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Research Article

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Regional Myocardial Downregulation of the Inhibitory Guanosine Triphosphate-binding Protein ($G_i\alpha_2$) and β -Adrenergic Receptors in a Porcine Model of Chronic Episodic Myocardial Ischemia

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Abstract

Regional myocardial ischemia is associated with increased levels of adenosine and norepinephrine, factors that may alter activation of the β -adrenergic receptor (β AR)-G protein-adenylyl cyclase pathway in the heart. We have used the ameroid constrictor model to determine whether alterations in myocardial signal transduction through the β AR-G protein-adenylyl cyclase pathway occur in the setting of chronic episodes of reversible ischemia. Pigs were instrumented with ameroid occluders placed around the left circumflex coronary artery. 5 wk later, after ameroid closure, flow and function were normal in the ischemic bed, but flow ($P = 0.001$) and function ($P < 0.03$) were abnormal when metabolic demands were increased. The ischemic bed showed a reduction in myocardial β AR number ($P < 0.005$). Despite regional downregulation of myocardial β AR number, adenylyl cyclase activity was similar in the ischemic and control beds. Quantitative immunoblotting showed that the cardiac inhibitory GTP-binding protein, $G_i\alpha_2$, was decreased in the ischemic bed ($P = 0.02$). In contrast, the cardiac stimulatory GTP-binding protein, $G_s\alpha$, was increased in endocardial sections from the ischemic bed ($P = 0.05$). Decreased $G_i\alpha_2$ content was associated with decreased inhibition of adenylyl cyclase. Reduced $G_i\alpha_2$ content, in conjunction with increased $G_s\alpha$ content in the endocardium, may provide a means by which adrenergic activation is maintained in the setting of chronic episodic myocardial ischemia. (*J. Clin. Invest.* 1993. 92:2644-2652.) Key words: adenylyl cyclase • G proteins • myocardial ischemia • angina pectoris • adrenergic signaling

Introduction

Recurrent reversible ischemia without infarction characterizes angina pectoris, a syndrome associated with coronary artery disease. The molecular and functional effects of repeated transient bouts of ischemia upon the heart are unknown, and embody the objective of this study. Using an animal model we have examined the effects of chronic ischemia on myocardial adrenergic function. Previous studies have been limited to acute severe ischemia leading to myocardial infarction, and no model appropriate to the specific problems encountered by patients with angina pectoris, in which chronic episodes of isch-

emia occur, has been available. We have used a method that produces gradual but complete coronary artery occlusion, resulting in a region of myocardium with minimal infarction that is perfused by collateral vessels (1, 2). The collateral-dependent region has normal function at rest, but, because of insufficient vascular reserve, has abnormal function when myocardial oxygen demands are increased. Important changes in myocardial β -adrenergic receptor (β AR)¹ expression have been found in animal models after complete coronary artery occlusion leading to myocardial infarction (3-7). In general, β AR upregulation is observed, a sequelae perhaps of externalization of cytosolic receptors previously sequestered by energy-dependent processes. The effects of chronic episodic myocardial ischemia without infarction on myocardial β AR expression have not been elucidated. Alterations in signal transduction in non-infarcted myocardium subjected to repeated bouts of reversible ischemia may reflect adaptive mechanisms of potentially more interest than those associated with acute severe injury and cellular disintegration.

While there is general agreement that β AR number is increased during acute severe ischemia leading to myocardial infarction, there are disagreements regarding changes in adenylyl cyclase activity. Some studies have found increased isoproterenol-stimulated adenylyl cyclase activity after acute severe myocardial ischemia (5), but most find decreased activity. Karliner et al. (6) recently evaluated β AR expression and adenylyl cyclase activity after 1-2 h of coronary occlusion in dogs. β AR-mediated and stimulatory GTP-binding protein (G_s)-mediated cAMP production were 30-50% reduced in the ischemic regions of the left ventricle. These differences in effects of acute ischemia on adenylyl cyclase activity may represent different stages of altered β AR expression. Early changes may include externalization of receptors that are coupled and responsive to agonist stimulation. Continued ischemia and agonist exposure may result in receptor uncoupling, desensitization, and decreased adenylyl cyclase response. In conscious dogs Susanni et al. (7) found that 45-60 min of coronary artery occlusion resulted in increased β AR number but decreased adenylyl cyclase activity in the ischemic zone. There also was decreased cardiac G_s in the ischemic region, a possible factor in the mechanism of decreased adenylyl cyclase activity. The effects of chronic episodic ischemia without infarction or heart failure upon β AR expression, G protein quantity and function, and adenylyl cyclase activity have not previously been addressed.

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1. Abbreviations used in this paper: β AR, β -adrenergic receptor; G_i , inhibitory GTP-binding protein; G_s , stimulatory GTP-binding protein; ICYP, [¹²⁵I]iodocyanopindolol; LAD, left anterior descending; LCx, left circumflex; QNB, [³H]quinclidinyl benzyllate.

It is reasonable to expect that repeated bouts of myocardial ischemia may ultimately lead to important alterations in signaling pathways in the heart. For example, norepinephrine concentrations increase in regions of myocardium that are ischemic, primarily due to impaired uptake and clearance of norepinephrine, a process that depends upon adequate ATP stores (8). It can be surmised that myocytes in the region of ischemia may be subjected to transient high concentrations of norepinephrine. Adenosine is also released in high concentrations from ischemic myocytes (9). These two compounds increase during myocardial ischemia and presumably then interact with β ARs and A_1 -adenosine receptors. These receptors are coupled with effectors through GTP-binding proteins: G_s links β ARs with adenylyl cyclase, and the inhibitory GTP-binding protein (G_i) links the A_1 -adenosine receptor with effectors that include adenylyl cyclase (10). Previous studies from our laboratory and others have shown that transient or sustained agonist stimulation with norepinephrine or adenosine analogues can result in β AR downregulation, alterations in G_s , and downregulation of G_i (11–13). Since β AR expression, G protein function, and adenylyl cyclase activity are important determinants of cardiac function, determining the effects of chronic bouts of regional myocardial ischemia, like that observed in patients with angina pectoris, is of clinical importance.

We therefore tested the hypothesis that chronic episodic ischemia would be associated with decreased myocardial β AR number and decreased cardiac G_i content in ischemic regions compared with regions of myocardium not susceptible to ischemia. The ameroid constrictor model is well suited to address these questions, since each animal serves as its own control, with a normally perfused and normally functioning region (the left anterior descending [LAD] coronary artery bed) adjacent to an inadequately perfused and abnormally functioning region (the left circumflex [LCx] coronary artery bed [1, 2]). The model thereby provides the ability to resolve changes in biochemical and molecular measurements that might not otherwise be detected due to variability between animals.

Our data indicate β AR downregulation but preservation of adenylyl cyclase activity in the region of myocardium subjected to chronic episodic ischemia. In addition, in the ischemic region, $G_{i\alpha_2}$ is reduced, and, in endocardium, $G_{s\alpha}$ is increased. These adaptations may provide an important mechanism by which β AR-stimulated adenylyl cyclase activity is preserved.

Methods

Animals. Experimental animals were seven sexually mature, (6 mo old, 36 ± 7 kg) Yucatan minipigs (*sus scrofa*). Four additional animals (22 ± 3 kg) were used as controls to determine whether regional differences in G proteins occur normally between the LAD and LCx beds. All animals were obtained from the same vendor and handled in an identical manner. The protocol was in accordance with National Institutes of Health guidelines for the use of animals in research, and was approved by the Animal Use Committee at the Veteran's Affairs Medical Center, San Diego and University of California, San Diego.

Surgical procedure. Initial acclimatization (8 d) was performed so that the animals would remain relaxed for 1 h in a sling (duration of hemodynamic data collection), and perform brief treadmill walking. After acclimatization, animals received ketamine (33 mg/kg, i.m.) and atropine sulfate (0.1 mg/kg, i.m.) followed by thiamylal sodium (100 mg/kg, i.v.). Animals underwent endotracheal intubation and halo-

thane (0.5–1.5%) was delivered by a pressure-cycled ventilator for the duration of the surgical procedure. A thoracotomy was performed for instrumentation and an ameroid was placed around the proximal LCx. Catheters were placed in the left atrium and aorta, providing a means to measure blood flow (microsphere technique), and to monitor arterial blood pressure. A Konigsberg high-fidelity pressure manometer was placed in the left ventricular cavity through an apical incision. Wires were sutured on the heart to permit electrocardiogram (ECG) recording and atrial pacing. Ultrasonic micrometers measuring transmural thickness were placed in the LCx (ischemic) bed and in the LAD (control) bed. Appropriate placement of the crystals was determined during thoracotomy by occluding the artery (LCx or LAD) and confirming that wall thickening decreased. The ultrasonic crystal micrometers provided data regarding regional function in two areas of the left ventricle.

Evaluation of myocardial blood flow and function. Every 7 d after surgery, hemodynamic and regional functional data were collected so that the progression of ischemia could be followed at rest and during treadmill running. Treadmill grade and speed were increased gradually so that heart rate reached 220 beats per minute (bpm) and remained relatively steady. Data recorded included: ECG, wall thickening (in both ischemic and control regions), and phasic and mean pressures from the left atrium and aorta. A 30-s collection in the basal state was followed by a 30-s collection during exercise at a heart rate of 220 bpm. Radioactive microspheres were injected at rest and during moderate treadmill exercise to establish transmural myocardial blood flow deficits in the ischemic bed (1, 2, 14). Data analyses were conducted upon the mean values obtained from 10 continuous cardiac cycles of the acquired data. Data selection was made based upon the integrity of the signal and absence of motion artifact. Signals from the sonomicrometer signals were calibrated, amplified, and converted to distance (in mm) as previously described (1, 2). End-diastole was defined as the point of initial positive dP/dt , which was obtained by differentiating the high-fidelity left ventricular pressure waveform. End-systole was taken as the point 20 ms before peak negative dP/dt . Percent wall thickening (%WTh) was calculated as:

$$\frac{\text{end-systolic wall thickness (mm)} - \text{end-diastolic wall thickness (mm)}}{\text{end-diastolic wall thickness (mm)}} \times 100.$$

Myocardial blood flow was determined by the radioactive microsphere technique as previously reported (1, 2, 14). Transmural samples from the relevant beds were divided into inner (endocardial), outer (epicardial), and middle thirds, and blood flow to each third as well as transmural flow were determined. Transmural sections were taken in areas adjacent to the sonomicrometer crystals, thereby making certain that blood flow measurements would be from an area of myocardium that would correlate with the functional measurements in each bed.

Terminal surgery. 36 ± 7 d after ameroid placement animals were anesthetized, intubated, and midline sternotomies made. Animals were killed 48 h after the last episode of exercise-induced ischemia to assure that changes in β AR expression and G protein function were not simply the result of acute ischemia. The heart was removed, rinsed in saline (4°C), and the coronary arteries were rapidly perfused with saline (4°C). The LCx was identified, and the ameroid was located and examined to confirm complete closure. The sonomicrometer crystals were identified and their appropriate position confirmed. Transmural myocardial samples were obtained adjacent to the crystal micrometers in the ischemic and control beds, taking great care to avoid areas of adhesion or fibrosis. Transmural samples for microsphere counting were obtained adjacent to the samples obtained for biochemical studies, and it was subsequently documented that the ischemic bed was underperfused during treadmill exercise. Myocardial samples were then frozen (-80°C). Time from heart removal to cold perfusion was < 5 min; samples were placed in liquid nitrogen within 15 min of extraction of the heart.

Assessment of myocardial infarction. The extent of myocardial infarction in the LCx region was assessed by histological morphometric analyses conducted on transmural sections from two areas of the LCx bed in each of five animals. Samples were formalin fixed and stained with Masson's trichrome. 5- μ m sections were assessed for fibrosis and area of fibrosis was calculated with a computer (9825B; Hewlett-Packard Co., Palo Alto, CA) as previously reported (1). The arithmetic mean of the amount of infarction from all sections within the LCx region was then determined. A total of 30 sections underwent quantitative morphometric assessment (endocardial, midwall, and endocardial sections from each of two transmural sections obtained from each of five animals).

Membrane preparation. Frozen (-80°C) transmural samples were powdered in a stainless steel mortar and pestle (also -80°C), placed in Tris buffer, glass-glass homogenized, and contractile proteins were extracted (0.5 M KCl; 20 min, 4°C). The pellet of a 45,000 g centrifugation was resuspended in buffer, and radioligand binding experiments, adenylyl cyclase studies, and G protein studies were performed.

Radioligand binding studies. β -Adrenergic receptors were identified using the radioligand [^{125}I]iodocyanopindolol (ICYP; 5–700 pM) in experiments conducted on crude membrane preparations as previously described (11, 15). Muscarinic cholinergic receptors were identified using the radioligand [^3H]quinuclidinyl benzylate (QNB; 2–200 pM) in a final volume of 3.0 ml as previously described (15). Determinations of the K_i for isoproterenol and the proportion of β ARs displaying high or low affinity binding were performed in competition binding experiments by incubating 100 pM ICYP with 10^{-10} to 10^{-4} M (–)isoproterenol in the absence of guanine nucleotide as previously reported (11). Protein concentrations were determined by the method of Bradford (16), and assessment of membrane protein yield per milligram crude membrane homogenate was performed using the cardiac sarcolemmal membrane marker, K^+ -stimulated *p*-nitrophenylphosphatase (K^+ -pNPPase) following the method of Bers (17). Previous experiments have established that receptors are not lost to the supernatant in our membrane preparations and that receptor number is unaffected by thoracotomy alone (11, 15, 18). We have previously documented that β AR numbers in the LCx and LAD beds are similar in control animals (our unpublished data).

Adenylyl cyclase assays. Methods were modified from Salomon (19) as previously reported (11, 18). The following agents were used to stimulate cAMP production (final concentrations): isoproterenol (10 μM), Gpp(NH)p (100 μM), AIF (10 mM), and forskolin (100 μM). We found that cAMP production under these conditions was linear with respect to time and protein concentration, and that 3-isobutyl, 2-methylxanthine (1.0 mM), adenosine deaminase (5 U/ml), or both had no effect on basal or maximally stimulated cAMP production. Previous experiments established that adenylyl cyclase activity does not distribute to the supernatant of a 45,000-g centrifugation in our membrane preparation, and that thoracotomy alone does not influence adenylyl cyclase activity (11).

Construction of fusion protein expression vectors. $\text{Gi}\alpha_2$ cDNA was digested by BamHI and EcoRI. The resultant 960-bp fragment was purified from agarose gel, then ligated with BamHI/EcoRI-digested pGEX-3X using T4 DNA polymerase. A partial cDNA fragment of $\text{Gs}\alpha$ was obtained by PCR using two gene-specific oligonucleotides, Gs5P₁ (5'-GACGGGATCCCACTACTGCTACCCCTCA-3') and Gs3P₁ (5'-TGGGAATTCCTTCTTAGAGCAGCTC-3'), as PCR primers. Each oligonucleotide was used in the PCR at 25 pmol with 5 ng of $\text{Gs}\alpha$ cDNA in a 100- μ l reaction volume. Thermus aquaticus (Taq) DNA polymerase was purchased from Cetus Corp. (Emeryville, CA). The amplified DNA fragment was digested by BamHI and EcoRI, then ligated with BamHI/EcoRI-digested pGEX-3X using T4 DNA ligase.

Expression and purification of fusion proteins. The constructs described above (pGEX- $\text{Gi}\alpha_2$ and pGEX-GsF7) were used to transform competent *Escherichia coli* strain NM522 (Stratagene, La Jolla, CA). The transformed bacteria were inoculated in 1 liter of LB ampicillin medium, then expression of fusion proteins (pGEX- $\text{Gi}\alpha_2$, 42.5 kD; and pGEX-GsF7, 31 kD) was induced by the addition of 0.1 μM iso-

propyl β -D-thiogalactosidase (IPTG). After a 3-h induction at 25°C , bacteria were harvested, then lysed by sonication (twice for 30 s). All subsequent purification procedures were performed at 4°C . Lysates were applied on 2 ml column of glutathion sepharose (Pharmacia LKB, Piscataway, NJ), then washed thoroughly with PBS. Adsorbed protein then was eluted from the resin with 14 aliquots of 10 mM Tris-HCl buffer (pH 9) containing 1 mM EDTA and 50 mM reduced glutathion (1 ml/aliquot). 5 μ l from each fraction was subjected to 10% SDS-PAGE to monitor the protein elution profile. The peak fractions were combined and dialyzed against PBS, then concentrated using an ultrafiltration unit. Protein concentration was determined by amido-black staining. Purity of GST- $\text{Gi}\alpha_2$ was estimated to be nearly 100%; purity of GST-GsF7 was estimated to be 43% by densitometric scanning of final products of SDS-PAGE.

Quantification of $\text{Gs}\alpha$ and $\text{Gi}\alpha_2$ by immunoblotting. Assessment of cardiac α s and α i subunits of Gs and Gi, respectively, was conducted using standard SDS-PAGE and immunoblotting techniques as previously described (20, 21). Briefly, 100 μg of protein from each supernatant and pellet fraction of a 45,000-g centrifugation of crude myocardial homogenate derived from appropriate endocardial and epicardial samples was subjected to electrophoresis on a 10% denaturing gel for 4 h at 30 mA. For quantification of cardiac α subunits, glutathion-S-transferase, 26.5 kD (GST)- $\text{Gs}\alpha$ subunit fusion proteins (GST-GsF7 and GST- $\text{Gi}\alpha_2$; 31 and 42.5 kD) were purified and quantitated (22), then used at four different dilutions per gel for standard curve generation. Low molecular mass standards were also included on each gel. Proteins were electroblotted onto nitrocellulose membranes (Amersham, Amersham UK) for 14 h, at 70 V, 4°C (23). Transfer efficiency was recorded by photocopies of membranes dyed with reversible Ponceau staining, and gel retention was checked with Coomassie blue staining. Background blocking was accomplished by incubating membranes in Tris-buffered saline (TBS; pH 7.5) with 2% nonfat dry milk, for 2 h, 25°C . Purified primary antibodies (NEN, Boston, MA) (rabbit anti-G proteins: RM/1 $\text{Gs}\alpha$; AS/7, transducin, $\text{Gi}\alpha_1$, $\text{Gi}\alpha_2$) were diluted 1:600 in 15 ml of TBS with 0.05% Tween-20 (TTBS, pH 7.5) and 1% nonfat dry milk, and membranes were incubated for 14 h at 4°C (24). Autoradiographic detection of bands was performed by incubating membranes in 75 ml TTBS with 1% nonfat dry milk and 15×10^6 cpm ^{125}I -protein A (NEN) for 2 h at 25°C followed by thorough sequential washes in TTBS, and placing against x-ray film (Kodak X-OMAT AR) for 5 d (-70°C). The 45- and 31-kD bands for $\text{Gs}\alpha$ and GST-GsF7, respectively, were removed from the membranes with background controls for gamma counting. Likewise, the 39- and 42.5-kD bands for $\text{Gi}\alpha_2$ and GST- $\text{Gi}\alpha_2$ were removed and counted. Using the specific activity of the GST fusion protein standards and the specific activity of the sample bands of interest, the tissue content of G protein was calculated (21). $\text{Gs}\alpha$ and $\text{Gi}\alpha_2$ cDNA were provided by Dr. A. Gilman (Texas Health Sciences Center, Dallas, TX) and Dr. Kaziro (Tokyo University, Tokyo, Japan). The fusion protein vector, pGEX3X, was obtained from Pharmacia LKB.

Reconstitution assay for Gs. We modified a reconstitution assay using cyc-lymphoma cells (which lack Gs) for use with porcine myocardial membranes (25) as previously described (11, 18). Reconstitution assays were performed on undiluted 1% cholate extract and several serial dilutions, giving a wide range of protein content. We have found that intrinsic adenylyl cyclase activity in extract and cyc-membranes is negligible, and that NaF stimulation of cyc-membranes yields no cAMP production. In preliminary studies we found cAMP production to be proportional to the amount of extract added (from 1–120 μg), and the rate of cAMP synthesis is linear with time for 40 min. We performed assays on extracts from experimental animals side by side with appropriate control extracts, using the same batch of cyc-membranes in order to minimize the potential confounding influence of variation in catalytic subunit concentration in cyc-membranes. Data are expressed as picomoles cAMP produced per 10 min as a function of membrane protein used.

Oxotremorine inhibition of adenylyl cyclase. To determine whether the apparent decrease in $\text{Gi}\alpha_2$ had a functional biochemical correlate,

we examined the ability of oxotremorine to inhibit adenylyl cyclase (26). Assays were conducted as described for determination of adenylyl cyclase activity, using 10 μM isoproterenol and 100 μM GTP (together) as a stimulant. Then, progressively increased concentrations of oxotremorine were added to the reaction vessel (10^{-9} to 10^{-4} M), and cAMP production was measured.

Statistics. Data are expressed as mean \pm 1 SEM. Specific measurements were compared between the ischemic (LCx) and control (LAD) beds in each animal using Student's *t* test for paired data. Dose-response data were analyzed by one-way analysis of variance. The null hypothesis was considered unlikely when $P < 0.10$, and rejected when $P < 0.05$ (two tailed).

Results

Validation of the model. Fig. 1 confirms that the model represents myocardial ischemia. Endocardial blood flow in the two beds was not different in the basal state (control, 1.0 ± 0.2 ml/min per g; ischemia, 0.8 ± 0.2 ml/min per g; $P = 0.23$). However, during moderate treadmill exercise, endocardial blood flow increased in the control bed (basal, 1.0 ± 0.2 ; exercise, 2.1 ± 0.3 ; $P < 0.002$), but did not change in the ischemic bed (basal, 0.8 ± 0.2 ; exercise, 1.2 ± 0.2 ; $P = 0.21$). Endocardial blood flow was greater in the control bed than in the ischemic bed during exercise (control, 2.1 ± 0.3 ml/min per g; ischemia, 1.2 ± 0.2 ml/min per g; $P < 0.001$). Percent wall thickening did not change in the control bed during moderate treadmill exercise (basal, $38 \pm 6\%$; exercise, $43 \pm 3\%$; $P = 0.48$). In contrast, function in the ischemic bed decreased significantly from rest to moderate exercise (basal, $27 \pm 5\%$; exercise, $19 \pm 4\%$; $P < 0.03$). Thus, side by side in each animal there is a normally perfused and normally functioning bed adjacent to an abnormally perfused and abnormally functioning bed. The end-diastolic wall thickness in the LCx bed was similar at initial placement of the crystals and at the end of the study (initial, 10.7 ± 0.5 mm; final, 10.9 ± 0.8 mm), confirming that no significant wall thinning occurred during the course of the study, providing evidence against infarction. Quantitative microscopic analyses found minimal fibrosis in the ischemic bed of

the animals studied (*vide infra*). These data establish that the model represents episodic ischemia associated with regional contractile dysfunction without significant infarction.

Assessment of myocardial infarction. Quantitative morphometric histological analyses conducted on multiple sections from the LCx bed showed that a minimal degree of fibrosis was present ($4 \pm 1\%$). This represents $< 1\%$ infarction of the left ventricle, and confirms prior reports using these same techniques in pigs (1).

Radioligand binding studies. Fig. 2 shows the results of radioligand binding experiments with ICYP performed in six animals. Data shown were obtained from a mean of three saturation isotherms/tissue per animal, performed with triplicate points for each of eight concentrations of ICYP. βAR number was decreased in the ischemic bed in all animals. The extent of βAR downregulation was 29% (control, 74 ± 9 fmol/mg; ischemia, 57 ± 9 fmol/mg; $P < 0.005$). K_d for ICYP was decreased (that is, the affinity of the radioligand for the βAR was increased) in membranes from the ischemic bed (control, 68 ± 17 pM; ischemia, 45 ± 7 pM; $P = 0.01$). Muscarinic cholinergic receptor number was similar in both beds (control, 55 ± 5 fmol/mg; ischemia, 61 ± 8 fmol/mg; $P = 0.63$). The affinity of receptors for QNB was also similar (control, 95 ± 28 pM; ischemia, 121 ± 13 pM; $P = 0.24$). Mean r^2 values for the Scatchard analyses were 0.92 ± 0.09 .

The proportion of receptors showing high affinity binding for isoproterenol, examined in five animals, was somewhat decreased in the ischemic bed (control, $80 \pm 12\%$; ischemia, $42 \pm 17\%$; $P < 0.06$). Receptors displaying high affinity binding had a similar K_i for isoproterenol in both beds (control, 22 ± 17 nM; ischemia, 29 ± 8 nM). In contrast, receptors displaying low affinity binding had an increased K_i (a lower affinity for isoproterenol) in the ischemic bed (control, 1 ± 1 μM ; ischemia, 15 ± 8 μM ; $P = 0.03$). Thus, the ischemic bed tended to have fewer coupled receptors; uncoupled receptors had reduced affinity for isoproterenol (Fig. 3). Therefore, based on these data, one would predict reduced adenylyl cyclase stimulation in the ischemic bed. Instead, we found preserved adenylyl cyclase activ-

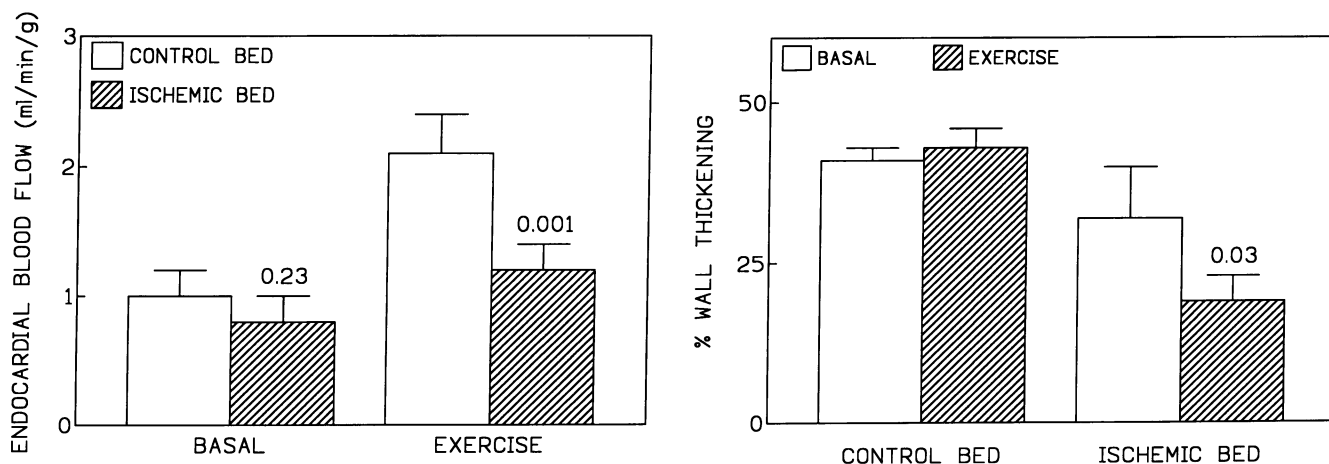


Figure 1. Endocardial blood flow and transmural wall thickening before (heart rate = 95 ± 7 bpm) and during moderate intensity exercise (heart rate = 220 ± 5 bpm). (Left) Endocardial blood flow in the two beds was not different in the basal state but was greater in the control bed than in the ischemic bed during exercise. While flow increased substantially during exercise in the control bed ($P < 0.002$), flow did not increase in the ischemic bed ($P = 0.21$). (Right) During exercise, transmural wall thickening remained constant in the control bed, but decreased in the ischemic bed. Data were obtained from six animals. Bars represent mean values; error bars denote 1 SEM.

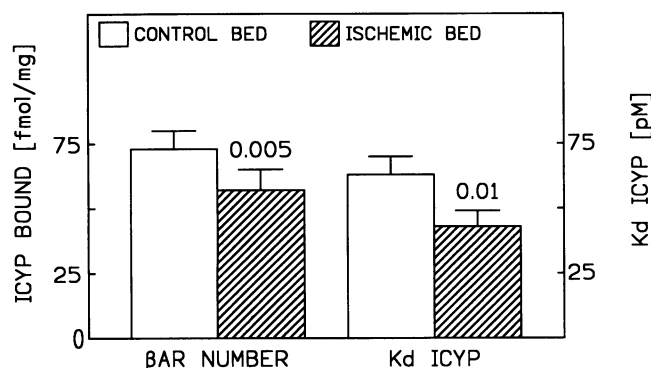


Figure 2. Radioligand binding (ICYP) was used to identify β ARs in membranes from transmural samples of myocardium from control and ischemic beds from each animal. The ischemic bed showed β AR downregulation, but the affinity for ICYP was increased (decreased K_d). Bars represent mean values from six animals; error bars denote ± 1 SEM.

ity. These data underscore the potential importance of reduced $G_{i\alpha_2}$ content in the ischemic bed as a mechanism by which normal signal transduction is preserved.

We were concerned that scattered fibrosis or focal hypertrophy might alter sarcolemmal membrane yield after the induction of episodic ischemia, thereby potentially artifactually lowering receptor number. We therefore used the specific activity of K^+ -pNPPase as a marker for sarcolemmal membrane (17) to confirm that expression of receptor binding per milligram protein was an appropriate means to measure loss of receptors. There was no significant difference in specific activities of this marker between crude membrane homogenates from the two beds (control, 622 ± 105 nmol/mg per h; ischemia, 589 ± 75 nmol/mg per h; $P = NS$). These data suggest that sarcolemmal membrane yield is not altered in the ischemic bed.

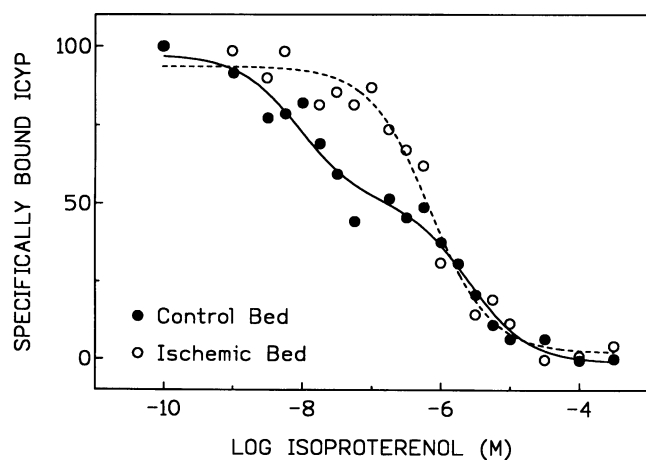


Figure 3. Competitive binding assays, performed to assess the proportion of β ARs displaying high and low affinity binding for (-)-isoproterenol. The ischemic bed had a reduced proportion of receptors with high affinity binding for isoproterenol, as indicated by a rightward displacement of the competition curve. Data from a representative animal are shown; data from five animals are presented in the text.

Adenylyl cyclase activity. β AR-dependent and Gs-dependent stimulation of adenylyl cyclase were unchanged in the ischemic as compared to the control bed (Table I), despite significant downregulation of β AR number in the ischemic bed. Basal values were similar in left ventricular membranes from either the control or ischemic beds after chronic episodic ischemia. There were small reductions in cAMP production in the ischemic bed, none of which were statistically significant. Thus, whether stimulated through the β AR, through Gs, or more directly through the catalytic subunit of adenylyl cyclase, net cAMP production was unchanged between the two beds.

Quantification of $G_{i\alpha_2}$ and $G_{s\alpha}$ by immunoblotting. To determine whether preserved adenylyl cyclase activity in myocardial membranes from the ischemic bed, despite β AR downregulation, was associated with altered levels of cardiac GTP-binding proteins (Gs and $G_{i\alpha_2}$), quantitative immunoblotting was performed. Quantitative immunoblotting using a purified antibody specific for $G_{i\alpha_2}$ (Fig. 4) showed reduced amounts of $G_{i\alpha_2}$ in membranes from the ischemic bed (control, 990 ± 132 pmol/g wet weight; ischemia, 625 ± 130 pmol/g wet weight; $P = 0.02$). Experiments performed on the endocardial and epicardial halves of transmural sections from both beds showed that downregulation of $G_{i\alpha_2}$ was uniform across the ventricular wall in the ischemic bed, and that $G_{i\alpha_2}$ was evenly distributed across the wall in the control bed.

Quantitative immunoblotting was also performed using purified antibodies against $G_{s\alpha}$ (Fig. 4), which showed that the quantity of $G_{s\alpha}$ was not different in transmural sections from the ischemic bed (control, 247 ± 64 pmol/g wet weight; ischemia, 320 ± 64 pmol/g wet weight; $P = 0.12$). However, experiments performed on the endocardial and epicardial halves of transmural sections showed an 82% increase in $G_{s\alpha}$ in the ischemic endocardium ($P = 0.05$). When the Gi/Gs ratio was calculated, based upon the quantitative immunoblotting data, there was a striking decrease in this ratio in the ischemic bed ($P < 0.005$; Fig. 5).

Assessment of Gs by reconstitution. To assess the biological activity of Gs in the ischemic region, we performed reconstitution assays using cholate extracts (Gs rich) from the ischemic and control beds of each animal (Fig. 6). These studies, unlike the quantitative immunoblotting studies, were conducted on tissue obtained from transmural sections; the endocardial/epi-

Table I. Stimulation of Adenylyl Cyclase in the Control (LAD) and Ischemic (LCx) Beds

| | LAD bed | LCx bed | P |
|-----------|------------------------|--------------|------|
| | <i>pmol/mg per min</i> | | |
| Basal | 102 \pm 13 | 84 \pm 7 | 0.24 |
| GTP + ISO | 58 \pm 7 | 48 \pm 3 | 0.12 |
| Gpp | 187 \pm 20 | 177 \pm 13 | 0.45 |
| AIF | 171 \pm 16 | 158 \pm 11 | 0.18 |
| Forskolin | 518 \pm 36 | 485 \pm 29 | 0.16 |

Data represent cAMP produced ± 1 SEM, and are net values (basal subtracted). $P =$ LAD bed vs. LCx bed, two-tailed t test; LAD, left anterior descending (control bed); LCx, left circumflex (ischemic bed); GTP, 100 μ M guanosine triphosphate; ISO, 10 μ M isoproterenol; Gpp, 100 μ M 5'-guanylyimidodiphosphogluconate; AIF, 10 mM aluminum fluoride; forskolin, 100 μ M forskolin. Data were obtained from ischemic and control beds of seven animals.

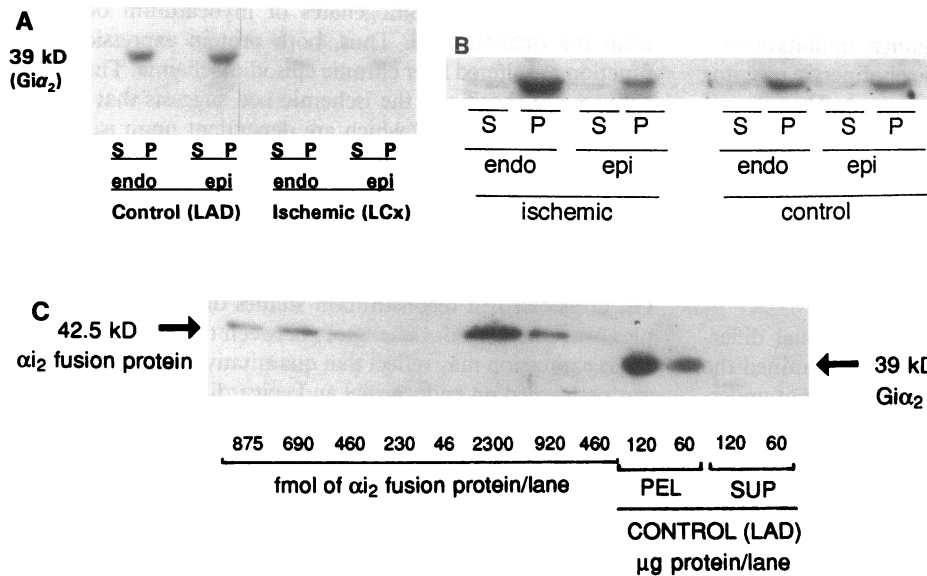


Figure 4. Autoradiogram of immunoblot using purified antibodies specific for $G_{i\alpha_2}$ in crude membrane homogenates of endocardial (*endo*) and epicardial (*epi*) samples of the control bed (*left*) and the ischemic bed (*right*) from a representative animal. 60 μg of protein from the soluble (*S*) and membrane-bound (*P*) fractions was examined for each preparation to evaluate labile G protein activity. This autoradiogram indicates that the ischemic bed had lower $G_{i\alpha_2}$ content than the control bed, and that there was no detectable difference in $G_{i\alpha_2}$ content between endocardium and epicardium. (*B*) Autoradiogram of immunoblot using purified antibodies to identify $G_{s\alpha}$ in crude membrane homogenates of endocardial (*endo*) and epicardial (*epi*) samples of the ischemic bed (*left*) and the control bed (*right*). 60 μg of protein from the

soluble (*S*) and membrane-bound (*P*) fractions was examined for each preparation to evaluate labile G protein activity. This autoradiogram indicates that the endocardial region of the ischemic bed had substantially greater $G_{s\alpha}$ content than the endocardial region of the control bed, while $G_{s\alpha}$ content in the epicardial region of the two beds was similar. (*C*) Autoradiogram of immunoblot using purified antibodies specific for $G_{i\alpha_2}$ against varied concentrations of fusion protein (GST- $G_{i\alpha_2}$) for quantitative immunoblotting (*first eight lanes*). The right side of the blot shows the soluble (*SUP*) and membrane-bound (*PEL*) fractions of two concentrations of crude homogenates of pig left ventricle. These findings indicate that twice the protein applied yields twice the visual signal, and that unlike $G_{s\alpha}$, $G_{i\alpha_2}$ is not detected in the supernatant fraction.

cardial distribution of reconstituted $G_{s\alpha}$ activity was not examined. Aluminum fluoride stimulation ($G_{s\alpha}$ dependent) of sarcolemmal membrane extracts from both beds resulted in similar levels of cAMP production, suggesting that the functional activity of $G_{s\alpha}$ was unaltered in transmural samples from the isch-

emic bed. Assessment of the distribution of $G_{s\alpha}$ between supernatant (presumably cytosolic) and pellet (presumably membrane associated) of the myocardial homogenates was performed (20). These data show that there was no alteration in the distribution between supernatant (cytosolic) and pellet (membrane associated) $G_{s\alpha}$ activity between the ischemic and control beds. Furthermore, within the ischemic region, there was no difference in proportion of $G_{s\alpha}$ in the supernatant vs. pellet between the endocardial and epicardial regions.

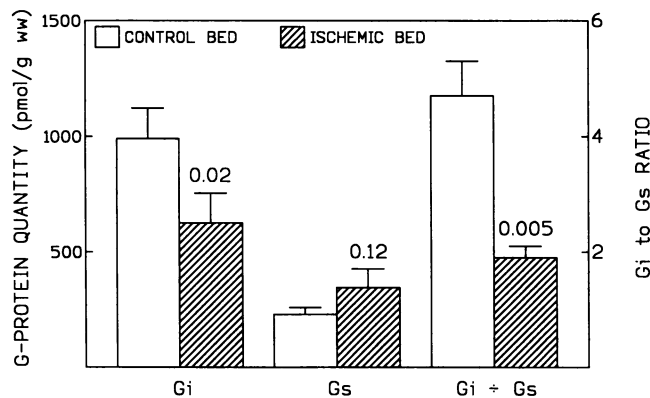


Figure 5. Quantitative immunoblots showed significant reductions in $G_{i\alpha_2}$ content in transmural membrane homogenates from the ischemic bed (37% decrease). Reduced $G_{i\alpha_2}$ was found in both endocardial and epicardial samples in the ischemic bed. $G_{s\alpha}$ content in transmural samples from the ischemic bed was mildly increased ($P = 0.12$), predominantly the result of increased $G_{s\alpha}$ content in the endocardial portion of the ischemic bed (82% increase; $P = 0.05$). The G_i/G_s ratio was markedly diminished in the ischemic bed. Data were obtained from endocardial and epicardial halves of control and ischemic beds from each of seven animals for $G_{i\alpha_2}$ and from each of four animals for $G_{s\alpha}$. Bars represent mean values, error bars denote 1 SEM.

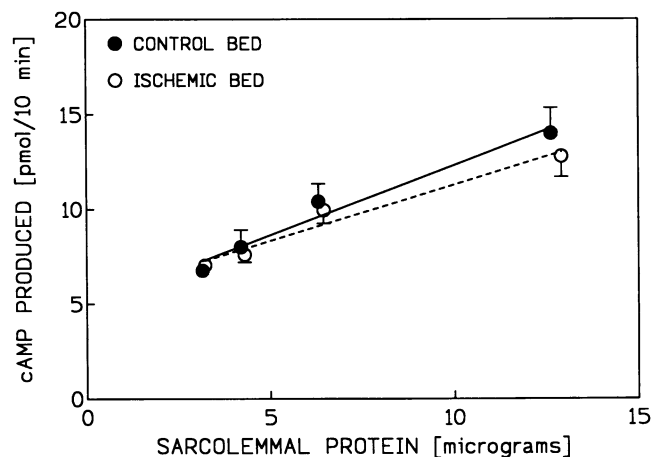


Figure 6. Reconstitution of $G_{s\alpha}$ into cyc-lymphoma cell membranes. Chololate extracts ($G_{s\alpha}$ rich) of left ventricular membrane homogenates prepared from transmural samples from the control and ischemic beds were stimulated (aluminum fluoride, 10 mM), and cAMP production was measured. Reconstituted $G_{s\alpha}$ activity was similar in membranes from both beds. Points represent mean \pm 1 SEM ($n = 4$).

Oxotremorine inhibition of adenylyl cyclase. Oxotremorine, a muscarinic cholinergic receptor agonist, inhibits adenylyl cyclase through $G_{i\alpha_2}$ (26). Muscarinic cholinergic receptor number was similar in the two beds (vide supra). Therefore, if a decrease in $G_{i\alpha_2}$ is relevant biochemically, the ability of oxotremorine to inhibit adenylyl cyclase should be decreased. The data shown in Fig. 7 support this conclusion. Thus, not only is $G_{i\alpha_2}$ decreased in the region of the heart subjected to chronic episodic ischemia, but membrane homogenates from the ischemic regions display less functional inhibition of cAMP production by agonists that are transduced through Gi.

Control animals. To determine whether regional differences in G protein content exist normally, we examined the LCx and LAD beds from control animals who had not undergone ameroid placement or prior thoracotomy. In immunoblotting studies, we found no difference in Gi content (LAD, $5,047 \pm 134$ arbitrary densitometry units; LCx, $4,582 \pm 334$ arbitrary densitometry units; $P = 0.22$) or in Gs content (LAD, $1,401 \pm 108$ arbitrary densitometry units; LCx, $1,344 \pm 100$ arbitrary densitometry units; $P = 0.71$). Because the immunoblots showed no significant differences, we did not conduct quantitative assays with fusion proteins on the control samples. Therefore, the differences between the two beds are not due to native regional differences, but result from chronic episodic ischemia.

Discussion

The key finding of this study is that chronic episodic ischemia results in important alterations in the myocardial β AR-G protein-adenylyl cyclase pathway that are specific to the region of myocardium that is susceptible to ischemia. Specifically, there is downregulation of β AR number, but preservation of adenylyl cyclase activity in the ischemic region. This is associated with reduced cardiac $G_{i\alpha_2}$ content in the ischemic region. The decrease in $G_{i\alpha_2}$, shown by quantitative immunoblotting, is associated with decreased ability of oxotremorine to inhibit

adenylyl cyclase in homogenates of myocardium obtained from the ischemic bed. Thus, both protein expression and function are altered after chronic episodic ischemia. That these changes are specific to the ischemic bed suggests that factors derived regionally, and which are dependent upon ischemia, underlie their molecular mechanisms.

Myocardial Gs α content also is altered in this model. Using quantitative immunoblotting, we found a tendency for Gs to increase in the ischemic bed, which was significant in the endocardial portion of the bed. However, the biological activity of Gs, as assessed in reconstitution studies on transmural samples, was unchanged. Differences between these two measures of Gs expression may reflect that quantitative immunoblotting was performed on endocardial and epicardial halves, while reconstitution studies were performed only on transmural samples. Therefore, an increase in reconstituted Gs α activity in the endocardium may have been missed.

This is the only study, to our knowledge, that has examined β AR expression, G protein function, and adenylyl cyclase activity in the setting of chronic episodes of myocardial ischemia without infarction or heart failure. An extensive literature has revealed important alterations in the β AR-Gs-adenylyl cyclase pathway in the setting of severe myocardial ischemia leading to myocardial infarction (3-7). Increased myocardial Gi content has been reported in patients with heart failure (27), where ischemia might be expected. However, our data were obtained from animals with normal ventricular function; alterations in Gi content in clinical heart failure may reflect the effects of heart failure per se rather than the effects of ischemia. These prior studies are germane for people with acute myocardial infarction and heart failure, but are not necessarily relevant for patients with angina pectoris, who experience repeated bouts of reversible myocardial ischemia. We believe that the current model, in which a region of the myocardium is perfused by collateral vessels and in which, during times of increased metabolic demands, myocardial perfusion is inadequate, is a reasonable representation of angina pectoris. It is therefore of interest that there are striking changes in this key signal transduction pathway in the heart after repeated bouts of reversible ischemia.

To assure ourselves that the changes encountered were not simply the reflection of regional differences in the heart, we examined the LCx and LAD beds from control animals who had not undergone ameroid placement or prior thoracotomy. We found that there was no evidence for alterations in G protein content between these two regions. Therefore, we believe that the alterations that we describe are the result of episodic ischemia per se. An additional concern was that our results might simply reflect alterations in the degree of fibrosis or in cell viability between the two regions. We found that K^+ -pNPPase activity, a sarcolemmal membrane enzyme (17), was unaltered between the two beds, suggesting that the sarcolemmal membrane yield between the two beds was similar. Furthermore, microscopic analyses of the LCx bed revealed minimal infarction. Thus, the changes we describe here are very likely due to alterations in viable myocytes, brought on by recurrent episodes of myocardial ischemia.

The degree of myocardial β AR downregulation in the ischemic bed was relatively small but very consistent. These data suggest strongly that the ischemic stimulus is the factor that leads to β AR downregulation in the ischemic bed. It seems plausible that increased levels of norepinephrine resulting from

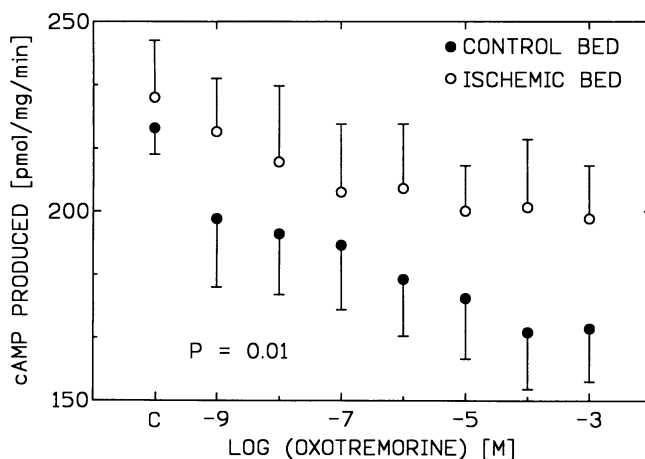


Figure 7. The ability of oxotremorine to inhibit adenylyl cyclase was examined in the control bed and ischemic bed of four animals. Stimulated adenylyl cyclase activity (10μ M isoproterenol and 100μ M GTP) was not different between the two beds. However, when oxotremorine was added, adenylyl cyclase activity was inhibited to a greater degree in the control bed. The data are consistent with a functional deficit of Gi in the ischemic bed. Points represent mean values, error bars denote 1 SEM.

regional ischemia (8) may underlie the changes in receptor number. Sustained myocardial ischemia has been shown to be associated with increased interstitial levels of myocardial norepinephrine, thought to result from decreased uptake 1 clearance of norepinephrine in the setting of decreased ATP stores, since uptake 1 clearance is energy dependent (8). We know of no suitable method to measure cardiac interstitial norepinephrine content during ischemia in conscious animals. However, data obtained in other models (8) support our speculation that increased norepinephrine is mechanistically important in the pathogenesis of reduced β AR numbers in the ischemic bed. Further studies will be required to elucidate the precise molecular mechanism for β AR downregulation in this model.

Recent reports showed that treatment of adipocytes with adenosine receptor agonists resulted in decreased pertussis toxin substrate and Gi (13), suggesting that prolonged adenosine receptor activation may be important in the regulation of $G_{i\alpha_2}$. Our data are consistent with this proposed mechanism. Since chronic episodic ischemia is associated with transient increases in adenosine, increased adenosine receptor activation is expected. Adenosine is released abundantly during myocardial ischemia (9), and may serve to inhibit adenylyl cyclase activity (10). Adenosine also may diminish the effects of agonist stimulation of β AR during ischemia by a mechanism independent of presynaptic inhibition, through a Gi-linked myocyte adenosine receptor (28). Decreasing adenylyl cyclase stimulation in the setting of myocardial ischemia through mechanisms linked with adenosine production provides an elegant means by which a product of ischemia can serve to attenuate further ischemia. It is of interest, therefore, that we find evidence for decreased $G_{i\alpha_2}$ in chronic episodic ischemia. Since adenosine may decrease adenylyl cyclase activation through an adenosine receptor linked with Gi (28), then reduction of $G_{i\alpha_2}$ would hamper this potential advantage. Similarly, if the presynaptic inhibition of norepinephrine release is transduced by Gi, a reduction in $G_{i\alpha_2}$ may serve to yield a net increase in adrenergic drive (29). A reduction in cardiac $G_{i\alpha_2}$ seems a poor adaptation, leaving the ischemic myocyte unprotected from ongoing, potentially damaging adrenergic stimulation. On the other hand, a feature of the model under study is that function in the ischemic bed is normal at rest. Increased adrenergic responsiveness conferred by decreased $G_{i\alpha_2}$ may, theoretically, be elemental in maintaining basal function in the ischemic bed. That adenylyl cyclase activation remained normal, despite decreased β AR number and decreased high affinity binding also suggests that decreased Gi content in the ischemic bed may be a method by which function remains normal.

In S49 lymphoma cells the stoichiometry of elements of the β AR-G protein-adenylyl cyclase pathway suggests that G protein levels exceed both β AR number and levels of adenylyl cyclase (30). If the porcine cardiac myocyte also has a molar excess of G proteins, could a 37% decrease in transmembrane $G_{i\alpha_2}$ content, or an 80% increase in endocardial $G_{s\alpha}$ content, as we report here, be biologically important? We previously found that a 42% increase in right atrial Gs was associated with a 57% increase in the efficacy of isoproterenol to stimulate heart rate, suggesting that levels of cardiac Gs may influence signal transduction efficiency (31). In animal models of heart failure, alterations in the cellular compartmentation of Gs occur, which may influence adrenergic signaling (21). In clinical dilated heart failure, depressed adrenergic responsiveness is associated with increased cardiac Gi content (27). The precise relation-

ship between cardiac Gi content and signal transduction through the β AR-G protein-adenylyl cyclase pathway remains to be established. Although β AR expression and the amount of adenylyl cyclase may set limits upon maximal responsiveness, our data suggest that levels of G proteins may have important effects upon signal transduction efficiency.

In conclusion, we have found that chronic episodic myocardial ischemia is associated with pronounced changes in signal transduction through the myocardial β AR-Gs-adenylyl cyclase pathway in a manner that is specific for the region of the heart subjected to ischemia. We found regional downregulation of myocardial β AR number. Despite myocardial β AR downregulation, adenylyl cyclase activity is preserved. The cardiac inhibitory GTP-binding protein ($G_{i\alpha_2}$) was decreased in the ischemic bed, and the cardiac stimulatory GTP-binding protein ($G_{s\alpha}$) was increased in the endocardium of the ischemic bed, findings that may contribute to preserved adenylyl cyclase activity. Determining the precise molecular mechanisms for these changes should prove interesting and quite relevant for patients with angina pectoris.

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