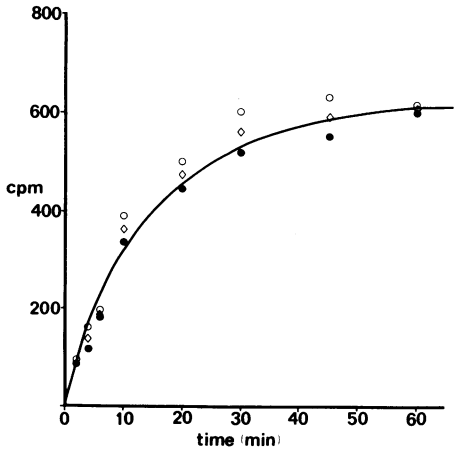

Figure 8. Time course of labeling of pertussis toxin substrate (G_i plus G_0) of solubilized sarcolemma from two normal (*open symbols*) and one failure (*filled symbols*) animal is shown. Note that saturation is nearly achieved by 30 min.

norepinephrine stores (5, 23). However, in a model of total cardiac denervation, where sympathetic denervation is more complete than in this heart failure model, we detected only a 20% decrease in muscarinic receptor density (24). Furthermore, in terms of the parasympathetic system, Roskoski et al. (25) have previously reported in an experimental model of heart failure in the guinea pig, that parasympathetic innervation as assessed by choline acetyl transferase levels, was reduced per unit weight, but not reduced in the entire ventricle. Their findings parallel our observations for muscarinic receptor density, which was reduced per milligram protein in the current investigation, but was not reduced in the entire ventricle, since hypertrophy had almost doubled the ventricular weight.

In the present investigation, agonist binding studies with carbachol and oxotremorine were also performed. Computer modeling of the binding data with carbachol indicated that there were three affinity states for the muscarinic receptor in the normal LV: superhigh, high, and low. In LV failure, agonist inhibition of [^{3}H]QNB binding confirmed the reduction in the total number of muscarinic receptors with specific reduction in high affinity sites and virtual absence of the superhigh affinity sites. With oxotremorine, only high and low affinity sites were observed in the normal LV, and there was a decrease in high affinity receptors in the failing LV. Similar modeling of agonist competition curves to three sites for carbachol and to two sites for oxotremorine has been reported previously (17). The mechanism underlying this difference has not been determined. As with the β-adrenergic receptor and other hormone receptors coupled to the stimulatory GTP protein, G_s, it has been postulated that the agonist high affinity state of the muscarinic receptor represents the "coupled" form of the receptor. Again, as with stimulatory hormone receptors, guanine nucleotides shift these receptors to a uniform low affinity state. Whether the ternary complex model of hormone, receptor and G protein, which represents the high affinity β-receptor agonist binding state and which dissociates in the presence of guanine nucleotides, can also explain the shifts observed in muscarinic receptor binding, has yet to be determined.

We had expected, in fact, that a reduction in the levels of G_i might be detected for two reasons. First, the specific loss of the high affinity form of the muscarinic receptor as discussed above suggested a potential deficiency in coupling to an inhibitory G protein. Secondly, our group has previously reported on a similar finding for the β-receptor, i.e., loss of high affinity β-agonist binding sites (despite an increase in β-adrenergic receptors as detected by antagonist binding) and loss of adenylate cyclase stimulation via both β-adrenergic receptors and sodium fluoride or Gpp(NH)p stimulation (5). Recently, we have confirmed that the level of the GTP stimulatory protein, G_s, is reduced by > 50% in these membranes, whether quantitated by cholera toxin labeling or by reconstitution into S49 cyc-membranes (26). However, in the case of the muscarinic receptor, a parallel decrease in its coupling protein, G_i, was not found. One possibility based on these observations is that some primary modification of the receptor itself, either pre- or posttranslational, has occurred in heart failure, which leads to the loss of that portion of the receptor pool able to bind agonists with high affinity. The mechanism of modification might involve a posttranslational event such as phosphorylation. A second possibility is that, although the density of the GTP-

binding proteins G_i and G_0, which have been shown to couple to the muscarinic receptor, is not reduced in heart failure, their functional activity in coupling the muscarinic receptor to adenylate cyclase may be abnormal. Recent evidence suggests that the population of the stimulatory G protein, G_s, for example, is not homogeneous, but rather represents isoforms generated from alternative splicing of a single RNA transcript (27, 28). It is possible then that a reduction in the activity of the GTP inhibitory proteins might occur if different isoforms of G_i are expressed in heart failure. Recently, highly homologous forms of G_i, likely encoded for by at at least three distinct genes, have been identified (29). Alternatively, posttranslational modification of these proteins could have occurred. For example, G_i alpha can be phosphorylated by protein kinase C. Because this reaction has not been demonstrated to occur in the intact cell (30), its physiological significance, however, remains in question.

We also attempted to directly examine this inhibitory pathway by measuring the ability of Gpp(NH)p to inhibit forskolin-stimulated adenylate cyclase activity, thus bypassing the muscarinic receptor. However, unlike the inhibitory effect reported in other systems including liver and $LLCPK_1$ cells (31), no significant inhibition of forskolin-mediated adenylate cyclase stimulation in sarcolemma is observed over a wide range of Gpp(NH)p concentrations (unpublished data).

To determine if the alterations in muscarinic receptors in heart failure were of functional significance, we compared the extent of muscarinic inhibition of adenylate cyclase activity (9) in normal and failing LV. Depression of methacholine-mediated adenylate cyclase inhibition in the LV sarcolemma from failing hearts is readily apparent from Fig. 6, which shows that at any concentration of methacholine, adenylate cyclase was inhibited by only a fraction of that observed in the normal LV. Statistical evaluation of the rate of change in adenylate cyclase activity with increasing concentrations of methacholine was carried out by regression analysis. The slopes for normal and LV failure were tenfold different, $P < 0.001$. Clearly the decrease in both basal and stimulated adenylate cyclase activity in heart failure makes such a comparison problematic. Nevertheless, even at a 100-fold greater concentration of methacholine, significantly less inhibition of adenylate cyclase activity was observed in LV failure as compared with normal LV. For example, if adenylate cyclase activity is stimulated with 0.3 μM isoproterenol and then inhibited by 100 μM methacholine, the inhibition was significantly less in the failing LV (58±17 pmol/mg per min) than that seen in the normal LV with 1 μM methacholine (210±40 pmol/mg per min). Under such conditions, the absolute number of muscarinic receptors occupied by agonists in the heart failure membranes would be comparable with that in the normal membranes.

In summary, this is the first study to demonstrate (*i*) a reduction in the density of cardiac muscarinic receptors, (*ii*) an alteration in agonist binding, as well as (*iii*) depression of muscarinic inhibition of adenylate cyclase activity, in a chronic animal model of LV failure. While a defect in the muscarinic receptor pathway distal to the receptor may also exist, the levels of G_i were not reduced in failure. However, the decrease in muscarinic receptor density and, in particular, the high affinity agonist-binding component could explain previous observations that parasympathetic control of cardiac function is defective both in animals (4) and in patients (2, 3) with heart failure.

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