Enhanced $\alpha_1$-Adrenergic Responsiveness in Cardiomyopathic Hamster Cardiac Myocytes

Relation to the Expression of Pertussis Toxin–Sensitive G Protein and $\alpha_1$-Adrenergic Receptors

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The pathogenesis of the myopathy occurring in the heart of the cardiomyopathic strain of the Syrian hamster is not well understood but is believed to be associated with abnormal calcium handling by myopathic cells. The purpose of this study was to determine whether the cardiomyopathy occurring in strain BIO 14.6 animals is associated with an enhanced $\alpha_1$-adrenergic receptor–mediated rise in cytosolic calcium, whether a pertussis toxin–sensitive G protein is involved in coupling the $\alpha_1$-adrenergic receptor to changes in intracellular calcium and whether enhanced $\alpha_1$ responsiveness is associated with an increase in the level of expression of the $\alpha_1$-adrenergic receptor or in the pertussis toxin–sensitive G protein or proteins. To test the hypothesis that the cardiomyopathic state is associated with a greater $\alpha_1$-receptor–mediated rise in cytosolic calcium, we studied the effect of phenylephrine (in the presence of propranolol) on time-averaged cytosolic calcium concentration ([Ca$^{2+}$]) in isolated cardiac myocytes from cardiomyopathic and age-matched control hamsters. Phenylephrine caused a greater increase both in time-averaged [Ca$^{2+}$], (an increase of 38.8±8% versus 12.3±3%, $p<0.01$) and in contractility (+181±22% versus +35±9%, $p<0.01$) in cardiomyopathic than in normal cardiac myocytes. Exposure to pertussis toxin (200 ng/ml for 3 hours) attenuated the $\alpha_1$-adrenergic receptor–mediated increase in contractility and time-averaged [Ca$^{2+}$], in both cardiomyopathic and normal cells. The level of pertussis toxin–sensitive G protein, as determined by pertussis toxin–mediated $[^3]$PADP-ribosylation, was 2.6-fold higher in cardiomyopathic versus normal hamster hearts. The density of $\alpha_1$-adrenergic receptors, as measured by the antagonist radioligand $[^3]$H]prazosin and the affinity of the receptor for agonist and antagonist were similar in myopathic and normal heart membranes. Thus, in cardiac myocytes from hamsters, the $\alpha_1$-adrenergic receptor–mediated effects on [Ca$^{2+}$], and contractility appear to be mediated by a pertussis toxin–sensitive G protein or proteins. In myocytes from cardiomyopathic hamsters, these $\alpha_1$-adrenergic effects were increased in magnitude, as was the level of pertussis toxin–sensitive G protein, but there was no measurable alteration in the density or ligand binding properties of $\alpha_1$-adrenergic receptors. (Circulation Research 1990;67:1182–1192)
hanced positive inotropic response to α1-adrenergic receptor stimulation. Myopathic animals, at all stages of the disease including the prenecrotic phase, also exhibit increased intracardiac turnover of norepinephrine and the enzymes of the catecholamine biosynthetic pathway. The density of radioligand binding sites for α- and β-adrenergic receptors has been reported to be increased in the dystrophic myocardium. Finally, chronic administration of the α1-adrenergic antagonist prazosin has been reported to prevent the development of cardiomyopathy.

Stimulation of the myocardial α1-adrenergic receptor leads to an increase in the slow inward Ca2+ current and a rise in cytosolic Ca2+ concentration ([Ca2+]c). Therefore, α1-adrenergic receptor stimulation could contribute to Ca2+ overload, which, in turn, might lead to cardiac myocyte damage.

The purposes of this study were 1) to test the hypothesis that stimulation of α1-adrenergic receptors results in a greater increase in [Ca2+]c in cardiomyocytes from cardiomyopathic compared with normal hamsters, 2) to determine whether a pertussis toxin (PT)-sensitive G protein is involved in coupling the α1-adrenergic receptor to an increase in [Ca2+]c, and a positive inotropic response, and 3) to determine whether the enhanced responsiveness to α1-adrenergic stimulation is associated with an increased level of α1-adrenergic receptors or PT-sensitive G protein.

Materials and Methods

Cardiac Myocyte Isolation

Eight-month-old male BIO 14.6 cardiomyopathic and FIB control hamsters were obtained from Biobreeders, Fitchburg, Mass. Both the cardiomyopathic and control hamsters were maintained under identical conditions on a normal laboratory animal diet with tap water ad libitum.

Normal and cardiomyopathic hamsters were anesthetized with ether, and the hearts were rapidly removed. After cannulation of the aorta, hearts were perfused with oxygenated, warm (37°C) low-calcium Krebs-Henseleit bicarbonate-buffered solution (pH 7.30) containing (mM) NaCl 118, KCl 4.7, CaCl2 0.6, MgSO4 1.20, KH2PO4 1.20, NaHCO3 2.50, and glucose 15. After 8 minutes of perfusion to clear blood from the heart, the same buffer solution was used except that the perfusate was changed to nominally zero calcium. The heart ceased contraction after change to this medium. Then, 0.03% collagenase and 0.015% hyaluronidase were added to this calcium-free medium and recirculated through the heart. Perfusion was continued for 30 minutes, during which time the heart became swollen and pale. The ventricles were removed, cut into small pieces (2 mm3), and placed in a flask containing 0.03% collagenase, 0.015% hyaluronidase, 0.001% trypsin, and 1.0 mM CaCl2 in Krebs-Henseleit physiological solution. The tissue fragments were shaken in an orbital shaking (120 cycles/min) water bath at 37°C for 15 minutes. Tissue pieces were then placed in a calcium-free buffer containing 0.05% collagenase and 0.03% hyaluronidase. The tissue pieces were pipetted up and down 10 times with a 5-ml plastic pipette, the tip of which was cut back so that the diameter of the orifice was approximately 6 mm. Warm (37°C) oxygenated calcium-free buffer was added to the tube, and the cells were centrifuged at 400 rpm for 1 minute. The cells were then washed twice using the same method. Disaggregation of a single heart produced approximately 5 × 106 cells from a cardiomyopathic or healthy hamster. The cells were then suspended in physiological buffer of the desired calcium concentration. At this point, 90–95% of cardiomyopathic cells and greater than 95% of normal cells were rod shaped. After 5 minutes in this buffer, an additional 5% of cells from either preparation became rounded. The percentages of rod-shaped cells remained stable for the subsequent 4 hours.

Measurement of Contractility

For contractility measurements, single adult cardiomyocytes were attached to glass coverslips with collagen (Vitrogen 400, Palo Alto, Calif.) and placed in a specially designed chamber that permitted continuous flow of superfusing medium. The chamber was placed on the stage of an inverted phase-contrast microscope (Diavert, Leitz, Wetzlar, FRG) enclosed in a Lucent box with controlled temperature (37°C). The cells were electrically stimulated at a frequency of 1.5 Hz using a platinum electrode. The pulse was square wave, 4 msec in duration, and 30% above threshold voltage (typically 5 V). Light-dark contrast at the edge of the cell provided a marker for measurement of the amplitude of motion along the axis of shortening. Movement of the cell was monitored using a video motion detector that provided new position data every 16 msec. The signal was relayed to a recorder (Honeywell Inc., Fort Washington, Pa.). Cells chosen for observation always contracted from an attachment point at the center of the cell such that both freely moving ends of the cell shortened with stimulation.

Mean [Ca2+]c Measurement

Time-averaged free [Ca2+]c of single cardiac myocytes from cardiomyopathic and normal control hamsters was measured using the Ca2+-sensitive fluorescent dye fura 2, as previously described. Fura 2 is a fluorescent tetracarboxylate chelator that exhibits a spectral shift in excitation maximum on binding calcium. Cells attached to glass coverslips were incubated with 2 mM fura 2-AM for 12 minutes at 30°C, and then washed for 5 minutes in HEPES-buffered medium to remove extracellular and bound dye. Glass coverslips with attached cells were placed in a perfusion chamber specifically designed to fit the stage of a phase-contrast microscope (Nikon Inc., Garden City, N.Y.) and superfused with oxygenated HEPES-buffered medium at 37°C. The microscope was attached to a Fluorolog 2 instrument (SPEX
Industries, Inc., Edison, N.J.) with excitation wavelengths of 340 and 380 nm and an emission wavelength of 505 nm. The two excitation wavelengths alternated once every second. The emission signals were stored in separate memories of a SPEX DataMate microcomputer.

Cells chosen for \([\text{Ca}^{2+}]_{i}\) measurement were electrically stimulated using platinum electrodes as described above. Contraction that occurred stimulated vision were confirmed before, during, and after each experiment. At the end of each experiment, background autofluorescence from a cell not loaded with fura 2 was subtracted from the original signals. In most cases, the background signal was less than 1% of the fluorescence signal from the fura 2–loaded cell. To calibrate the signals to represent actual \([\text{Ca}^{2+}]_{i}\) values, cells were superfused with HEPES-buffered medium containing ionomycin (3 \(\mu\)M) and digitonin (20 \(\mu\)g/ml) to obtain the maximum fluorescence, and then with \([\text{Ca}^{2+}]_{i}\)-free medium containing 5 mM EGTA to obtain the minimum fluorescence signal. With the equation formulated by Grynkiewicz et al.\(^{13}\) the 340 nm/380 nm fluorescence intensity ratio was transformed into \([\text{Ca}^{2+}]_{i}\) values. The use of the ratio (340 nm/380 nm) minimized problems with variable dye concentration, dye leakage, and cell thickness. Under the conditions of our studies, exposure to 3 \(\mu\)M fura 2 for 10 minutes reduced the amplitude of cell motion by approximately 15%. Washing of cells produced no further decline in cell motion. Any cell in which fura 2 decreased the amplitude of cell motion by more than 15% was not used.

**ADP-Ribosylation in the Presence of Pertussis Toxin**

Homogeneous populations of isolated myocytes from normal or cardiomyopathic hamster hearts were suspended in oxygenated HEPES-buffered medium and incubated at 37°C with or without 200 ng/ml PT at 33°C for 3 hours. Cells were then centrifuged at 3,000 rpm for 10 minutes and resuspended in buffer containing (mM) Tris HCl 50, EDTA 1, sucrose 75, dithiothreitol 1, and NaCl 75. Cells were broken by repeated freezing and thawing and homogenized using a Dounce homogenizer. Homogenates were incubated in the presence of \([^{32}\text{P}]\)NAD and pertussis toxin for \([^{32}\text{P}]\)ADP-ribosylation of PT-sensitive G proteins. The reaction was carried out at 37°C for 40 minutes. The assay mixture contained 10 \(\mu\)M NAD\(^{+}\), 2 \(\mu\)Ci \([^{32}\text{P}]\)NAD, 2.5 mM ATP, 2 mM GTP, 10 mM isoniazide, 10 mM thymidine, 2 mM MgCl\(_2\), 1 \(\mu\)g PT, 10 mM phosphocreatine, 0.84 IU creatine phosphokinase, and tissue homogenate (30 \(\mu\)g proteinreaction assay) in a total volume of 50 \(\mu\)l. The reaction was stopped by the addition of 2% sodium dodecyl sulfate in Laemmli sample buffer followed by boiling for 1 minute. Analysis of protein bands was performed on 11% polyacrylamide gels prepared according to Laemmli.\(^{14}\) Dried gels were exposed to Kodak XAR film with an enhancing screen for 1–2 days at −70°C. Molecular weights were estimated by plotting the distance migrated for standard proteins stained with Coomassie blue versus the logarithm of their molecular weights. Standards included soybean trypsin inhibitor, carbonic anhydrase, egg ovalbumin, bovine serum albumin, and phosphorylase \(b\) with molecular weights of 21,500; 31,000; 42,699; 66,200; and 97,400, respectively. The amount of \([^{32}\text{P}]\)ADP-ribosylation was inversely related to the amount of G protein ADP-ribosylated from endogenous NAD in the intact myocyte.

For comparison of levels of PT-catalyzed \([^{32}\text{P}]\)ADP-ribosylation in homogenates of cardiomyopathic and healthy control hearts, homogenates of both types of hearts were incubated in the presence of the same concentrations of PT and \([^{32}\text{P}]\)NAD\(^{+}\) before electrophoresis and autoradiography. The peaks corresponding to the 40-kDa band obtained by densitometric scanning were cut out and weighed. The weight of the peak from a cardiomyopathic heart was normalized to that of the peak from a control heart.

**\([^{3}\text{H}]\)Prazosin Binding**

\([^{3}\text{H}]\)Prazosin binding was performed on a particulate fraction of myocardium. Briefly, after the animal was anesthetized with ether, the heart was rapidly excised, placed in ice-cold phosphate buffered saline and rinsed free of blood. The atria and great vessels were discarded, and the ventricular tissue was coarsely minced with scissors and homogenized in 0.25 M sucrose in a polytron (Brinkmann Instruments, Inc., Westbury, N.Y.). The homogenate was centrifuged at 1,500g for 10 minutes at 4°C, and the pellet was discarded. The supernatant was centrifuged at 48,000g for 20 minutes at 4°C, and the pellet was resuspended in assay buffer consisting of (mM) Tris HCl 50 and MgCl\(_2\) 10, pH 7.50, at 20°C. The binding assay was then performed, as previously described,\(^{15}\) in a total volume of 1.0 ml containing \([^{3}\text{H}]\)prazosin (0.05–1.0 nM), the particulate fraction (approximately 1 mg/ml), and other agonists or antagonists, as indicated. Incubations (30 minutes at 30°C) were terminated by adding 4.5 ml assay buffer (room temperature) and immediately filtering the mixture through a GF/C glass fiber filter (Whatman Inc., Clifton, N.J.) followed by two more washes with assay buffer (4.5 ml each). Specific binding was defined as the counts displaced by 10 \(\mu\)M phenolamine and was equivalent to specific binding as defined by 1 mM l-norepinephrine. At the \(K_d\) concentration of \([^{3}\text{H}]\)prazosin, specific binding averaged 80±3% of total binding. Saturation binding curves for \([^{3}\text{H}]\)prazosin binding were analyzed by nonlinear curve fitting using the LIGAND program as described by Munson and Rodbard.\(^{16}\)

**Materials**

\(^{4}\text{Ca}^{2+}\) and \([^{3}\text{H}]\)prazosin were obtained from New England Nuclear, Boston. Hyaluronidase, trypsin, propranolol, phenylephrine, and l-norepinephrine were from Sigma Chemical Co., St. Louis. Collage-
nase and deoxyribonuclease were purchased from Worthington Biochemical, Malvern, Pa. PT was purchased from List Biological Laboratories, Inc., Campbell, Calif.

Statistical analysis was performed using Student's two-tailed $t$ test and two-way analysis of variance. Where appropriate, simple comparisons were made by paired $t$ test. For concentration-effect curves, the slopes were determined by log-logit transformation and linear regression analysis. Results are expressed as mean±SD.

**Results**

**Effects of Phenylephrine on Myocyte Contractility**

Electrically stimulated isolated ventricular myocytes contracting at 1.5 Hz and superfused with oxygenated HEPES-buffered physiological medium (pH 7.35, 37°C, 0.6 mM CaCl$_2$) responded to propranolol (1 μM) with a significant decrease in the amplitude of contraction of $-25±3\%$ ($n=15$, $p<0.05$) in cardiomyopathic cells and a similar decrease of $-25±2\%$ in normal cells ($n=15$, $p<0.05$). Phenylephrine (10 μM) in the presence of 1 μM propranolol caused an increase in contractile amplitude in both cardiomyopathic and age-matched normal cardiac myocytes (Figure 1). Propranolol at 1 μM completely inhibited the contractile response to 100 nM isoproterenol, which in the absence of propranolol produced a 70% increase in the amplitude of cell motion, indicating that the observed effect of phenylephrine in the presence of propranolol was mediated via an $\alpha$-adrenergic receptor. Prazosin (1 μM) fully antagonized the 10 μM phenylephrine-induced positive inotropic response, further indicating that the effect of phenylephrine was mediated by $\alpha$-adrenergic receptors of the $\alpha_1$ subtype (Figure 2). In cardiomyopathic cells, the extent of the $\alpha_1$-adrenergic receptor-mediated increase in contractility (+180±22%, $n=15$) was substantially greater than that observed in normal cells (35±9%, $n=15$, $p<0.01$ versus cardiomyopathic cells) (Figure 2). Despite a significantly lower basal amplitude of contraction in cardiomyopathic myocytes (10.1±2.4 μm, $n=15$) than normal myocytes (17.7±3.2 μm, $n=15$, $p<0.01$), the absolute contractile amplitude observed in response to phenylephrine in cardiomyopathic cells (28±3 μm, $n=15$) exceeded that occurring in the presence of the same concentration of phenylephrine in normal myocytes (21±2 μm, $n=15$). The $EC_{50}$ for the phenylephrine-induced positive inotropic responses were similar in cardiomyopathic and normal hamster myocytes (10.6±1.0 μM, $n=15$ for myopathic cells; 9.9±0.8 μM, $n=15$, $p>0.05$ versus myopathic cells).

**Effect of $\alpha_1$-Adrenergic Stimulation on Time-Averaged Cytosolic Calcium Levels**

To determine whether the $\alpha_1$-adrenergic agonist-induced increase in amplitude of cell motion was associated with a change in $[Ca^{2+}]_c$, we examined the effect of phenylephrine (in the presence of 1 μM propranolol) on the time-averaged fluorescence intensity ratio in cells loaded with fura 2 and paced at

![Figure 1](image1.png)  
**Figure 1.** Contractile response to phenylephrine. Isolated ventricular myocytes from normal (NH; top panel) and cardiomyopathic (CM; bottom panel) hamsters were electrically stimulated at 1.5 Hz while superfused with HEPES-buffered medium (pH 7.35, 37°C) containing 0.6 mM calcium. After equilibration, cells were perfused with buffer containing 1 μM dl-propranolol for 2.5 minutes (to steady state) and then switched to medium containing 10 μM phenylephrine and 1 μM dl-propranolol. Changes in the amplitude of cell motion as judged by the distance a point on the cell edge moves along the axis of shortening during contraction and relaxation were monitored. This tracing is typical of five similar experiments.

![Figure 2](image2.png)  
**Figure 2.** Prazosin antagonism of the phenylephrine-induced contractile response. Contractile responses to phenylephrine (10 μM) in cardiomyopathic (●) and normal (○) myocytes, and to phenylephrine plus prazosin (1 μM) in myopathic (▲) and normal (●) myocytes are shown. Cells were pretreated with 1 μM propranolol for 2.5 minutes and then superfused with phenylephrine plus propranolol or phenylephrine and prazosin plus propranolol. The amplitude of cell motion at each time point after exposure to drug as indicated has been normalized to the initial basal amplitude as 100%. Each point represents the mean±SD of 15 experiments, with cells from five normal and five myopathic hearts.
1.5 Hz. Baseline time-averaged [Ca\(^{2+}\)]\(_i\) in normal cardiac myocytes (270±34 nM, n=10) was significantly less than in cardiomyopathic myocytes (470±56 nM, n=10, p<0.01). Propranolol (1 \(\mu\)M) did not cause a measurable change in [Ca\(^{2+}\)]\(_i\) in either cardiomyopathic or normal cells. In normal cells, phenylephrine (10 \(\mu\)M) caused a significant 17.2±3% increase in time-averaged [Ca\(^{2+}\)]\(_i\) (from 270±34 nM to 302±53 nM, n=10, p<0.05 versus basal). However, in cardiomyopathic myocytes, the same concentration of phenylephrine induced a much greater increase in [Ca\(^{2+}\)]\(_i\), from 470±56 to 680±73 nM. This percent increase of +48%±8% (n=10) was significantly greater than that observed in normal myocytes (p<0.01) (Figures 3 and 4). Moreover, phenylephrine caused a gradual rise in [Ca\(^{2+}\)]\(_i\) over a period of 7.8±1.1 minutes (n=10) in normal myocytes (Figure 3), whereas the phenylephrine-induced increase in the myopathic cell was much more rapid (0.9±0.1 minute, n=8, p<0.001). Washout of drug produced a slow decrease in [Ca\(^{2+}\)]\(_i\) over an average of 7 minutes in normal cells. In the cardiomyopathic cells, the [Ca\(^{2+}\)]\(_i\) recovery to control levels appeared to be slightly but not significantly more rapid (with an average of 6 minutes). Prazosin (1 \(\mu\)M) fully antagonized the phenylephrine-induced increase in [Ca\(^{2+}\)]\(_i\) in both groups (data not shown). These data provide further evidence that the physiological responsiveness to \(\alpha\)_1-adrenergic stimulation is greater in cardiomyopathic than in age-matched normal cardiac myocytes.

**Effect of Pertussis Toxin on the \(\alpha\)_1-Adrenergically Stimulated Increase in Contractility and [Ca\(^{2+}\)]\(_i\).**

To examine whether a PT-sensitive G protein is involved in coupling the \(\alpha\)_1-adrenergic receptor to the increase in contractility or [Ca\(^{2+}\)]\(_i\), in both normal and cardiomyopathic hamster cardiac myocytes, we examined the effect of PT (200 ng/ml for 3 hours) on responses to phenylephrine. Exposure of pertussis-intoxicated cells to dl-propranolol (1 \(\mu\)M) produced a statistically significant decrease in amplitude of cell motion in cardiomyopathic myocytes (−23±5%, n=8, p<0.05) as well as in normal controls (−25±3%, n=8, p<0.05). In sharp contrast to the results observed in cells not exposed to PT, exposure of the pertussis-intoxicated cells to phenylephrine (10 \(\mu\)M) did not produce any detectable increase in contractile amplitude in either the cardiomyopathic (Figure 5) or normal myocytes (data not shown).

Incubation of homogenates from cardiac myocytes from both normal and cardiomyopathic hamster hearts in the presence of PT and [\(^{32}\)P]NAD resulted in the incorporation of [\(^{32}\)P]ADP-ribose into a band migrating with an apparent molecular mass of 40 kDa (Figure 6, top panel). In cells treated with PT (200 ng/ml for 3 hours), the extent of ADP-ribosylation of PT-sensitive G protein by endogenous NAD was determined by quantifying the amount of PT-catalyzed [\(^{32}\)P]ADP-ribosylation of unreacted G protein in homogenates of these cells. Homogenates of cardiomyopathic cells preincubated in the presence or absence of 200 ng/ml PT were incubated with PT and [\(^{32}\)P]NAD. The level of PT-catalyzed [\(^{32}\)P]ADP-ribosylation in membrane homogenates of both control and pertussis-intoxicated cells were quantitated by

**Figure 3.** Effect of \(\alpha\)_1-adrenergic agonist exposure on the time-averaged cytosolic calcium level. Typical changes in the fura 2 fluorescence ratio (340 nm/380 nm) converted to [Ca\(^{2+}\)], produced by propranolol (PRO; 1 \(\mu\)M) and propranolol plus phenylephrine (PHE; 10 \(\mu\)M) are shown for normal (NH; top panel) and cardiomyopathic cells (CM; middle panel). Cells loaded with fura 2 (see "Materials and Methods") were superfused with HEPES-buffered medium (pH 7.35, 37°C) and electrically stimulated at 1.5 Hz. When the fluorescence ratio had stabilized (2.5 minutes), myocytes were switched to medium containing 10 \(\mu\)M phenylephrine in the presence of 1 \(\mu\)M dl-propranolol, and changes in the fluorescence ratio were monitored continuously. The bottom panel shows time-averaged [Ca\(^{2+}\)]\(_i\) in a cardiomyopathic cell pretreated with pertussis toxin (PT; 200 ng/ml, 3 hours) and exposed to 10 \(\mu\)M phenylephrine plus 1 \(\mu\)M dl-propranolol after 5 minutes of exposure to dl-propranolol. At the end of each experiment, myocytes were perfused with ionomycin (IONO) and digitonin to allow full equilibration of extracellular and intracellular calcium until the fluorescence ratio reached a maximal value. Then, cells were perfused with calcium-free medium containing 5 mM EGTA until the fluorescence ratio reached a minimum value (middle panel). [Ca\(^{2+}\)]\(_i\) values were then calculated from these fluorescence ratios by using the appropriate equation (see "Materials and Methods"). Representative tracings, similar to 10 replicate experiments, are shown.
laser densitometric scanning of the autoradiogram. These results indicated that in pertussis-intoxicated cardiomyopathic cells, PT-mediated $[^{32}P]$ADP-ribosylation of the 40-kDa protein was nearly absent (Figure 6, bottom panel), indicating that the protein was already ADP-ribosylated by the endogenous NAD$^+$. Similarly, there was virtually no labeling of the 40-kDa band by PT in pertussis-intoxicated normal myocytes (data not shown). Thus, treatment of the myocyte with 200 ng/ml PT at 33°C for 3 hours resulted in virtually complete ADP-ribosylation of sensitive G protein by endogenous NAD$^+$.

Comparison of the Levels of Pertussis Toxin–Induced $[^{32}P]$ADP-Ribosylation in Membrane Homogenates From Hearts of Normal and Cardiomyopathic Hamsters

The level of PT-sensitive G protein in homogenates from normal hearts was compared with that in homogenates from cardiomyopathic hearts. The level of PT-sensitive G protein in hearts of cardiomyopathic hamsters, as assayed by quantitation of the extent of $[^{32}P]$ADP-ribosylated 40-kDa protein, was significantly higher than in hearts of normal hamsters (Figure 6, top panel). The relative levels of PT-sensitive G protein in both normal and cardiomyopathic hearts were quantified by scanning the 40-kDa bands with a laser densitometer (Figure 6, middle panel). The peaks corresponding to the 40-kDa bands were cut out and weighed, demonstrating a 1.6-fold increase (+63±12%, n=5, p<0.01) in the level of PT-sensitive G protein in hearts of cardiomyopathic hamsters compared that of normal hearts.

$\alpha_1$-Adrenergic Receptor Density and Affinity

To determine whether the enhanced $\alpha_1$-adrenergic responsiveness in cardiomyopathic hearts was associated with an increase in $\alpha_1$-adrenergic receptor density or altered agonist binding affinity, $[^{3}H]$prazosin binding and displacement studies were performed using a particulate membrane fraction from normal or cardiomyopathic hearts. $[^{3}H]$Prazosin identified a single high-affinity binding site in preparations from both normal and cardiomyopathic hearts ($K_d$: normal, 0.07±0.03 nM; cardiomyopathic, 0.07±0.02 nM; n=10; p=NS). The density of $\alpha_1$-adrenergic receptors in cardiomyopathic and normal hearts was not different (B$_{max}$: normal, 37±8 fmol/mg protein; cardiomyopathic, 41±6 fmol/mg protein; n=10; p>0.3) (Table 1).

Norepinephrine competition for the $[^{3}H]$prazosin binding site was best fit by a one-site model in both normal and cardiomyopathic heart membranes (Figure 7). The IC$_{50}$ for norepinephrine was similar in normal and cardiomyopathic hearts (normal, 1.07±0.29 μM, n=6; cardiomyopathic, 1.18±0.45 μM, n=6). Norepinephrine binding was not affected by Gpp(NH)p (100 μM) in either normal or cardiomyopathic membrane preparations (Figure 7).

Discussion

Previous studies$^{8,11}$ demonstrated that $\alpha_1$-adrenergic stimulation produced a significantly greater positive inotropic response in electrically driven papillary muscles from cardiomyopathic hamsters compared with age-matched normal hamsters. Because the $\alpha_1$-adrenergically mediated positive inotropic effect in bovine cardiac tissue is reported to be associated with increased calcium entry,$^{10}$ and because calcium overload has been postulated to be related to the pathogenesis of cardiomyopathy in Syrian hamsters,
whether α1-adrenergic stimulation is associated with enhanced contractility in cardiomyopathic hamsters. Because a PT-sensitive G protein appears to couple α1-adrenergic receptors to an increase in contractility in isolated ventricular myocytes (D. Kim, B.T. Liang, and T.W. Smith, unpublished observations, 1988), and because the failing myocardium appears to have an increased level of PT-sensitive G protein,17 we also examined whether a PT-sensitive G protein couples the α1-adrenergic receptor to an increase in contractility and cytosolic calcium level in hamster myocytes. We further tested the hypothesis that enhanced responsiveness to α1-adrenergic stimulation in the cardiomyopathic hamster is associated with an alteration of α1-adrenergic receptor density or affinity, or of the PT-sensitive G protein.

The use of isolated single cardiac myocytes from normal and cardiomyopathic hamsters enabled us to measure [Ca²⁺], and to correlate it with contractile behavior and facilitated the treatment of cells with PT. Our findings demonstrate that α1-adrenergic receptor stimulation causes increases in both contractility and time-averaged [Ca²⁺], that are antagonized by prazosin in isolated hamster myocytes, that the α1-adrenoceptor–mediated increase in contractility and [Ca²⁺] is significantly greater in the cardiomyopathic than in age-matched normal control myocytes, and that this increase in responsiveness to
Table 1. [3H]Prazosin Binding in Membrane Preparation From Cardiomyopathic and Normal Hamster Hearts

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<th>KD (nM)</th>
<th>Bmax (fmol/mg protein)</th>
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<tr>
<td>Control (n=10)</td>
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<tr>
<td>Cardiomyopathic (n=10)</td>
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<td>40.5±6.2</td>
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<td>p</td>
<td>&gt;0.9</td>
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Data are mean±SD.

α1-adrenergic receptor stimulation is associated with an increased level of a 40-kDa PT-sensitive G protein.

Pathologically, the cardiomyopathic hamster heart is characterized by myocytolytic or contraction band necrosis, myocyte hypertrophy, and fibrosis, characteristics similar to those of catecholamine-induced myocardial damage.18,19 Prior studies reported an increased sensitivity of the microvasculature of the cremaster muscles of cardiomyopathic hamsters to topically applied norepinephrine in vivo20 and an enhanced cardiac positive inotropic response to α1-adrenoceptor stimulation.7 Furthermore, the development of features of cardiomyopathy can be prevented by administration of the α1-adrenergic antagonist prazosin to preneocrotic juvenile hamsters.3 These observations have led to the hypothesis that enhanced α1-adrenergic sensitivity may be related etiologically to the genesis of the myocardial lesions observed in the later stages of this genetically determined syndrome.3,7

Our results with the calcium-sensitive dye fura 2 indicate that the extent of the α1-adrenergically mediated increase in [Ca2+]i in cardiac myocytes of cardiomyopathic hamsters was fourfold greater than that in normal myocytes. However, a cause-and-effect relation between cellular calcium overload and the enhanced α1-adrenergic receptor-mediated increase in [Ca2+]i cannot be established from these data. It is of interest that α1-adrenergic stimulation has been reported to cause an increase in the slow inward calcium current,10 establishing another possible link between enhanced α1-adrenergic sensitivity and excess cellular calcium. These data also provide a plausible mechanistic explanation for the beneficial effect of the α1-adrenergic antagonist prazosin in this experimental model.3

In both myopathic and normal myocytes, the increase in [Ca2+]i induced by phenylephrine is smaller relative to the response to isoproterenol at agonist concentrations that produce equal increases in contractile amplitude. These results are consistent with the observation that the magnitude of the increase in slow inward Ca2+ current induced by phenylephrine is smaller than that induced by a concentration of isoproterenol that produces a quantitatively similar positive inotropic effect20,21 and supports the hypothesis that a mechanism other than an increase in slow inward Ca2+ current is involved in the α1-adrenergically mediated positive inotropic response. Of note, Blinks and Endoh22 have found that α1-adrenergic stimulation leads to sensitization of contractile proteins to Ca2+. A β-adrenergic receptor-mediated decrease in contractile element sensitivity to Ca2+ could also play a part.

In this study, the density of α1-adrenergic receptors, as assessed by [3H]prazosin binding, and the affinity of α1-adrenergic receptors for [3H]prazosin and epinephrine are not significantly different in membranes from normal and cardiomyopathic hamsters. These findings differ from those of Karliner et al8 and Kobayashi et al,9 who found increases in the density of α1-adrenergic receptors in cardiomyopathic heart membranes. Kobayashi et al8 found no difference in α1-receptor density in 21-day-old hamsters but a 29% mean increase in 70-day-old animals. Karliner et al8 found a 35% increase in cardiomyopathic hamsters that appear to have been from 6 to greater than 15 month old. Because the age of animals in our study (8 months) was intermediate between the ages of those studied by Karliner et al8 and Kobayashi et al9 per se does not seem to explain the difference in findings. Increased α1-adrenergic responsiveness could also be a reflection of an increase in the affinity of the receptor for agonist. However, the 50% inhibitory concentrations for epinephrine were similar in normal and myopathic membranes. Because the evaluation of α1-adrenergic agonist binding affinity in membranes may fail to detect an alteration that would be evident in intact cells,23 we cannot exclude the possibility that there is an alteration in agonist binding properties.

Because a PT-sensitive G protein was involved in coupling α1-adrenergic receptor occupancy to an increase in contractility (D. Kim, B.T. Liang, and T.W. Smith, unpublished data, 1988) from adult rat cardiac myocytes and phosphatidyl-inositol hydrolysis in neonatal24 but not adult rat cardiac myocytes,25
we postulated that a PT-sensitive G protein might also be involved in coupling the α1-adrenergic receptor to its functional effect in hamster cardiac myocytes and that an alteration in the level of this PT-sensitive G protein (or proteins) might contribute to the enhanced α1-adrenergic responsiveness. PT exposure indeed abolished the ability of phenylephrine to cause an increase in either the contractility or the [Ca2+]i, in both cardiomyopathic and normal myocytes, confirming the important role of G protein in coupling the α1-adrenergic receptor to its functional effects.

To document the completeness of the PT effect, we quantified the level of PT-mediated [32P]ADP ribosylation in membrane homogenates prepared from both control and PT-treated cells. PT-catalyzed [32P]ADP ribosylation was nearly absent, indicating that the α subunit of the G protein was already ADP-ribosylated by endogenous NAD+ in intact myocytes. Thus, PT-sensitive G protein is involved in coupling the α1-adrenergic receptor to an increase in contractility and [Ca2+]i, in both normal and cardiomyopathic myocytes.

The role of a G protein in the coupling of myocardial α1-adrenergic receptors has been suggested by the observation that guanine nucleotides can alter agonist binding affinity, causing a shift from high to low affinity.15,26 However, this effect appears to be small in magnitude when compared with Gt-coupled receptors and has not been found consistently in all studies.27 In the present experiments with hamster myocardium, agonist binding curves are already steep and shifted to the right and model best to a single low-affinity site, reminiscent of the effect of guanine nucleotides. Because we were unable to find any difference between myopathic and nonmyopathic hearts, this indicates that under the specific conditions of this experiment we cannot demonstrate an altered effect of guanine nucleotide on agonist binding. It is conceivable that extensive washing of the tissues might expose a high-affinity agonist binding site. However, the lack of an evident shift in agonist binding affinity in the present study (Figure 7) may reflect a relatively high level of endogenous guanine nucleotide binding.

Next, we examined the possibility that the enhanced responsiveness mediated by the G protein–coupled α1-receptor is associated with, and possibly caused by, an increased level of expression of PT-sensitive G protein. PT mediated the incorporation of [32P]ADP-ribose into a 40-kDa band in heart membrane homogenates from both the cardiomyopathic and the normal control hamster hearts. The expression level of this 40-kDa PT-sensitive protein, as determined by the level of PT-catalyzed [32P]ADP-ribosylation in membrane homogenates, was 1.6-fold higher in cardiomyopathic than in normal control hamster hearts. While the method of PT-catalyzed [32P]ADP-ribosylation does not quantitate directly the absolute level of PT-sensitive proteins in tissues, it provides a measure of the relative levels of sensitive G protein when levels of [32P]ADP-ribosylation of different samples are compared directly in the same [32P]ADP-ribosylation reaction. Previous studies from this laboratory28,29 demonstrated that levels of [32P]ADP-ribosylation of PT-sensitive G proteins in cardiac membrane homogenates did not increase further in the presence of increased concentrations of PT (up to 60 μg/ml in the assay mixture) or increased time of incubation (up to 90 minutes). Furthermore, the extent of [32P]ADP-ribosylation increased linearly as a function of the amount of cell homogenate added to the reaction mixture up to a limit of 70 μg protein. The amount of protein used in the present study was in the linear range of the curve relating the labeling of G protein to the amount of total added protein. Adding the detergents lubrol (0.01%, wt/vol), alamethicin (25 μg/ml), or digitonin (0.01%, wt/vol), while increasing the level of PT-mediated [32P]ADP-ribosylation in both normal and myopathic heart membranes, did not change the percent increase in the level of the G protein in the cardiomyopathic heart (data not shown). However, PT labeling only provides information on the G protein available for ADP-ribosylation, and the level of G protein labeled is probably only a small fraction of the total amount of G protein.30 The present data must be interpreted with caution in this context. The toxin labeling of G protein may also represent primarily a functional property of the G protein. Finally, because multiple isoforms of PT-sensitive G protein are present in the heart (e.g., Gαi2, Gαi3, Gαs, and possibly others), it is possible that the increase in the level of [32P]ADP-ribosylation in the 40-kDa band region represents the appearance of a new Ga species or a selective increase in a particular Ga isoform.

Because PT-sensitive G protein is also coupled to muscarinic cholinergic and A1 adenosine receptors in the heart,31–33 the level of PT-catalyzed [32P]ADP-ribosylation in membrane homogenates would be expected to represent G proteins coupled not only to α1-adrenergic receptors but also to muscarinic and A1 adenosine receptors. Thus, the 1.6-fold increase in the level of PT-sensitive G protein in the cardiomyopathic hamster heart cannot be assumed to represent exclusively an increase in the PT-sensitive G protein coupled to the α1-adrenergic receptor. Conversely, the increase in G protein that is coupled to the α1-adrenergic receptor may be higher than 1.6-fold. Thus, our findings indicate that the increase in α1-adrenergic receptor–mediated functional responsiveness in cardiomyopathic cells is associated, perhaps causally, with an increase in the level of G protein through which the α1-adrenergic physiological response is coupled. Our data are of interest in relation to those of Feldman et al,19 who reported an increased level of a 40-kDa PT-sensitive G protein in failing human myocardium compared with normal controls, indicating that an increased level of PT-sensitive G protein may be common to both genetically determined and acquired forms of heart failure. The present study demonstrates enhanced α1-adren-
ergic responsiveness and increased expression of \( \alpha \)-adrenergic receptor–coupled G protein in cardiomyopathic hearts and is also consistent with the view that \( \alpha \)-adrenergic receptors from failing human hearts are coupled to a GTP-binding protein.\(^{34} \)

The mechanism responsible for increased PT-sensitive G protein levels in cardiomyopathic hamster hearts is not clear. Myocardial norepinephrine levels are increased in the prenecrotic stage of this process, presumably because of some abnormality of neural control or catecholamine metabolism.\(^{3} \)

The demonstrated relationship between cardiac innervation and expression of G in the neonatal rat heart\(^{35} \) supports the possibility that the observed increase in PT-sensitive G protein is in some way related to altered sympathetic tone. We conclude that \( \alpha \)-adrenergic stimulation causes a greater contractile and cytosolic calcium response in isolated cardiac myocytes from cardiomyopathic hamsters than in age-matched normal cells. PT-sensitive G protein is involved in the \( \alpha \)-adrenergic response in both cardiomyopathic and normal cells, and enhanced \( \alpha \) responsiveness in cardiomyopathic myocytes is associated with an increase in PT-sensitive G protein levels, but there is no significant difference in \( \alpha \)-adrenergic receptor number or agonist affinity. The possibility that increased PT-sensitive G protein levels may be involved in the calcium overload that occurs in the cardiomyopathic hamster heart and may play a part in the etiology of myocardial failure deserves further study.

References


KEY WORDS: cardiomyopathic hamster • α₁-adrenergic receptor • pertussis toxin-sensitive G protein • cardiac contractility • intracellular calcium
Enhanced alpha 1-adrenergic responsiveness in cardiomyopathic hamster cardiac myocytes. Relation to the expression of pertussis toxin-sensitive G protein and alpha 1-adrenergic receptors.

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