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Inefficient Muscarinic Transduction in Cardiomyopathic Syrian Hamsters

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P. CHIDIAC, A. NAGY, M. J. SOLE AND J. W. WELLS. Inefficient Muscarinic Transduction in Cardiomyopathic Syrian Hamsters. *Journal of Molecular and Cellular Cardiology* (1991) **23**, 1255–1269. Regulation of cyclic AMP (cAMP) production and muscarinic binding were studied in highly washed left ventricular membranes from spontaneously cardiomyopathic Syrian hamsters (TO strain). Basal production of cAMP was decreased relative to that in random-bred (RB) controls, with proportionally similar decreases in stimulated production elicited by 7β-deacetyl-7β-(γ-N-methylpiperazino)-butyryl forskolin and by the β-adrenergic agonist isoproterenol. GTP-stimulated production of cAMP was inhibited fully by the muscarinic agonist carbachol in tissue from controls, but only partially in tissue from TO hamsters. Total muscarinic binding, as revealed by N-[^{3}H]methylscopolamine, was similar in the two strains. Competition between carbachol and the radioligand revealed at least three classes of sites for the agonist, the apparent affinities of which were insensitive to the disease. Upon the addition of guanylyl imidodiphosphate (GMP-PNP, 0.1 mM), there was a disease-dependent redistribution such that the sites appeared to be predominantly of low affinity for the agonist in RB tissue and predominantly of medium affinity in TO tissue. The potency of GMP-PNP in mediating the change in carbachol binding apparently was unaffected by the disease. The loss of muscarinic regulation of cAMP production in TO left ventricular tissue appears to reflect a disease-related change in the coupling of muscarinic receptors to inhibitory GTP-binding proteins.

KEY WORDS: Adenylate cyclase; Muscarinic acetylcholine receptors; β-adrenergic receptors; G-proteins; 7β-deacetyl-7β-(γ-N-methylpiperazino)-butyryl forskolin.

[1] *The abbreviations used are*: G protein, GTP-binding protein; G_s, the stimulatory regulatory GTP-binding protein of adenylate cyclase; G_i, the inhibitory regulatory GTP-binding protein of adenylate cyclase; TO, TO strain of cardiomyopathic Syrian hamster, derived from BIO 53.58; RB, random-bred control hamster; buffer A, sucrose (0.32 M), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, 20 mM), EDTA (1.0 mM), bacitracin (0.2 mg/ml), pH 7.4; buffer B, HEPES (5.0 mM) EDTA (1.0 mM), bacitracin (0.2 mg/ml), pH 8.0; buffer C, HEPES (50 mM), NaCl (100 mM), EDTA (1.0 mM), EGTA (1.4 mM), dithiothreitol (1.0 mM), bovine serum albumin (0.1 mg/ml), soybean trypsin inhibitor (16 μg/ml), lima bean trypsin inhibitor (16 μg/ml), leupeptin (9 μg/ml), alamethicin (14 μg/ml), pH 7.45; buffer D, HEPES (50 mM), NaCl (100 mM), $MgSO_4$ (3.0 mM), dithiothreitol (1.0 mM), EDTA (1.0 mM), EGTA (1.4 mM) and bovine serum albumin (0.1 mg/ml), pH 7.45; DMB-forskolin, 7β-deacetyl-7β-(γ-N-methylpiperazino)-butyryl forskolin; cAMP, cyclic adenosine monophosphate; NMS, N-methylscopolamine; GMP-PNP, guanylyl imidodiphosphate.

[2] *The coefficients of eq 4 are defined as follows*:

$$C_4 = a,\ C_3 = a(3f + [R]_t) + b[A]K_p,\ C_2 = a\{3f^2 + [R]_t(2K_p + 3[P]_t)\} + [A]K_p(2bf + e[R]_t) + c[A]^2K_p^2,$$

$$C_2 = a\{3f^2 + [R]_t(2K_p + 3[P]_t)\} + [A]K_p(2bf + e[R]_t) + c[A]^2K_p^2,$$

$$C_1 = a\{f^3 + f[R]_t(K_p + 3[P]_t)\} + [A]K_p\{bf^2 + e[R]_t(K_p + 2[P]_t)\} + [A]^2K_p^2(cf + d[R]_t) + [A]^3K_p^3,$$

$$C_0 = [P]_t[R]_t(af^2 + ef[A]K_p + d[A]^2K_p^2);$$

where $a = K_{A1}K_{A2}K_{A3}$, $b = K_{A1}K_{A2} + K_{A2}K_{A3} + K_{A1}K_{A3}$,

$c = K_{A1} + K_{A2} + K_{A3}$, $d = F_{A1}K_{A1} + F_{A2}K_{A2} + F_{A3}K_{A3}$,

$e = K_{A1}K_{A2}(F_{A1} + F_{A2}) + K_{A2}K_{A3}(F_{A2} + F_{A3}) + K_{A1}K_{A3}(F_{A1} + F_{A3})$, $f = K_p + [P]_t$,

and $F_{Ai} = [R_i]_t/[R]_t$, $[R]_t = \Sigma_{i=1}^n[R_i]_t$, $[R_i]_t = [R_i] + [AR_i] + [PR_i]$,

$[P]_t = [P] + [PR]_t$, $[PR]_t = \Sigma_{i=1}^n[PR_i]$, $\Sigma_{i=1}^n F_{Ai} = 1$

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Introduction

Heart failure is known to be accompanied by changes in autonomic cardiovascular regulation [6, 11, 12, 14], including increased sympathetic tone and decreased parasympathetic control. Those changes are believed in turn to contribute to the progression of the disease [11]. Many reports have documented changes in sympathetic regulation; in contrast, parasympathetic dysfunction has received comparatively little attention, although it is known to occur in human heart failure [6].

Genetically transmitted cardiomyopathy in the Syrian hamster differs from induced models of the disease in that it arises spontaneously; thus, it has been an important paradigm of human disease. The hamster model reveals several biochemical abnormalities that are related to autonomic neurohumoral transduction, including reduced activity of choline acetyltransferase [20], abnormal production and distribution of catecholamines [31], and decreased stimulation of adenylate cyclase by forskolin, sodium fluoride, and β-adrenergic agonists [15, 34]. Irregularities in adenylate cyclase regulation also have been reported in humans [5, 9, 27], in turkeys inbred for congestive cardiomyopathy [32], and in dogs with induced left ventricular failure [34, 35]. It is unknown whether or not defective regulation of adenylate cyclase in the various spontaneous and induced models of cardiomyopathy arises through a common mechanism.

Adenylate cyclase is under the dual control of two G proteins[1]: G_s stimulates the enzyme, and G_i inhibits [2]. In cardiac tissue, G_s and G_i are activated through β-adrenergic and muscarinic receptors, respectively. G proteins therefore may be implicated in one or more of the disease-related defects in autonomic function [14]. Decreases in total myocardial G_s have been reported in patients with congestive heart failure [13], in dogs with induced pressure-overload left ventricular failure [18], and in pigs with volume-overload hypertrophy (reference 45 in 12); increases in G_i have been reported in human heart failure [9, 25]. Other studies have failed to detect changes in cardiac G_s in human heart failure [9, 28], or in G_i in induced canine left ventricular failure [34]. Impaired stimulation of adenylate cyclase thus tends to be accompanied by decreased G_s or by increased G_i [12, 28]; either possibility implies a decrease in the ratio of G_s to G_i [12]. In contrast, a study on pre-necrotic cardiomyopathic hamsters found no change in the level of either G_s or G_i, in spite of a decrease in adenylate cyclase stimulation through G_s [15]. Differences among reports may arise in part from differences among the various models of heart failure. It has been pointed out, however, that there are uncertainties associated with the quantitative measurement of G proteins [14]; moreover, only a fraction of the total G_i or G_s within the membrane may have access to muscarinic or adrenergic receptors, respectively [7, 17]. The relevance of reported changes in quantities of G_s and G_i to the loss of regulation of adenylate cyclase in heart failure thus is unclear.

Although impaired autonomic cardiovascular control in heart failure appears to involve altered G protein-mediated transduction, the molecular basis of the changes is poorly understood. The purpose of this study was to investigate the interrelationships among adenylate cyclase and the proteins that govern its function, particularly G_i, G_s, and the muscarinic receptor, during the late stages of heart failure in the cardiomyopathic Syrian hamster.

Materials and Methods

Chemicals

[^{3}H]NMS (87 Ci/mmol) and [^{3}H]cAMP were purchased from New England Nuclear. [α-^{32}P]ATP was purchased from Amersham. Unlabeled adenyl nucleotides, creatine phosphokinase, muscarinic and adrenergic ligands, and protease inhibitors were purchased from Sigma Chemical Co. Guanyl nucleotides and HEPES were purchased from Boehringer Mannheim. DMB-forskolin was purchased from Calbiochem-Behring Corp, La Jolla, CA. All other chemicals were reagent grade or better.

Animals

Cardiomyopathic Syrian hamsters (TO, a strain originally derived from BIO 53.58) and age-matched random-bred (RB) controls were obtained from breeding colonies at the

University of Toronto or from Bio-Breeders Inc, Fitchburg, MA. Diseased animals were in the late stages of heart failure, as characterized by marked cardiac dilation, hepatic and pulmonary congestion, and subcutaneous edema.

Preparation of tissue

Animals were decapitated, the aortas were clamped, and the hearts were flushed with ice-cold buffer A. Left ventricles and interventricular septa were removed and diced. The tissue then was homogenized (Brinkman Polytron, setting 7 for 11 s) in approximately 15 volumes of buffer A and centrifuged for 15 min at 113 000 g; the resulting pellets were stored at −75°C. *Washing procedure*: Pellets were resuspended in buffer B (Brinkman Polytron, setting 7 for 11 s) and centrifuged for 15 min at 72 000 g; this procedure was repeated three times, with the force of the final centrifugation increased to 131 000 g. The washed pellets were stored at −75°C until assayed for either adenylate cyclase activity or muscarinic binding properties. Protein was assayed according to the procedure of Lowry *et al.* [*19*] using bovine serum albumin as the standard.

Activity of adenylate cyclase

Washed pellets were resuspended (Potter-Elvehjem tissue blender) in buffer C. Enzymic activity was measured according to reported procedures [*26*], with the substitution of HEPES (50 mM) for tris(hydroxymethyl) aminomethane and of magnesium sulfate (3 mM) for magnesium acetate and with the additional inclusion of theophylline (0.1 mM) in the reaction mixture; the final concentration of protein was 1.8 mg/ml. The reaction was allowed to proceed for 10 min at 30°C. Assays of enzymic activity at graded concentrations of carbachol and isoproterenol were conducted in the presence of 0.1 mM GTP; the responses were fully inhibitable by atropine and propranolol, respectively. Assays within each experiment were performed in triplicate, and the results were averaged to obtain the means for subsequent analyses; the standard error of the mean was less than 1.5% for approximately half of the values and less than 2.0% for approximately two thirds of the values.

Binding of muscarinic ligands

Washed membranes were resuspended in buffer D to a final protein concentration of 0.5 mg/ml, and bound [^{3}H]NMS was measured following equilibration of ligands and membranes for 2 hr at 30°C; further details have been described by Wong *et al.* [*36*]. The binding properties of cardiac muscarinic receptors from Syrian hamsters can be unstable in crude homogenates and in preparations of membranes washed without appropriate protection (Green, M. A., Vigor, A., and Wells, J. W., unpublished observations). For example, the time-dependent binding of [^{3}H]NMS achieves a maximum within 30 min and decays thereafter with a half-time that can be increased or decreased by prior washing of the membranes. Various protease inhibitors have been included in the washing buffer and screened for their effect on stability in subsequent binding assays; with membranes prepared as described above, binding is stable for at least 4 hr at 30°C. Crude and washed membranes exhibit the same capacity for [^{3}H]NMS provided that the time of equilibration is short relative to the half-time for eventual loss of binding in the former.

In assays performed at graded concentrations of [^{3}H]NMS, non-specific binding was taken as total binding in the presence of 0.01 mM unlabeled NMS; the value increased linearly with the concentration of radioligand up to at least 7 nM. In assays performed at graded concentrations of carbachol, saturating concentrations of the agonist routinely reduced total binding of the radioligand to levels indistinguishable from those in controls containing 0.01 mM NMS. Binding in the absence and presence of GMP-PNP (0.1 mM) was measured concomitantly, and aliquots of membranes were drawn from the same suspension. Individual measurements of binding within each experiment were made in quadruplicate; the radioactivity of each sample was measured twice, and the results were averaged to obtain means for subsequent analyses. The standard error of the mean was less than 1.0% for approximately

half of the values and less than 2.0% for approximately three quarters of the values.

The cocktail used for the determination of adenylate cyclase activity contained several ingredients which were absent from the buffer used in the binding assays (i.e., buffer D): namely, cAMP, ATP, theophyline, creatine phosphate, creatine phosphokinase, soybean trypsin inhibitor, lima bean trypsin inhibitor, leupeptin, and alamethicin. Control studies indicated that the additional ingredients had no discernible effect on parameters associated with the binding of carbachol (e.g., K_{Ai} and F_{Ai} in eq 4 below).

Analysis of data and statistical procedures

All parameters were estimated by non-linear regression as described previously [36]; values at successive iterations of the fitting procedure were adjusted according to the algorithm of Marquardt [23]. Experiments at graded concentrations of the radioligand were weighted as described previously [36]; for all other measurements, standard errors tended to be a constant percentage of the mean, and the data were weighted accordingly.

Hill coefficients (n_H) were estimated by fitting eq 1 to the data from individual experiments.

$$Y_{obsd} = Y_{[X]\to\infty} + (Y_{[X]=0} - Y_{[X]\to\infty}) \frac{EC_{50}^{n_H}}{EC_{50}^{n_H} + [X]^{n_H}} \quad (1)$$

For measurements of binding at graded concentrations of the radioligand, Y_{obsd} was taken as the difference between total binding in the absence of unlabeled NMS and the fitted estimate of non-specific binding; values were corrected for the difference in depletion of the radioligand between the two measurements [30]. The concentration of unbound radioligand ([X]) was taken as the difference between total radioligand and the corrected estimate of specific binding. For other experiments, the expression was fitted to the data taken as measured, and [X] represents the concentration of the ligand being varied [36]. Individual estimates of n_H from triplicate experiments were averaged to obtain the means (± S.E.M.).

Total binding of [^{3}H]NMS (B_{obsd}) at graded concentrations of the radioligand (P) was analyzed in terms of a single class of sites according to the expression $B_{obsd} = [PR] + NS([P]_t - [PR])$; NS is the fraction of unbound radioligand that appears as non-specific binding, and $[PR]$ is the concentration of radioligand-receptor complex. Specific binding ($[PR]$) was calculated according to eq 2, in which A represents the unlabelled form of NMS; the affinity of A for the receptor is assumed to be indistinguishable from that of P. The appropriate root was evaluated by Newton's method [1]. No statistical advantage was gained by assuming two classes of sites with an expression analogous to eq 2.

$$[PR]^2([P]_t + [A]_t) - [PR][P]_t(K_P + [R]_t + [P]_t + [A]_t) + [R]_t[P]_t^2 = 0 \quad (2)$$

For each strain, estimates of affinity (K_P) and capacity ($[R]_t$) were determined from simultaneous analyses involving four sets of data acquired in the absence of nucleotide and four sets of data acquired in the presence of 0.1 mM GMP-PNP; the nucleotide had no discernible effect on affinity in either strain, and K_P therefore was held common for all sets of data within each strain. Values of $[R]_t$ were assigned separately to individual sets of data. GMP-PNP had no consistent effect on capacity from one experiment to the next; estimates of $[R]_t$ in the presence and absence of the nucleotide therefore were averaged within each experiment, and the numbers presented are the means (± S.E.M.) from four experiments with each strain.

The production of cAMP at graded concentrations of isoproterenol, DMB-forskolin, and carbachol, and the change in muscarinic binding at graded concentrations of GMP-PNP were analyzed in terms of eq 3. The independent variable [X] represents the concentration of the ligand being varied; K_{Si} and F_{Si} (i = 1, 2, or 3) represent the potency of the ligand with respect to process i and the corresponding fraction of the specific signal ($Y_{[X]=0} - Y_{[X]\to\infty}$), respectively. The value of the final term in the expression was set equal to one (i.e., $I_p = 1$ and $I_q = 0$) for all experiments except those with DMB-forskolin. The production of cAMP at graded concentrations of DMB-forskolin showed a reduced response at the highest concentrations of the ligand, and the decrease was described by the final term of eq 3. Inhibition was assumed to be complete (i.e., $Y_{obsd} = Y_{[X]=0}$ as $[X]\to\infty$), and the values

of I_p and I_q therefore were set equal to zero and one, respectively. Under these circumstances, the parameter $Y_{[X]\to\infty}$ represents the maximal production of cAMP expected in the absence of the inhibitory process, and K_F is the concentration of X that achieves half-maximal inhibition of the virtual signal.

$$Y_{obsd} = Y_{[X]\to\infty} + (Y_{[X]=0} - Y_{[X]\to\infty}) \left\{ \sum_{i=1}^{n} \frac{F_{Si}[X]}{[X] + K_{Si}} \right\} \left\{ I_p + \frac{I_q K_F}{[X] + K_F} \right\} \quad (3)$$

Competition between carbachol (A) and [^{3}H]NMS (P) for the receptor (R) was analyzed according to eq 4[2], which depicts a model wherein three classes of non-interacting sites (R_i, i = 1, 2, or 3) are equal in affinity for the radiolabeled antagonist (K_P) but differ in affinity for the agonist. The parameter F_{Ai} represents the fraction of total sites ($[R]_t$) that bind the agonist with the equilibrium dissociation constant K_{Ai} (i.e., $[A][R_i]/[AR_i] = K_{Ai}$). [^{3}H]NMS and carbachol enter the expression as total and free concentration, respectively. Total specific binding of the radioligand was calculated by substituting the value of $[PR]_t$ obtained from eq 4 into the expression $B_{obsd} = [PR]_t + NS([P]_t - [PR]_t)$. The appropriate root was evaluated by Newton's method [*1*].

$$[PR]_t^4 C_4 - [PR]_t^3 C_3 + [PR]_t^2 C_2 - [PR]_t C_1 + C_0 = 0 \quad (4)^2$$

As pointed out previously [*17*], the notion of distinct and non-interconverting sites disregards the effects of guanyl nucleotides and other properties revealed in the binding of muscarinic agonists. Similarly, the origin of the dispersion of data observed for the regulation of adenylate cyclase by carbachol, isoproterenol, and DMB-forskolin is unknown at present. Thus, all descriptions of data reported herein should be considered empirical in nature.

The estimates of affinity (K_A), potency (K_S) and the relative signal (F_A, F_S) were obtained from simultaneous analyses of three sets of data, and the accompanying errors reflect the range over which the sum of squares is insensitive to the value of the parameter. Estimates of the absolute signal (i.e., $Y_{[X]=0}$ and $Y_{[X]\to\infty}$, $[R]_t$ and NS) were unique to individual sets of data, and individual values were averaged to obtain the mean (± S.E.M.) for experiments performed under the same conditions.

Statistical procedures were carried out as described previously [*36*]. Data acquired under each set of conditions initially were fitted to the appropriate equation with a single value of K; the effect of additional parameters on the sum of squares of weighted residuals was tested for significance by using the F-statistic. The F-statistic also was used to test for the effect of common parameters in analyses of pooled data acquired under different conditions and with different strains. Mean parametric values were compared using the t-statistic.

Results

Activity of adenylate cyclase

Throughout the present study, a rectangular hyperbola has been inadequate to describe the regulation of adenylate cyclase, as indicated by Hill coefficients significantly less than one (Table 1). The production of cAMP in RB and TO tissue at graded concentrations of carbachol, isoproterenol, and DMB-forskolin therefore was interpreted in terms of multiple contributions to the total response, as described by eq 3.

Preliminary experiments indicated that the GTP-stimulated, forskolin-stimulated and carbachol-inhibited production of [^{32}P]cAMP increased linearly with time for at least 15 min, with the lines passing through the origin in every case. Moreover, experiments on fresh left ventricular tissue homogenized and assayed in buffer C yielded Hill coefficients less than one for carbachol, with agonist potencies comparable to those observed in washed membranes. The complexity of the response therefore does not appear to be an artifact of the treatment of the membranes, nor can it be attributed to a kinetic anomaly or to the delayed attainment of a steady state.

Muscarinic inhibition of cAMP production was decreased in absolute and in relative terms in TO as compared to RB tissue. In each case, the data are described adequately by assuming two inhibitory components (eq 3, $n = 2$; Fig. 1). Either or both of the lower potency and the relative amount of inhibition associated with each potency could be held common to the data from the two strains without a significant

TABLE 1. Parametric values for the regulation of cAMP production in control (RB) and cardiomyopathic (TO) hamsters.

	DMB-Forskolin		Isoproterenol		Carbachol	
	RB	TO	RB	TO	RB	TO
n_H	0.69 ± 0.01	0.64 ± 0.01	0.88 ± 0.11	0.89 ± 0.04	0.71 ± 0.04	0.60 ± 0.05
$-\log K_{S1}$	5.50 ± 0.05		7.03 ± 0.13		5.96 ± 0.09	6.42 ± 0.16
$-\log K_{S2}$	4.33 ± 0.08		6.31 ± 0.28		4.79 ± 0.09	
$-\log K_{S3}$	2.64 ± 0.59		-		-	
F_{S1}	0.11	0.07	0.68	0.49	0.53	
F_{S2}	0.25 ± 0.27	0.18 ± 0.19	0.32 ± 0.23	0.51 ± 0.25	0.47 ± 0.05	
F_{S3}	0.64 ± 0.34	0.75 ± 0.26	-	-	-	

Hill coefficients (n_H) were determined according to eq 1, and each value is the mean from 3 experiments analyzed independently (± S.E.M.). Other parameters are from eq 3, and the values for each ligand were estimated from a simultaneous analysis of the data from 6 experiments (3 experiments per strain); data from the two strains were assigned common or separate values of K_S and F_S as shown. The value of F_{S3} was fixed at zero for isoproterenol and carbachol.

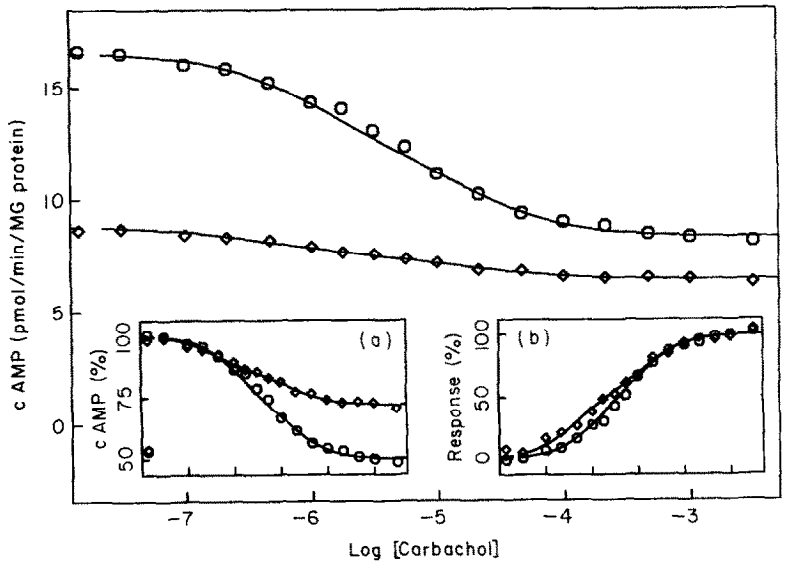

FIGURE 1. Effect of carbachol on the GTP-stimulated production of cAMP (0.1 mM) in RB (○) and TO (◇) washed left ventricular membranes. Experimental procedures are described in Materials and Methods. Data were analyzed according to eq 3, and the parametric values are listed in Table 1. Points represent averages from three separate experiments for each strain. *Insets* The same data are replotted in (a) as percentages of the maximum fitted signal for each strain, and in (b) normalized to the fitted estimates of $Y_{[X]=0}$ and $Y_{[X]\to\infty}$ for individual experiments taken as 0% and 100%, respectively.

increase in the sum of squares ($P>0.13$, Fig. 1); in contrast, the higher potency appeared to differ ($P=0.009$). Three potencies thus are required to describe all of the data in both strains (Table 1). The relative signal associated with the lowest potency is the same in both strains, while the component of higher potency appears to have been transformed by the disease into a state more sensitive to the agonist.

The dose-response data for isoproterenol also require a two-component model with either RB or TO membranes. Either the stimulatory potencies [$P=0.86$; Fig. 2(a)] or the relative fractions of enzymic activity associated with each potency ($P=0.88$) can be

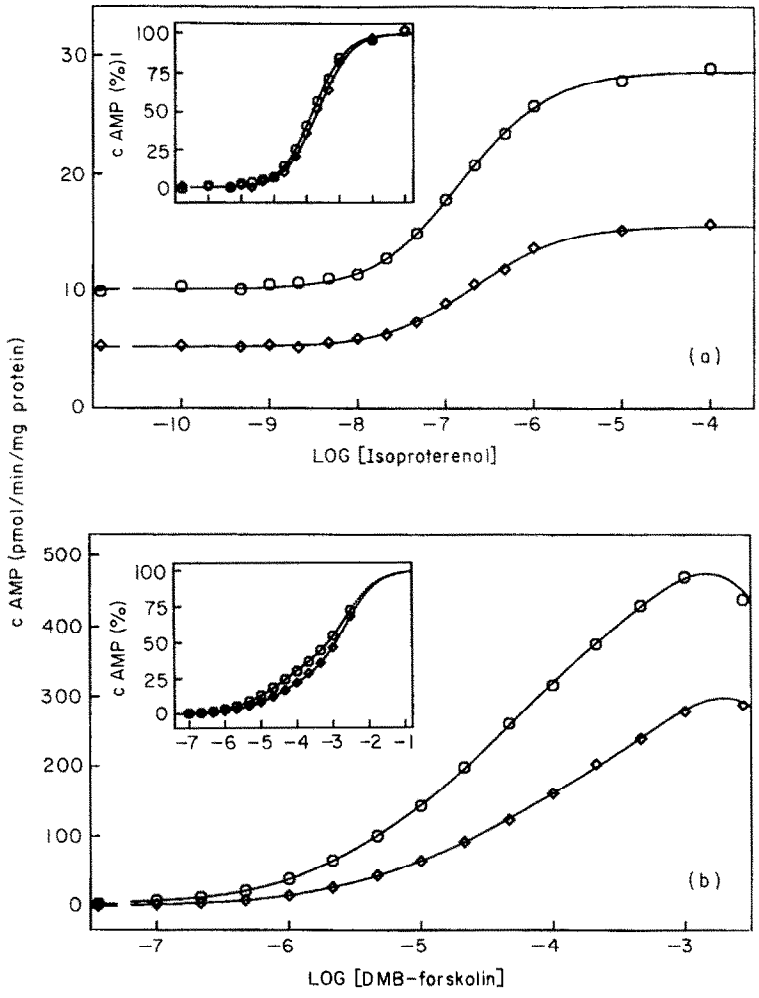

FIGURE 2. Effects of isoproterenol (a) and DMB-forskolin (b) on the production of cAMP in RB (○) and TO (◇) washed left ventricular membranes. Experimental procedures are described in Materials and Methods. Points represent averages from three separate experiments for each strain. (a) Data were analyzed according to eq 3, and the fitted values of K_S and F_S are listed in Table 1. *Inset* Data were normalized taking the fitted estimates of $Y_{[X]=0}$ and $Y_{[X]\to\infty}$ for individual experiments as 0% and 100%, respectively. (b) Data were analyzed according to eq 3 on the assumption that the inhibitory component is common to the two strains ($-\log K_F = 2.39 \pm 0.57$); the values of other parameters are listed in Table 1. *Inset* Data from individual experiments were transformed according to eq. 3 to the values expected in the absence of the inhibitory process; the adjusted values were normalized to the fitted values of $Y_{[X]=0}$ and $Y_{[X]\to\infty}$ taken as 0% and 100%, respectively, and then averaged to obtain the means shown in the figure.

held common between the two strains without a significant increase in the sum of squares. If both the potencies and their relative fractions are held common, an increase occurs ($P<0.02$).

The dose-response data for DMB-forskolin require at least four components for adequate description. For both strains, the data can be described well by assuming three stimulatory processes and one inhibitory process; the inhibition was evident only at high concentrations of DMB-forskolin (≥ 1 mM), and hence is not well characterized. Either the stimulatory potencies [$P = 0.16$; Fig. 2(b)] or the relative fractions of enzymic activity associated with each potency ($P = 0.24$) can be held common

TABLE 2. Regulation of left ventricular cAMP production in control (RB) and cardiomyopathic (TO) hamsters.

Treatment	pmol cAMP/mg protein/min			Proportion of basal rate		
	RB	TP	P	RB	TO	P
Basal	6.35 ± 0.42	3.61 ± 0.09	0.003	1.0	1.0	-
1.0 mM DMB-Forskolin	470 ± 29	279 ± 12	0.004	73.3 ± 4.5	77.2 ± 1.5	0.46
0.1 mM GTP	11.94 ± 0.87	6.73 ± 0.18	0.002	1.88 ± 0.04	1.86 ± 0.01	0.70
0.1 mM GTP + 0.01 mM Isoproterenol	35.60 ± 2.20	17.98 ± 0.56	0.001	5.61 ± 0.16	4.98 ± 0.10	0.03
0.1 mM GTP + 1.0 mM Carbachol	5.91 ± 0.23	5.19 ± 0.20	0.076	0.94 ± 0.04	1.49 ± 0.03	<0.001

Levels of cAMP were measured as described in Materials and Methods; values reported are means (± S.E.M.) of 3 separate experiments done in triplicate. Each experiment consisted of all treatments indicated done on the same day with the same preparation of RB or TO membranes. Values of P were determined according to Student's t-test.

between the two strains; holding both the potencies and their relative fractions common leads to a large increase in the sum of squares ($P<0.0001$). The maximum production of cAMP expected in the absence of the inhibitory process (i.e., $Y_{[X]\to\infty}$ in eq 3) is 1066 ± 7 and 744 ± 33 pmol/min/mg of protein for RB and TO tissue, respectively, for the fit shown in Figure 2(b); this decrease recalls the disease-related decrease in basal activity, as well as that in the maximal response to isoproterenol [Fig. 2(a)].

To compare the various enzymic responses more directly, production of cAMP was measured on the same day and with aliquots from the same preparation of tissue at single concentrations of DMB-forskolin, GTP, isoproterenol, and carbachol. Basal activity of adenylate cyclase and activity in the presence of GTP, isoproterenol, and DMB-forskolin all were reduced significantly in tissue from diseased animals (Table 2). If enzymic stimulation is expressed as a percentage of basal activity, there is little or no difference between the two strains. The loss of stimulation thus appears to arise from an overall loss of enzymic activity. In contrast, muscarinic inhibition of cAMP production in TO tissue was decreased both in absolute and in relative terms. Carbachol inhibited the GTP-stimulated activity completely in RB tissue (108 ± 5%) but only partially in TO tissue (50 ± 3%; $P=0.0005$), indicating that the reduced muscarinic response does not arise solely from the reduced activity of adenylate cyclase.

Muscarinic binding

Hill coefficients for the specific binding of the muscarinic antagonist [^{3}H]NMS were indistinguishable from one under all conditions, and binding was analyzed by assuming a single class of sites (eq 2). Simultaneous analyses of data from four experiments with and without GMP-PNP in each strain indicated a small but significant difference in the affinity of the radioligand between RB ($K_P = 700 \pm 20$ pM) and TO tissue ($K_p = 520 \pm 10$ pM; $P<0.001$). The nucleotide had no discernible effect on affinity or capacity in either strain. Maximal specific binding was similar in the two strains (RB, 136 ± 7 pmol/g of protein; TO, 164 ± 22 pmol/g of protein; $P=0.05$).

The specific binding of [^{3}H]NMS was inhibited by carbachol to yield Hill coefficients less than one under all conditions ($P<0.002$). Preliminary analyses indicated that three classes of sites are required for eq 4 to describe the binding of carbachol in tissue from either strain, either with or without 0.1 mM GMP-PNP, although the fraction of sites exhibiting high affinity for the agonist is poorly defined in RB tissue in the presence of the nucleotide. Parametric values from those fits suggested that disease-related differences in binding (Fig. 3) reflected the relative number of receptors ostensibly in one or another state of affinity for the agonist.

To characterize further the differences in muscarinic binding between TO and RB tissue, simultaneous analyses of all twelve sets

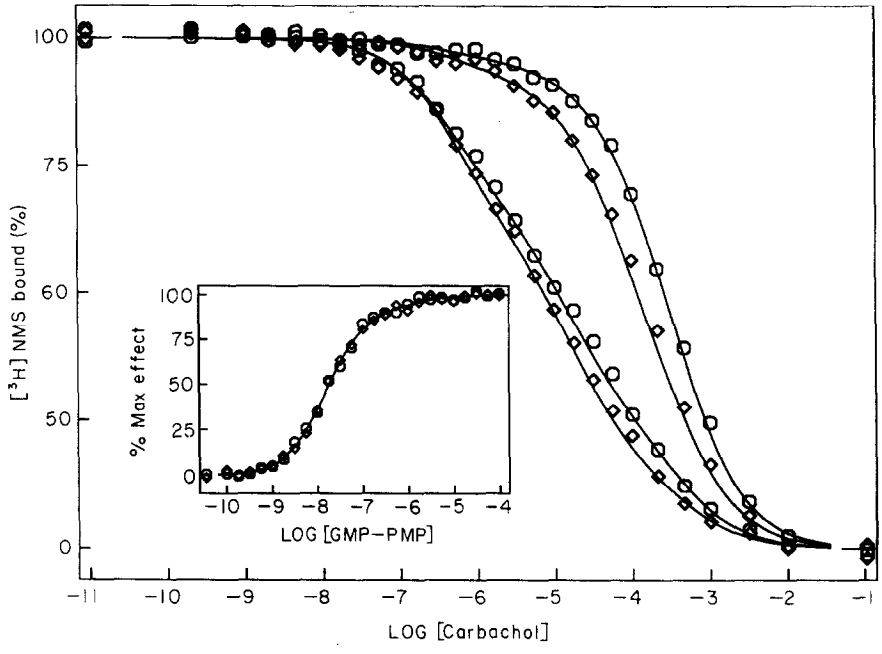

FIGURE 3. Binding of carbachol as measured by competition with [^{3}H]NMS (1.0 nM) for RB (○) and TO (◇) washed left ventricular membranes in the absence of nucleotide (two leftmost curves) and in the presence of GMP-PNP (0.1 mM) (two rightmost curves). Experimental procedures are described in Materials and Methods. Data from individual experiments were normalized to the fitted asymptotes determined by simultaneous analysis with eq 4 taken as 0% and 100%; points are the means for each of the four types of experiment. Parametric values are reported in Table 3. *Inset* Effect of GMP-PNP on the binding of carbachol in RB and TO washed left ventricular membranes. The tissue was incubated with 1 nM [^{3}H]NMS, 10 μM carbachol, and the nucleotide at the concentrations shown on the abscissa. All data were analyzed simultaneously according to eq 3, assuming two potencies per strain. Estimates of the absolute signal were unique to the data from individual experiments; other parameters were common to all of the data from both strains and are as follows: $-\log K_{S1} = 7.81 \pm 0.02$, $-\log K_{S2} = 5.88 \pm 0.30$, $F_{S1} = 0.92 \pm 0.01$, $F_{S2} = 0.08 \pm 0.01$. Values plotted on the ordinate represent the means from three experiments per strain and have been normalized to the fitted estimates of $Y_{[X]=0}$ and $Y_{[X]\to\infty}$ for individual experiments taken as 0% and 100%, respectively; the former thus represents the binding of 1 nM [^{3}H]NMS in the presence of 10 μM carbachol.

of data were performed with various parameters held common among the four experimental groups. Any or all of the values of K_{Ai} can be held common between the two strains without a significant increase in the sum of squares ($P>0.38$). Either or both of the values of K_{A1} and K_{A3} (high and low affinity, respectively) additionally can be held common between data acquired with and without GMP-PNP ($P>0.44$); in contrast, when K_{A2} is held common among all four experimental groups, either alone or in addition to K_{A1} and K_{A3}, the sum of squares is increased ($P<0.05$) relative to that when K_A is held common only between strains. Thus, the binding of carbachol is described adequately by a total of four dissociation constants, all of which are unaffected by the disease; GMP-PNP is without effect on K_{A1} or K_{A3}, but causes a 3.7-fold increase in K_{A2}. The nucleotide also effects a general redistribution of sites from higher affinity to lower affinity (Table 3).

To determine whether or not the relative capacity at any given site (F_{Ai}) was the same for both strains, individual values of F_{Ai} were held common in addition to the four values of K_{Ai}; values of P for the corresponding increases in the sum of squares are shown in Table 3. The disease appears to have little or no effect on the apparent number of receptors of medium affinity for carbachol in the absence of GMP-PNP ($F_{A2(-)}$), or on the number of sites of high affinity (F_{A1}) in either the presence or the absence of the nucleotide; the number of

TABLE 3. Inhibition of the specific binding of [^{3}H]NMS by carbachol.

	Strain		
	RB	TO	P
$-\log K_{A1}$	6.72 ± 0.11		
$-\log K_{A2(-)}$	5.22 ± 0.13		
$-\log K_{A2(+)}$	4.65 ± 0.11		
$-\log K_{A3}$	3.83 ± 0.08		
$F_{A1(-)}$	0.34	0.40	0.04
$F_{A1(+)}$	0.05	0.07	0.40
$F_{A2(-)}$	0.41 ± 0.04	0.45 ± 0.04	0.40
$F_{A2(+)}$	0.14 ± 0.11	0.55 ± 0.10	<0.001
$F_{A3(-)}$	0.26 ± 0.03	0.15 ± 0.03	<0.001
$F_{A3(+)}$	0.81 ± 0.10	0.38 ± 0.10	<0.001

Parameters were estimated simultaneously from a single analysis as described in the text. Each value of P indicates the level of significance (F-statistic) for the increase in the sum of squares of weighted residuals when that value of F_A is held common between strains in addition to the values of log K_A. Subscripts denote the absence (−) and presence (+) of GMP-PNP (0.1 mM).

sites of medium affinity in the presence of GMP-PNP ($F_{A2(+)}$) was increased threefold in the disease, while the number of sites of low affinity (F_{A3}) was decreased both in the presence and in the absence of the nucleotide. The disease thus affects the apparent distribution of muscarinic receptors among the various states of affinity, particularly in the presence of GMP-PNP; whereas the net effect of the nucleotide in RB tissue is an apparent redistribution to the state of low affinity, the state of medium affinity predominates in TO tissue.

The disease was without effect on the dose-dependence observed for the modulation of carbachol binding by GMP-PNP. The data require two components to describe the allosteric decrease in the binding of carbachol as indicated by an increase in the binding of [^{3}H]NMS at an agonist concentration of 10 μM. All values of K_S and F_S could be held common between strains without a significant increase in the sum of squares ($P = 0.89$; inset, Fig. 3).

Discussion

Isoproterenol, carbachol, and DMB-forskolin all regulate adenylate cyclase in a complex manner characterized by multiple inflections in the log dose-response profiles. The interpretation of muscarinic and β-adrenergic regulation of the enzyme is complicated by the involvement in each case of at least three constituents: receptor, G protein, and effector. The behavior observed at steady-state in the dose-response curves may be a manifestation of the same G protein-related phenomena responsible for the dispersion seen at equilibrium in the binding of agonists; alternatively, the patterns may arise from several forms of the enzyme contributing independently to the total signal. The former is suggestive of a more complex linkage than generally is envisaged between receptor and effector, with multiple forms of each receptor contributing to the response.

The complex stimulatory response to DMB-forskolin may arise from intrinsic or induced heterogeneity at the level of the enzyme; for example, it may be partly coupled to G_s or partly phosphorylated [*29*], with different forms contributing independently to the response. The relative inhibition of enzymic activity observed at DMB-forskolin concentrations greater than 1 mM may be attributable to an increase in the K_M of ATP for adenylate cyclase, which reportedly occurs at higher concentrations of forskolin [*29*].

Heart failure is accompanied by proportionally similar decreases in the basal and stimulated activities of adenylate cyclase in membranes from the aged cardiomyopathic hamsters used in the present study. It follows that whatever underlies the decrease in basal activity also may be responsible for the loss of responsiveness to isoproterenol, GTP, and

DMB-forskolin. The underlying cause itself remains unclear, owing in part to the uncertainty associated with total protein as a point of reference. If the changes are restricted to individual myocytes, the loss of activity may reflect a decrease in the amount of enzyme present, in the value of k_{cat}/K_M for the reaction, or both. Changes related to the composition of the plasma membrane are difficult to assess, since hamster cardiomyopathy is known to involve changes in the physical properties of the sarcolemma [*10, 16*] and in the levels of sarcolemmal markers [*10, 16, 21*]. Na^+/K^+-ATPase activity was reported to be decreased by 24% in UM-X7.1 hamsters at 40 days of age, with the decrease becoming greater in older animals [*21*]; in contrast, the activity of 5'-nucleosidase was reported to be increased by 66% in BIO 14.6 hamsters aged between 50 and 70 days [*10*], while a study on 40-day-old 53.58 hamsters found no change in either marker [*16*]. The similarity in the density of muscarinic receptors in TO and RB homogenates in the present study suggests that the proportion of sarcolemmal membrane in the two preparations is about the same, but the agreement may be fortuitous. A disease-related increase in the density of muscarinic receptors on individual myocytes may have been obscured, for example, by fibrosis and non-myocytic proliferation; differences in enzymic activity similarly may reflect different concentrations of myocytes in normal and cardiomyopathic tissue. In any event, changes in the enzymic response to isoproterenol and DMB-forskolin may not arise solely from differences in maximal response, depending upon the origin of the dispersion; slightly higher concentrations of both agents are necessary overall to activate the enzyme (insets, Fig. 2).

The muscarinic inhibition of adenylate cyclase in cardiomyopathic hamsters is compromised in a manner that suggests impaired communication between the receptor and the enzyme. As the capacity for [^{3}H]NMS was not decreased, the defect presumably arises from something other than a lack of receptors. In absolute terms, the loss of muscarinic response arises partly from the decrease in enzymic activity in response to GTP (Fig. 1, outer frame); the muscarinic defect remains evident, however, when inhibition is expressed as a percentage of the production of cAMP in the absence of carbachol [Fig. 1(a)]. Although enzymic activity was reduced by carbachol to similar absolute levels in both strains, the inability of the agonist to reverse fully the effect of GTP in TO tissue indicates a defect that is specifically muscarinic in nature; GTP-stimulated activity in normal tissue was reduced to a level equal to or slightly less than the basal rate, but that in TO membranes was reduced to a level which exceeded the basal rate by approximately 50% (Table 2). Both the stimulation and the inhibition of cardiac adenylate cyclase thus are compromised in TO hamsters. A loss of muscarinic function also has been reported to accompany a disease-related decrease in adenylate cyclase activity in a canine model of induced left ventricular failure [*34*].

The impairment of muscarinic signalling in hamster cardiomyopathy could stem from an altered relationship between G_i on the one hand and the effector, the receptor, or both on the other. In a study on human heart failure, disease-related decreases in both basal and stimulated adenylate cyclase activity were reversed by pertussis toxin, which inactivates G_i [*9*]. That observation was interpreted as an indication that the loss of enzymic activity was due to an increased propensity of G_i to inhibit both the stimulated and the unstimulated forms of the enzyme [*8, 9*]. A change in G_i therefore might account for all or part of the decrease in adenylate cyclase activity and for the loss of muscarinic function observed in the present study, although the mechanistic details remain unclear.

A common view of G protein-mediated transduction is that the G protein or a subunit thereof shuttles randomly between the receptor and adenylate cyclase [*2*]. In that context, disease-related changes in transduction could arise from changes either in the local concentration of G_i or in its ability to interact effectively with other components of the system. The former is difficult to rationalize with the concomitant losses in enzymic activity and muscarinic efficacy, which imply an increase and a decrease, respectively, in available G_i. This discrepancy suggests that an altered form of G_i, which is more capable of inhibiting adenylate cyclase but less able to mediate the signal from the muscarinic

receptor, presides in cardiomyopathy. A change in G_i capable of reducing the levels of both basal and stimulated cyclase activity also might be expected to influence the potency of agents that activate G_s, particularly if the two G proteins or subunits thereof bind to adenylate cyclase in a mutually dependent manner. The sensitivity of the enzyme to isoproterenol is decreased 1.4-fold in the present study ($P<0.02$), but it is unclear whether or not that change is consistent with the 40–50% decrease in the basal and stimulated activities of the enzyme. Such an assessment requires an explicit model based upon a likely mechanism, and the mechanistic events that link G protein-coupled receptors and their effectors remain speculative [*17*].

Disease-related changes in muscarinic binding are most pronounced at the sites of medium and low affinity for carbachol in the presence of GMP-PNP. The apparent redistribution of binding sites in response to the nucleotide was predominantly to the state of low affinity in RB tissue but to the state of medium affinity in TO tissue; cardiomyopathy thus appears to reduce the nucleotide-promoted conversion to the state of lowest affinity. The results of the binding studies therefore concur with the results of the cyclase experiments in suggesting that changes in G_i underlie the reduced muscarinic response. In myopathic tissue, the area between the binding curves in the absence and presence of the nucleotide is decreased by 14%, implying that less energy is available to modulate the behavior of the effector.

The concomitant reduction in muscarinic response and in the sensitivity of agonist binding to GMP-PNP shows an interesting parallel with the known correlation among muscarinic ligands between their inhibitory efficacy with respect to cardiac adenylate cyclase and the magnitude of the rightward shift effected by GTP [*7*]. Such an analogy is consistent with the notion that the efficiency of coupling between the receptor and the G protein is lower in TO tissue than in control tissue. Since the comparisons among agonists in normal tissue are free of any independent change in either the receptor or G_i [*7*], considerations based on the total numbers of either protein may be misleading with regard to the origin of disease-related effects.

Abnormalities associated with the regulation of adenylate cyclase have been studied in various models of heart failure (reviewed in 12). A common observation is that of decreased stimulation in response to β-adrenergic agonists [*5, 12, 27, 32, 33*]. Disease-related decreases in the number of β-adrenergic receptors are observed in most models, although increases also have been reported [*12*]; decreased enzymic stimulation in response to agents such as GTP, GMP-PNP, and NaF have been observed in the majority of studies where direct stimulation of G_s was measured [*12, 32, 33*]. Impaired β-adrenergic responsiveness thus appears to involve defects both at the level of the receptor and at the level of G_s, although the relative contribution of each change to the overall loss of enzymic function may vary from one model of heart failure to the next.

The present results agree with those of previous investigators with regard to the unstimulated production of cAMP. Decreased basal activity of adenylate cyclase previously has been reported to accompany the decreased response to β-adrenergic agonists in spontaneous cardiomyopathy in both humans [*5, 9, 27*] and turkeys [*32*], as well as in a canine model of induced pressure-overload left ventricular failure [*34, 35*]. It follows that changes in G_s may not account entirely for the reduced response of adenylate cyclase to agents such as guanyl nucleotides and NaF, at least in some forms of heart failure.

In a related strain of cardiomyopathic hamster (BIO 14.6), reduced production of cAMP in response to isoproterenol and forskolin has been observed in cardiac tissue before the appearance of pathologic abnormalities [*15*]. With the exception of an apparently normal response to GTP, which is surprising in light of the decreases observed with forskolin and isoproterenol, the results of that study are consistent with our own observations on aged TO hamsters. Those authors found no difference in the absolute levels of either G_s or G_i, and the reduced enzymic response was attributed to a defect in G_s [*15*]. An alternative hypothesis is suggested by the present results and by the observation that pertussis toxin can reverse the disease-related loss of stimulated production of cAMP in humans [*9*]: namely, that the changes in the

autonomic regulation of adenylate cyclase in young cardiomyopathic hamsters [*15*] and possibly in other models of heart disease are related to a change in the nature of G_i.

An increase in the concentration of mRNA encoding the α subunit of G_i (αG_i) has been found in cardiac tissue from humans with chronic congestive heart failure [*8*]; the change was due primarily to an increase in αG_{i3}, one of three known subtypes of αG_i. Although G_{i1}, G_{i2}, and G_{i3} appear to have similar affinities for GTPγS, it has been proposed that their relative amounts are important in determining the kinetics of signal transduction through G_i-coupled effectors [*4*]. Altered ratios of G_i subtypes thus could have an effect on the responsiveness of a cell to extracellular signals [*4*]; such changes might play a role in the muscarinic defect observed in the present study, as the response of adenylate cyclase to carbachol suggests the appearance of a novel regulatory component.

Congestive heart failure is characterized by compensatory responses designed to maintain perfusion pressure [*11*], and the levels of circulating noradrenaline, renin, and vasopressin are abnormally high [*11, 12, 31*]. Chronic stimulation of adrenergic and other receptors helps to maintain intravascular volume, but such activation also may cause further deterioration as a result of sustained vasoconstriction and volume overload [*11, 12*]. Decreased β-adrenergic function in heart failure appears to be related to the prolonged increase in β-adrenergic stimulation; the change may be adaptive insofar as it slows the progression of myopathy, but it also may contribute to the hemodynamic disorder. Decreased muscarinic function may arise as a consequence of hereditary cardiomyopathy; alternatively, it may be an independent adaptation designed to maintain intravascular volume and perfusion pressure, possibly in response to the adrenergic defect. If both defects share a common root, an adaptive change may have been optimized to achieve reduced but relatively stable levels of cAMP, or one defect may be merely a by-product of the other. Finally, it ought to be noted that cardiomyocytes stimulated to hypertrophy by pathologic, as opposed to physiologic, stimuli partially revert to the fetal phenotype for protein expression [*22, 24*]; the relationship of this reversion to autonomic functional changes is not yet known.

Overall, the data from this and other studies indicate that hamster cardiomyopathy is accompanied by reduced activity of adenylate cyclase and a reduction in the efficacy of muscarinic regulation. The muscarinic defect is not mechanistically dependent on the loss of activity *per se*. The loss of activity itself may be independent, or it may reflect the same change that underlies the muscarinic defect. The impaired muscarinic response appears to arise from a change in the nature of G_i and hence in its ability to mediate between the receptor and the enzyme. The reduced production of cAMP is observed both at the level of basal activity and as an attenuated response to stimulatory agents such as isoproterenol, GTP, and DMB-forskolin. Such a general loss of activity may reflect a decrease in the quantity or catalytic efficiency of the enzyme. An alternative or possibly contributing cause is the disease-related change in G_i that seems to underlie the impaired muscarinic response; both the present results and those of Feldman and coworkers [*8, 9*] are consistent with the notion that, in diseased tissue, G_i may be less responsive to muscarinic activation but otherwise more effective at reducing the prevailing activity of adenylate cyclase.

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