Biochemical Pharmacology, Vol. 53, pp. 347–356, 1997.

ELSEVIER

ISSN 0006-2952/97/$17.00 + 0.00
PII: S0006-2952(96)00717-4

Mechanisms of Adaptive Supersensitivity: Correlation of Guinea Pig Atrial Supersensitivity with Modifications in Adenylyl Cyclase Activity

Mark I. Roberts, Jeffrey M. Stadel,† Theodore J. Torphy,† William W. Fleming* and David A. Taylor*‡*

*Department of Pharmacology and Toxicology, West Virginia University Health Sciences Center, Morgantown, WV 26506-9223; and †Department of Pharmacology, SmithKline Beecham Pharmaceuticals, King of Prussia, PA 19406-0939, U.S.A.

ABSTRACT. The possibility that the cellular mechanism underlying adaptive supersensitivity in right and left atria of the guinea pig may involve either adenylyl cyclase or components of that transduction process was examined in left and right atria obtained from controls or guinea pigs chronically treated with reserpine. Adenylyl cyclase activity and the abundance of α-subunits of several G-proteins (i.e. G_s, G_i, and G_o) were quantified using standard techniques. Functional concentrations of G_s and G_i were compared in tissues from control and treated animals using pertussis- or cholera toxin-induced protein ribosylation. Chronic treatment with reserpine did not alter basal levels of adenylyl cyclase activity in left or right atrium but did increase significantly the ability of isoproterenol, 5′-guanylylimido diphosphate, and forskolin to activate adenylyl cyclase in the left atrium compared wtih the control. In contrast, treatment with reserpine increased the ability of only isoproterenol to active adenylyl cyclase in the right atrium. The increases in enzyme activation were not correlated with any detectable change in the concentrations of G-proteins or β-adrenoceptors. The correlation between the specificity of changes in responsiveness and increased activation of adenylyl cyclase suggests that the cellular mechanism that underlies the development of adaptive supersensitivity in the guinea pig myocardium may involve a modification of adenylyl cyclase. The data also support the idea that the development of enhanced responsiveness in cardiac muscle may not only involve more than one cellular mechanism but may even differ between right and left atrium and ventricles of the same species. *Copyright © 1997 Elsevier Science Inc.* BIOCHEM PHARMACOL 53;3:347–356, 1997.

KEY WORDS. adaptation; adenylyl cyclase; supersensitivity; cardiac muscle; G-proteins; guinea pig

The development of supersensitivity in a tissue following chronic interruption of its neural input ("*adaptive supersensitivity*") is a homeostatic response that occurs in a number of tissues including neurons and skeletal, smooth, and cardiac muscle (see Refs. 1 and 2). While the characteristics of the phenomenon vary among cell types and species, the change in sensitivity develops slowly over time and is qualitatively and quantitatively similar within a given tissue regardless of the procedure used to reduce the level of input. Furthermore, a single tissue may utilize more than one mechanism to fully express the phenomenon (see Refs. 1 and 3). To date, only three mechanisms have been clearly identified to be responsible for the development of adaptive supersensitivity: (1) an increase in receptor number; (2) a change in the electrophysiological properties of cells; and (3) an alteration in the intracellular transduction processes responsible for the action of an agonist following receptor occupation by that agent (see Ref. 1).

The guinea pig heart has been utilized extensively to investigate the cellular basis of adaptive supersensitivity. The alteration in neuronal input has been achieved in most cases through chronic treatment with reserpine [2, 4, 5]. Chronic reduction in neural input by reserpine leads to the development of enhanced sensitivity of ventricular muscle to the inotropic effects of isoproterenol, forskolin, and impromidine, a histamine H_2 receptor agonist [4]. The cellular mechanism underlying this nonspecific change in responsiveness has not been identified. However, the enhanced responsiveness has been associated with little or no change in the receptor population [4, 6] and with no overt modifications in the adenylyl cyclase system or the associated guanine nucleotide regulatory proteins [7].

In contrast to the ventricle, adaptive supersensitivity develops in the right atrium to the chronotropic and inotropic effects of only the β-adrenoceptor agonist isoproterenol [3, 8]. The left atrium, on the other hand, develops enhanced

‡ Corresponding author: Dr. David A. Taylor, Department of Pharmacology and Toxicology, West Virginia University Health Sciences Center, P.O. Box 9223, Morgantown, WV 26506-9223. Tel. (304) 293-3949; FAX (304) 293-6854.

Received 4 April 1996; accepted 2 August 1996.

responsiveness to the inotropic effects of not only isoproterenol but also the post-receptor agonists forskolin and GppNHp§ [4, 8]. The change in sensitivity of the right atrium is not associated with modifications in the β-adrenoceptor population [8] or in a significant elevation in the production of cAMP induced by isoproterenol [2]. The potential contribution of changes in the β-adrenoceptor population to the alteration in responsiveness observed in left atria following chronic reserpine treatment has not been explored. However, increased responsiveness of the left atrium has been correlated to a change in the production of cAMP [2], which could result from a change in the receptor population, adenylyl cyclase, phosphodiesterase, or other elements in the transduction process.

The present studies were undertaken to determine the cellular basis responsible for the alterations in cAMP production in the guinea pig atrium [2]. The experiments employed three agonists (forskolin, GppNHp, and isoproterenol) that are believed to interact with different components in the cascade leading to cAMP production. The action of GppNHp on adenylyl cyclase appears to be mediated through a guanine nucleotide regulatory protein [9, 10], while forskolin is thought to activate the cyclase directly [11, 12]. Isoproterenol, in contrast, activates adenylyl cyclase through a G-protein-coupled β-adrenoceptor [13]. Adenylyl cyclase activity was measured directly in the presence of phosphodiesterase inhibitors to assess whether the difference in the cAMP production resulted from a modification in product catabolism. Finally, the cellular and functional levels of G-protein α-subunits and the receptor population were quantified to assess the potential role of these other cellular components as contributors to the enhanced responsiveness. A preliminary account of some of these studies has been presented [14].

MATERIALS AND METHODS

Chemicals

[α-^{32}P]ATP (sp. act. 30 Ci/mmol) and [^{3}H]cAMP (sp. act. 36.1 Ci/mmol) were purchased from Dupont NEN Research Products (Boston, MA). GppNHp was purchased from Boehringer Mannheim (Indianapolis, IN). Dowex AG W50-X4, 200–400 mesh, was purchased from Bio-Rad (Richmond, CA). Other chemicals and drugs were purchased from the Sigma Chemical Co. (St. Louis, MO).

Pretreatment Schedule

Adult male albino guinea pigs (300–500 g) were randomly assigned to either a control (untreated) or a reserpine-treated group (animals received reserpine 0.1 mg/kg/day for 1 or 7 days). Reserpine used for injection was prepared from Serpasil® as previously described [15] which involved dissolution of Serpasil® into a vehicle composed of benzyl alcohol, citric acid, and Tween 80. Injectable solutions were derived from this stock solution by dilution into distilled water. The dose of reserpine used has been shown to induce 95% depletion of catecholamines within 24 hr [16] and lead to the development of supersensitivity [8, 17]. Guinea pigs were treated daily for 1 or 7 days prior to being killed, and weight was monitored daily.

Preparation of Atrial Membranes

Guinea pigs were stunned and then euthanized by exsanguination via incision of the carotid arteries. A thoracotomy was performed to expose the myocardium, and the entire heart was removed. The ventricles and atria were separated, rapidly rinsed in ice-cold salt solution and blotted, immediately wrapped in aluminum foil, and submerged in liquid nitrogen. The frozen tissues were stored at −70° until the time of the experiment. Crude membrane fragment preparations from the frozen atria were obtained according to the method described by McMurchie *et al.* [18]. Briefly, atria from five individual animals were thawed, pooled together, rapidly minced, and homogenized in 40 mL of ice-cold STEM buffer of the following composition: 250 mM sucrose; 20 mM Tris; 1 mM EDTA and 1 mM $MgCl_2$ with pH = 7.4 at 37°. Tissue homogenization was accomplished using three consecutive 30-sec bursts of a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) at a setting of "5." Homogenates were filtered through four layers of cheesecloth and centrifuged twice at 500 *g* for 15 min at 4°. Following the second centrifugation, the supernatant was discarded, and the pellet was resuspended in STEM buffer to yield a crude membrane fraction. Each homogenate was then divided into 10 aliquots of nearly 200 μL each and frozen at −70° until used for the cyclase assay. During the preparation of the crude membrane fraction and subsequent use in the adenylyl cyclase assay, homogenates were kept on ice at all times. The protein concentration of each homogenate was determined immediately prior to the assay using the method of Bradford [19]. It should be noted that while each individual homogenate contained tissue from five different animals, the homogenate was considered an "N" of 1 for statistical purposes.

Adenylyl Cyclase Assay

Adenylyl cyclase [ATP pyrophosphate-lysate (cyclizing)], EC 4.6.1.1, activity was assayed according to the method of Salomon [20] as previously described using ventricular muscle [7]. Crude membrane fraction preparations from four different atrial homogenates from each treatment group were incubated for 10 min at 37° in a reaction medium containing the following: 0.4 mM ATP; 0.1 mM Li_2GTP; 50 mM Tris; 5 mM $MgCl_2$; 0.1% BSA; 1 mM Na_2EDTA; 0.5 mM EGTA; 1 mM DTT; 1 mM cAMP; 1 mM isobutylmethylxanthine; an ATP-regenerating system composed of 13.5 mM phosphocreatine and 21.2 U/mL

§ *Abbreviations:* GppNHp, 5′-guanylylimido diphosphate; DTT, dithiothreitol; cAMP, cyclic AMP; and ICYP, iodocyanopindolol.

creatine phosphokinase and an appropriate concentration of agonist. [α-^{32}P]ATP was added to the reaction mixture to yield a final activity of 0.5 μCi/tube in a final volume of 50 μl/tube. Reactions were initiated by the addition of 10 μL of homogenate sample protein (diluted to a final protein concentration of 1 mg/mL) to the reaction tube. After the 10-min incubation period, the reaction was terminated by the addition of 100 μL of a Stop solution containing 2% SDS, 40 mM Na_2ATP, and 1.4 mM cAMP. The cAMP contained [^{3}H]cAMP (sp. act. 36.1 Ci/mmol at approximately 20,000 cpm/100 μL of Stop solution) for an internal standard to determine recovery after the chromatographic separation of ATP and cAMP. Following the addition of the Stop solution, each sample was boiled at 100° for 5 min.

[^{32}P]ATP and [^{32}P]cAMP were separated by sequential Dowex and alumina column chromatography as described by Salomon [20]. Appropriate eluates were collected, and aliquots were added to scintillation fluid and counted by liquid scintillation spectrometry for [^{32}P] and [^{3}H] using a Packard liquid scintillation counter. [^{32}P]cAMP generated was calculated as initial [^{3}H]cAMP added/[^{3}H]cAMP recovered · [^{32}P]cAMP recovered. Under the conditions described, [^{32}P]cAMP production was linear with membrane protein concentrations up to 100 μg/tube for at least 20 min.

Radioligand Binding Analysis of β-Adrenoceptors in Left Atria

Analysis of the number and affinity of left atrial β-adrenoceptors was carried out using the non-selective receptor antagonist, [^{125}I]ICYP (sp. act. 2000 Ci/mmol). The membrane preparations used in these studies were obtained by subjecting the crude membrane fractions described above to centrifugation at 20,000 *g* for 20 min at 4°. The resulting pellets were resuspended in the buffer using a vortex and a single 30-sec burst of a Polytron (setting "5"). The final particulate preparation contained a protein concentration of 10–20 μg protein/100 μL. The binding reaction was performed at 37° and was initiated by addition of 100 μL of membrane protein into 12 × 75 glass culture tubes containing ICYP and the buffer identified above. Incubations were performed in a Dubnoff incubator for 1 hr. Final concentrations of radioligand ranged from 5 to 150 pM, and all samples were assayed in duplicate. Specific binding for each concentration of labeled ICYP was defined as the difference between total binding and binding observed in the presence of 100 nM (DL)-propranolol. The reaction was terminated by rinsing the samples with 7 mL of cold buffer and filtering through Whatman GF/B glass fiber filters that had been soaked for at least 3 hr in buffer containing 0.1% polyethyleneimine using a Brandel cell harvester. Filters were removed and counted in a Beckman gamma counter. Specific binding routinely represented 60–70% of total binding. Saturation isotherms were analyzed using Rosenthal and Hill analyses performed through the EBDA binding analysis program (Biosoft).

[^{32}P]ADP-Ribosylation and Autoradiographic Analysis of G_s and G_i Proteins

TOXIN ACTIVATION. Pertussis toxin solution (1 μg/μL) was dissolved in 250 mM NaCl and 500 mM K_2HPO_4 (pH 7.5) and activated by the addition of an equal volume of a solution containing 40 mM DTT, 100 mM glycine (pH 8.0), and 2 mg/mL BSA. The mixture was incubated for 10 min at 30° prior to use. Cholera toxin was activated by incubating a solution containing cholera toxin (10 μg/μL) in buffer containing 500 mM K_2HPO_4 (pH 7.5), 2 mg/mL BSA, and 20 mM DTT for 30 min at 30° and then diluted 10-fold in buffer prior to use in the assay.

[^{32}P]ADP-RIBOSYLATION. Atria were homogenized at 4° with a motor-driven ground glass pestle and tube in a buffer containing K_2HPO_4 (50 mM; pH 7.5), EGTA (5 mM), soybean trypsin inhibitor (10 μg/mL), benzamidine (17 μg/mL), bacitracin (100 μg/mL), and phenylmethylsulfonyl fluoride (10 μM). To measure ADP-ribosylation induced by pertussis toxin, aliquots of atrial homogenates (25 μg of protein) were incubated for 3 hr at 30° in a total volume of 50 μL of buffer containing K_2HPO_4 (50 mM; pH 7.5), thymidine (20 mM), [α-^{32}P]NAD (5 μCi/assay), and pertussis toxin (5 μg/assay). Assays were terminated by adding 100 mL of stopping solution containing Tris-HCl (125 mM; pH 6.8), 4% SDS, 20% sucrose, 10% 2-mercaptoethanol, and 0.002% bromophenol blue.

To measure cholera toxin-mediated ADP-ribosylation, atrial homogenates (150 μg of protein) were incubated at 30° in a total volume of 50 μL of K_2HPO_4 (50 mM; pH 7.5), thymidine (20 mM), $MgCl_2$ (10 mM), GTP (0.1 mM), and [α-^{32}P]NAD (5 μM; 5 μCi/assay). The reaction was allowed to proceed for 30 min at 30° and was terminated in a manner similar to that described above for pertussis toxin-induced ADP-ribosylation.

Electrophoresis of membrane proteins through polyacrylamide was performed using the discontinuous system described by Laemmli [21]. Aliquots (100 μL) of the stopped samples were loaded onto polyacrylamide slab gels (separating gel of 7.5% acrylamide, stacking gel of 3% acrylamide). Electrophoresis was performed using a constant current of 30 mA for 5 hr. The gels were then stained overnight with Coomassie brilliant blue G-250, destained, and finally dried on filter paper. Autoradiography was performed on the dried gel at −60° using Kodak XAR-5 or SB-5 film. Densitometric analysis was carried out on the films using procedures similar to those described below for immunoblot analysis of G-proteins.

Immunoblot Analysis of G-Protein α-Subunits

Immunoblot analysis of the α-subunits of G-proteins was carried out using electrophoresis in a discontinuous system as described above and by Laemmli [21]. Proteins to be studied were obtained by centrifugation of the initial supernatant from the preparation of crude atrial membranes at 40,000 *g* for 30 min at 4°. The resulting pellet was re-

suspended in buffer and frozen. For SDS–PAGE, the frozen sample was thawed and resuspended in buffer, and size fractionation was performed on a 50-μg aliquot of membrane protein on a 10% acrylamide gel at 200 V for 5 hr at 4°. Protein concentrations applied to the gels were optimal for separation and fell within the linear range to ensure accurate comparative quantitation within each gel.

Immunoblot analysis was performed as described previously by O'Donnell *et al.* [22] and Roberts *et al.* [7]. Briefly, proteins separated by SDS–PAGE were transferred to nitrocellulose overnight at 30 V. The membrane was air dried, washed with buffer A (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100 and 0.1% SDS), and blocked with 3% BSA in buffer A for 60 min at 37°. After three consecutive 5-min washes in buffer A, the membrane was incubated at 37° for 1 hr in buffer A containing a 1/500 dilution of antiserum. For G_s, the anti-$G_{s\alpha}$ rabbit antiserum utilized by O'Donnell *et al.* [22] was employed while G_i analysis was accomplished using an anti-G_i antiserum (rabbit antiserum 8730), which recognizes the carboxyl terminus of $G_{i1-3\alpha}$ and was provided by Dr. David Manning, University of Pennsylvania. Attempts to quantify G_o levels using anti-G_o antiserum revealed levels of this protein that were near the lower limit of detection and, therefore, could not be quantitated accurately.

The nitrocellulose was washed three times in buffer A and then incubated with radioiodinated Protein A (0.4 μCi/mL) in buffer A for 60 min at 37°. Excess [^{125}I]protein A was removed by washing the membrane three times in buffer. Nitrocellulose membranes were dried and then exposed to Kodak XAR film with Dupont Cronex image-intensifying screens at −70° for 5 hr. Bands were quantified by a Research Analysis Systems image analyzer. Exposure times were controlled to provide the optimal quantitation and comparison between proteins obtained from the different treatment groups. Several different exposures of each film were made and quantified to ensure that the exposure times were optimal and submaximal. To be certain that both the protein and exposure levels were below saturation, proteins extracted from control tissues (S49 lymphoma cells for $G_{s\alpha}$ and rat cerebral cortex for $G_{i\alpha}$) were included as standards.

Statistical Analysis

Data are presented as the mean ± SEM of the picomoles of [^{32}P]cAMP produced per minute per milligram of protein. Basal values were determined in the absence of any agonist but with all other components of the reaction mixture. Comparisons between groups were made using an ANOVA followed by Student's *t*-test for unpaired samples. Concentration–response relationships were determined for a given agonist using membrane protein fractions derived from the same sample and conducted at the same time. Mean values of adenylyl cyclase activity were considered to be significantly different if $P \leq 0.05$. Characteristics of the β-adrenoceptors in left atria were compared between control and treated groups using an ANOVA followed by Student's *t*-test if indicated. Arithmetic mean values for the affinity (K_D) and density (B_{max}) were obtained from Scatchard and Hill analyses. Comparisons of $G_{s\alpha}$ levels were made using the values obtained from the image analysis system for samples from control and reserpine-treated tissues and by quantifying the percent of incorporation of [α-^{32}P]NAD into the specific proteins separated by SDS–PAGE.

RESULTS

Effects of Treatment with Reserpine on Adenylyl Cyclase Activity

EFFECT ON BASAL ENZYME ACTIVITY. Chronic treatment with reserpine did not alter significantly basal adenylyl cyclase activity in either left or right atria (Table 1, control). In right atrial preparations from untreated animals, the addition of ethanol, as the solvent for forskolin, did produce a small but significant increase in basal levels of enzyme activity (Table 1). While this incubation condition did tend to increase basal adenylyl cyclase activity in left atria from both treatment groups and right atria from animals treated with reserpine (Table 1), none of these differences

TABLE 1. Influence of reserpine treatment and incubation medium on basal adenylyl cyclase activity in atrial membrane preparations

		Adenylyl cyclase activity cAMP (pmol/mg protein/min)					
		Right atrium			Left atrium		
Group	Incubation condition	N	Untreated	Reserptine	N	Untreated	Reserpine
1.	Control	8	222 ± 14	237 ± 18	4	165 ± 21	267 ± 56
	Ethanol (0.7%)	8	277 ± 11*	308 ± 30	4	200 ± 17	298 ± 41
2.	Control	8	190 ± 8	203 ± 12	8 (7)†	290 ± 18	286 ± 20
	GppNHp (10^{-7} M)	8	285 ± 14*	322 ± 28*	4	529 ± 32*	654 ± 46*

Values provided represent mean cAMP produced ± SEM.

* Significantly different from control incubation conditions ($P \leq 0.05$).

† The "N" value for reserpine-treated left atria in control incubation conditions (2) = 7.

was statistically significant. The addition of GppNHp (10^{-7} M) to the incubation medium significantly increased levels of basal enzyme activity in both left and right atrial preparations from controls and from animals treated with reserpine (Table 1). The elevations in enzyme activity represented a 50–130% increase over basal activity with greater effects of GppNHp being found in left atria (Table 1). There was no statistically significant difference in nucleotide-induced elevations in enzyme activity between control preparations and preparations obtained from animals pretreated with reserpine (Table 1).

EFFECT ON AGONIST-INDUCED ENZYME ACTIVITY. Concentration-dependent elevations in cAMP production by adenylyl cyclase were observed in both right and left atria in response to forskolin (Fig. 1), GppNHp (Fig. 2), or isoproterenol (in the presence of 10^{-7} M GppNHp) (Fig. 3). In left atria, tissue homogenates obtained from animals pretreated with reserpine showed greater responsiveness to all agonists at the highest concentrations employed when compared with homogenates obtained from control animals. As expected, forskolin produced the greatest activation of the enzyme (note differences in vertical scales in Figs. 1–3). Incubation in the presence of increasing concentrations of GppNHp also led to substantial concentration-related increases in cAMP production that were significantly greater at concentrations above 10^{-6} M in tissues obtained from animals treated chronically with reserpine (Fig. 2). The isoproterenol concentration–response curves were carried out in the presence of GppNHp, 10^{-7} M, which improves responsiveness to isoproterenol. However, pretreatment with reserpine did not alter cAMP production induced by 10^{-7} M GppNHp in either right or left atria (Fig. 2 and Table 1).

Concentration-dependent activation of adenylyl cyclase in tissue homogenates of right atria was also observed in

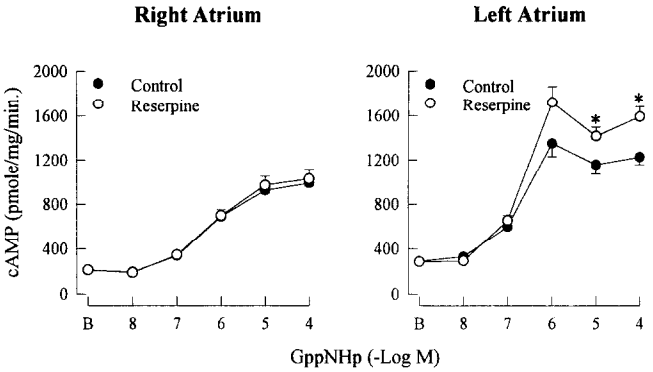

FIG. 2. Concentration–response relationship between GppNHp and adenylyl cyclase activity in membrane preparation of right and left atria from control (●) and reserpine-treated (○) tissues. Each data point represents the mean ± SEM. Key: (*) significantly different from control ($P < 0.05$).

response to increasing concentrations of forskolin (Fig. 1), GppNHp (Fig. 2), and isoproterenol (Fig. 3). Similar to the observation in left atria, forskolin and GppNHp both produced greater activation of the enzyme than did isoproterenol (compare vertical scales in Figs. 1 and 2 with Fig. 3). Treatment with reserpine did not elevate significantly the level of enzyme activation by either forskolin (Fig. 1) or GppNHp (Fig. 2) in right atrial preparations. However, tissue homogenates obtained from animals treated with reserpine did display small but significantly higher levels of enzyme activation in response to the two highest concentrations of isoproterenol (Fig. 3).

Influence of Reserpine Treatment on Left Atrial β-Adrenoceptors

Chronic treatment with reserpine did not alter the β-adrenoceptor population in tissue homogenates from left atria.

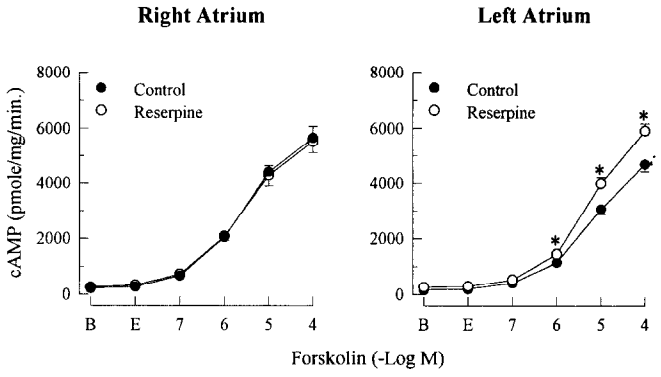

FIG. 1. Forskolin-stimulated adenylyl cyclase activity in crude membrane fragments of guinea pig right (N = 8) and left (N = 4) atrial tissues obtained from control (●) and reserpine-treated (○) animals. All data points except basal (B) were performed in the presence of 0.7% ethanol (E). Basal enzyme activities in the absence and presence of ethanol are given in Table 1. Each data point represents the mean ± SEM. Key: (*) significantly different from control ($P < 0.05$).

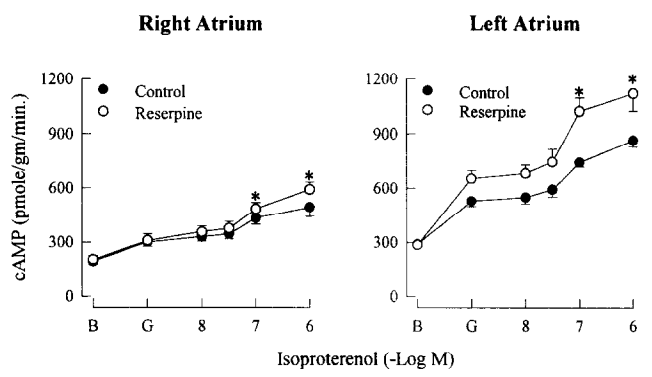

FIG. 3. Concentration–response relationship between isoproterenol and adenylyl cyclase activity in crude membrane fractions from right (N = 8) and left (N = 4) atria obtained from control (●) and reserpine-treated (○) tissues. All data points except basal (B) were performed in the presence of GppNHp [10^{-7} M; (G)]. Basal enzyme activity levels in the absence and presence of GppNHp are given in Table 1. Each data point represents the mean ± SEM. Key: (*) significantly different from control ($P < 0.05$).

The affinity and density of β-adrenoceptors were assessed by saturation isotherms and Scatchard analysis using [^{125}I]ICYP as the radioligand. As indicated in Table 2, there was no statistically significant difference in any measured parameter between control tissue homogenates and those obtained from animals treated with reserpine. The calculated Hill coefficients, 0.97 and 0.95, for control and reserpine-treated preparations, respectively, are consistent with an interaction of the ligand with a single site.

Effect of Chronic Treatment with Reserpine on Atrial G-Proteins

IMMUNOBLOT ANALYSIS OF α-SUBUNIT ISOFORMS OF G-PROTEINS. Tissue homogenates from either right or left atria were used to analyze the levels of $G_{s\alpha}$, $G_{i\alpha}$, and $G_{o\alpha}$ proteins. Each individual tissue was treated similarly so that comparable homogenates could be obtained. For each protein studied, the concentration of protein loaded onto each gel was equal among samples and below the saturating range for that protein as well as being in the linear range to permit quantitative comparisons using densitometric analysis. Positive controls were also included with each protein subunit. As previously observed in ventricular tissue homogenates [7], the levels of $G_{o\alpha}$ were very low and near the lowest level of detection (data not shown). However, atria did display measurable levels of both $G_{s\alpha}$ and $G_{i\alpha}$. The predominant form of $G_{s\alpha}$ in atria appeared to be the short isoform (Fig. 4) in contrast to the predominant form present in S49 mouse lymphoma cells that were used as the positive control (Fig. 4). The presence of measurable levels of $G_{i\alpha}$ was also confirmed by immunoblot analysis (Fig. 5) in which proteins obtained from rat cerebral cortex were simultaneously electrophoretically separated and subjected to analysis as the positive control. Quantitative densitometric analysis of reaction product for both $G_{s\alpha}$ and $G_{i\alpha}$ did not reveal any significant differences in the levels of these protein subunits in homogenates obtained from either control or animals treated with reserpine.

Analysis of G-Proteins by Toxin-induced ADP-Ribosylation

Cholera toxin-mediated ribosylation of G_s proteins and pertussis toxin-mediated ribosylation of G_i proteins were analyzed densitometrically. Tissue homogenates were obtained from animals treated with reserpine for 0, 1, or 7 days (Fig. 6, Table 3). The ribosylated proteins were separated by SDS–PAGE and compared with molecular weight standards. As illustrated in Fig. 6, levels of the ADP-ribosylated proteins were clearly measurable and conformed to the appropriate molecular weights of 45 kDa for G_s (Fig. 6A) and 41 kDa for G_i (Fig. 6B). Quantitative analysis of the separated proteins indicated that no significant differences could be measured in the percentage of [α-^{32}P]NAD incorporated into either G_s or G_i among any of the treatment groups (Table 3).

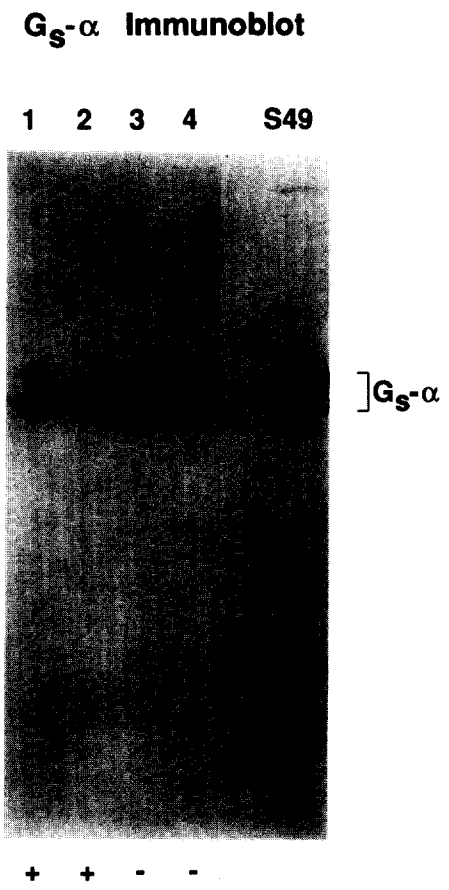

FIG. 4. Immunoblot of $G_{s\alpha}$ in membrane fractions of guinea pig atrial tissue. Lanes 1 and 3 represent fractions obtained from right atrium, while lanes 2 and 4 represent membrane fractions from left atria. Membrane fractions from S49 mouse lymphoma cells were included as a $G_{s\alpha}$ standard. Note that both the long and short splice variants of $G_{s\alpha}$ are present in all tissues. This experiment was repeated twice with comparable results.

TABLE 2. Influence of reserpine treatment on [^{125}I]ICYP binding characteristics in left atrial membranes

	K_D (× 10^{-12} M)	B_{max} (fmol/mg protein)	Hill coefficient (n_H)	Correlation coefficient	N
Untreated	81 ± 13	136 ± 28	0.97	0.98	8
Reserpine	43 ± 8	195 ± 44	0.95	0.98	5

Values of K_D and B_{max} are means ± SEM.

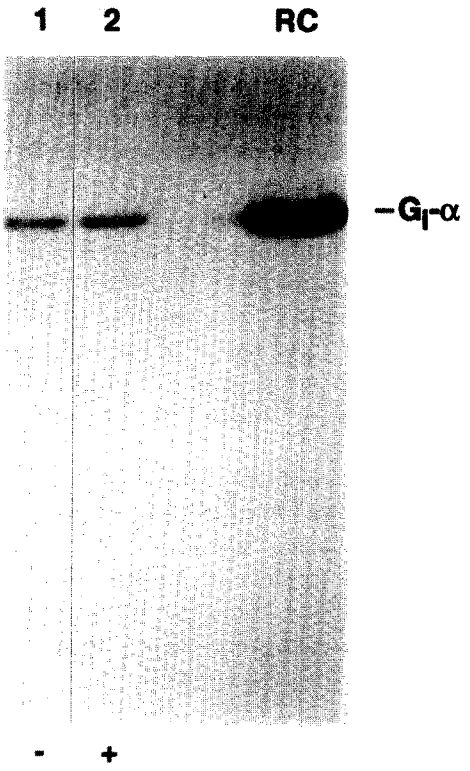

FIG. 5. Immunoblot of $G_{i\alpha}$ in membrane fractions of guinea pig left atrial tissue from control (lane 1) and from animals treated with reserpine for 7 days (lane 2). Membrane fractions obtained from rat cerebral cortex (RC) were also included as a G_i standard. Note that the levels of $G_{i\alpha}$ in atrial tissue are considerably less than those of the standard. This experiment was repeated twice with comparable results.

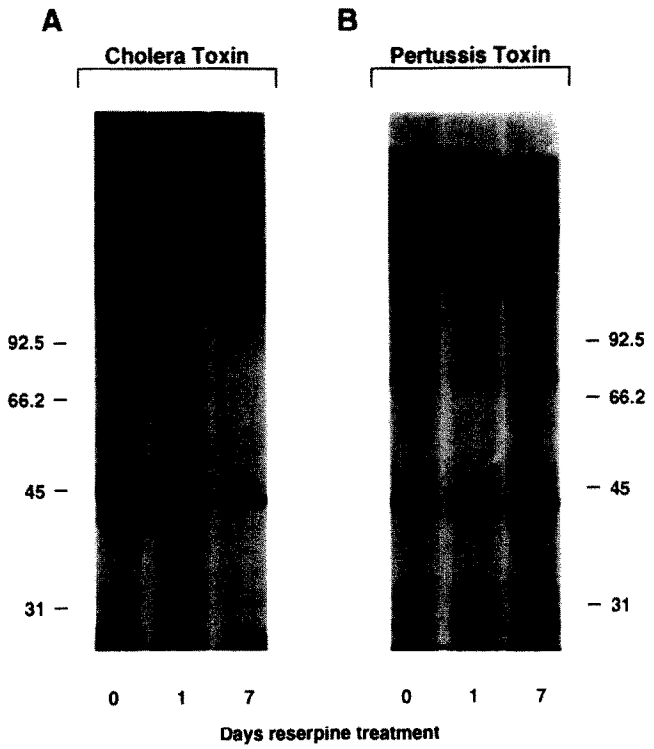

FIG. 6. Influence of reserpine treatment on cholera toxin- and pertussis toxin-induced ribosylation of G_s and G_i in left atrial membrane fractions from guinea pigs. Densitometric analysis of the [^{32}P] incorporation is provided in Table 3. Molecular weight standards are included to the left (panel A) or right (panel B) of the autoradiographs. Note that the molecular weight standards indicate a molecular weight of approximately 45 kDa for G_s (panel A) and 41 kDa for G_i (panel B). These data are representative of the results of 6 individual experiments included in Table 3.

DISCUSSION

Chronic treatment with reserpine did not alter significantly basal levels of adenylyl cyclase activity in either left or right atrial preparations. A similar lack of effect on basal adenylyl cyclase activity has been observed in papillary muscles of the rat following chronic treatment with propranolol, which would also lead to a reduced level of activity [21]. However, previous studies from this laboratory [7] investigating the role of changes in adenylyl cyclase activity in guinea pig ventricular muscle found a significant reduction in basal levels of enzyme activity following chronic treatment with reserpine.

In both right and left atria, clear concentration-related increases in the activation of adenylyl cyclase occurred with forskolin, GppNHp, and isoproterenol (in the presence of GppNHp). The magnitude of the responses differed markedly with an order of maximum efficacy of forskolin $\gg$ GppNHp $\gg$ isoproterenol and, in the case of GppNHp and isoproterenol, responses were greater in left than in right atria. These results are typical of what has been reported in most other tissues. An exception is the guinea pig ventricle, in which GppNHp does not activate adenylyl cyclase in membrane fragments [7]. Indeed, concentrations of GppNHp as high as 10^{-5} M, which increased markedly enzyme activity in atria, caused significant decreases in activity in ventricular preparations. The results of these two studies suggest that GppNHp has a predominant effect via G_s in atria and G_i in the ventricle.

In left atria, the activation of adenylyl cyclase by several agonists was greater in membrane preparations obtained from animals chronically treated with reserpine. Significantly greater elevations in cAMP production by adenylyl cyclase observed in response to forskolin, GppNHp, and isoproterenol after reserpine treatment are consistent with pharmacological data previously reported in this tissue [4]. These authors observed a significant increase in responsive-

TABLE 3. Influence of reserpine treatment on toxin-mediated ADP-ribosylation of guinea pig atrium

	Percent [α-^{32}P]NAD incorporation in G-proteins*		
Toxin	Control	1-Day	7-Day
Cholera	7.7 ± 1.1	7.3 ± 0.8	6.7 ± 0.3
Pertussis	6.5 ± 0.2	6.4 ± 0.2	6.4 ± 0.4

* Values expressed represent the percentage of total [α-^{32}P]NAD present that was incorporated into the 45 kDa (cholera toxin, G_s) or 41 kDa (pertussis, G_i) proteins. Each value is the mean ± SEM of 6 experiments.

ness of whole left atria to the inotropic effects of forskolin, GppNHp, and isoproterenol following reserpine treatment. Hawthorn *et al.* [4] observed a greater shift in responsiveness to isoproterenol and GppNHp than to forskolin, which suggested that mechanisms in addition to an alteration in adenylyl cyclase activation may play a role in the development of adaptive supersensitivity in left atria. One important result of the current studies is the observation that the change in responsiveness of the adenylyl cyclase system is present even when phosphodiesterase is blocked. These data strongly support the idea that the previously reported difference in cAMP production [2] is due to a modification in the production of cAMP rather than in the metabolism of the substance. Therefore, the possibility exists that the enhanced responsiveness observed following chronic treatment with reserpine may involve a cellular alteration within the transduction cascade from the β-adrenoceptors through the G-proteins to adenylyl cyclase. Such a change in responsiveness could be explained through an increase in the β-adrenoceptor population, an increase in G_s, a decrease in G_i, an increase in adenylyl cyclase, or any combination of these changes.

A quantitative evaluation of the β-adrenoceptor population and of G-protein levels did not reveal any significant differences between control preparations and preparations obtained from animals treated with reserpine in these cellular proteins. No statistically significant change in the β-adrenoceptor population was observed in the current study. While it is possible that modifications in the density of β-adrenoceptor subtypes may occur, it is unlikely that such a change would not be reflected in modification in the total receptor pool. However, the relatively small sample size and large variability around these observations do not permit an absolute exclusion of the possibility that a small change in the receptor population exists which could not be identified as statistically significant. In addition, the fact that the sensitivity of the left atrium is changed to agonists that do no require β-adrenoceptors for their action suggests that additional sites must also be involved.

Quantitative western blot analysis of left atrial G-protein abundance did not reveal any alterations in the level of $G_{s\alpha}$ or $G_{i\alpha}$ following chronic treatment with reserpine. Since G_o levels in left atria, like those of ventricular tissue [7], were just barely detectable, it is unlikely that significant differences exist in the levels of these proteins which could account for the development of enhanced responsiveness to several different agonists. The expression of low levels of G_o in guinea pig cardiac muscle is consistent with observations of low levels of this G-protein in the rat heart [23]. The data also indicate that the predominate isoform of $G_{s\alpha}$ present in the guinea pig myocardium is the short isoform. The presence of this lower molecular weight isoform is consistent with the lower receptor–G-protein-stimulated adenylyl cyclase activity in myocardial membranes observed in other studies [10, 24]. In addition, densitometric analysis of cholera toxin- and pertussis toxin-induced ribosylation of G_s and G_i subunits, respectively, also indicated that no significant differences in the levels of these G-proteins developed as a result of the cellular adaptation process. These data taken together suggest that the adaptive process of guinea pig atrial cells to chronic reductions in sympathetic input does not involve modification in either the amount or functional activity of cellular G-proteins. In view of the fact that the cellular effector of action appears to be the ligand-bound α-subunit [10, 25], it would be unlikely that changes in the β- or γ-subunits would occur under these conditions. However, the data cannot rule out the possibility that a modification in the production of these subunits develops in response to chronic reductions in sympathetic input.

Another possibility that could account for the quantitative discrepancy between data generated in whole tissues [4] and the data reported herein for membrane preparations may be the presence of multiple forms of adenylyl cyclase [26] which may be compartmentalized within the whole tissue. The data generated in membrane preparations are consistent with the enzyme being one or more of several types of adenylyl cyclase. Forms of adenylyl cyclase designated Types IV, V, or VI [26] display properties and distributions that are consistent with those observed in these studies with respect to responsiveness to $G_{s\alpha}$ and forskolin. Since multiple forms of adenylyl cyclase may concurrently exist in the same tissue and be differentially coupled to receptors, G-proteins, and/or protein kinases, the pharmacological responsiveness of the whole tissue to an agonist may differ from that observed in crude membrane preparations to the same agonist. Thus, it is possible that the greater elevation in responsiveness of whole left atrial preparations to isoproterenol and GppNHp compared with forskolin after treatment with reserpine [4] may reflect a difference in the tissue preparation rather than a mechanistic difference. As discussed above, the participation of a small but not statistically significant change in the receptor population or in the G-protein abundance may also contribute to the difference observed in responsiveness of the whole tissue. The increased activity of adenylyl cyclase observed in left atria, however, is qualitatively very consistent wtih those studies of whole tissue responsiveness in which elevated sensitivity was observed to forskolin, GppNHp, and isoproterenol.

Chronic treatment with reserpine did not alter basal levels of adenylyl cyclase activity in the right atrium nor did the treatment alter the ability of either forskolin or GppNHp to activate the enzyme. These data are also consistent with previous studies by Fleming and Taylor [2] which demonstrated to detectable differences in basal cyclic AMP production in right atria and no detectable difference in forskolin-mediated increases in either frequency of contraction or cAMP generation [2]. Forskolin and GppNHp did produce a significant elevation in adenylyl cyclase activity in both right and left atria. However, there was not a significant difference in the ability of the agonists

to activate the enzyme in preparations from control animals compared with those obtained from animals treated with reserpine. In contrast, activation of the enzyme by isoproterenol was enhanced slightly but significantly in right atrial preparations from animals treated with reserpine. These data are consistent with pharmacological studies of responsiveness of right atria to β-adrenoceptor activation. Torphy *et al.* [8] found a significant 2.3-fold leftward shift in the concentration–response curve to the chronotropic effects of isoproterenol in right atria. Furthermore, the change in responsiveness occurred in the absence of any detectable changes in the β-adrenoceptor population. In addition, Fleming and Taylor [2] found a significant 2-fold leftward shift in the concentration–response curve for the chronotropic effects of isoproterenol following chronic reserpine treatment in preparations that showed no significant increase in responsiveness to the chronotropic action of forskolin. Therefore, these data suggest that the cellular mechanism underlying the change in responsiveness of right atria must not reside at either the level of the receptor or the adenylyl cyclase and suggests some modification in coupling between the cellular effectors, e.g. the possibility that β-adrenoceptor-mediated modifications of ion channels directly coupled to G-proteins may be enhanced (see Ref. 25).

Western blot analysis of the G-proteins in the right atrium did not detect any significant differences in this tissue following treatment with reserpine (Figs. 5 and 6). The lack of enhanced responsiveness of adenylyl cyclase to forskolin suggests that no change in the total pool of adenylyl cyclases occurs during the adaptive process. There are a number of possibilities that exist to explain a significant and selective increase in responsiveness to isoproterenol in right atria after treatment with reserpine: (1) there might be an improvement in the coupling between the β-adrenoceptor, G_s, and the cyclase which allows for improved activation of adenylyl cyclase; or (2) there may be an elevation in the specific type of adenylyl cyclase that is coupled to the β-adrenoceptor. However, it should be recognized that because of the smaller supersensitivity to isoproterenol in right atria (~2-fold) [2, 8] than left atria (~5-fold) [4], the biochemical changes responsible for supersensitivity may simply be too small to detect with existing methods.

The data in the present study provide valuable new insight into the potential mechanisms that underlie the adaptation by cardiac tissues to chronic changes in the level of activity. In left atria, chronic treatment with reserpine evokes an adaptive alteration in cellular function which is expressed at the level of adenylyl cyclase, at the very least, since changes in responsiveness and enzyme activation occur in parallel. The possibility that the inotropic actions of the agonists employed depend on cAMP-dependent protein phosphorylation mediated by protein kinases would be consistent with a change in enzyme activation that closely parallels the observed differences in sensitivity. In contrast to the ventricle where no correlation could be made between changes in pharmacological responses and alterations in adenylyl cyclase activity [7], there is a high degree of correlation in these two entities in both the right and left atrium. The modification underlying the change in sensitivity does not appear to involve the cellular production of β-adrenoceptors in either the right or the left atrium and does not involve an alteration in the G-proteins associated with activation or inhibition of the cyclase. However, in left atrium the cellular adaptation does appear to involve adenylyl cyclase. In the right atrium, enhanced sensitivity does not appear to involve adenylyl cyclase directly but may be associated with changes in the coupling of the receptor, G-protein, and cyclase. At this point, there is no reason to exclude a change in coupling in the left atrium as well.

The data provided in this study also raise the possibility that mechanisms underlying the cellular adaptation to chronic changes in tissue activity may differ even within different compartments of the same tissue. It has long been recognized that the cellular mechanism underlying adaptive supersensitivity is not the same among different tissues (see Ref. 1). Furthermore, for any given tissue, more than one mechanism can contribute simultaneously to the functional change in responsiveness observed. The data from the present study suggest that the cellular mechanisms underlying adaptive supersensitivity differ in the right and left atria and ventricle in the guinea pig myocardium. Furthermore, these studies support the possibility that multiple mechanisms may work together in cardiac muscle to effect the change in tissue responsiveness. The data also provide an excellent correlation between the qualitative changes in tissue responsiveness and the activation of adenylyl cyclase by agonists in the right versus the left atria. The high degree of correlation supports the idea that, in left atrium, one site of change responsible for the development of adaptive supersensitivity is at the level of the adenylyl cyclase.

The work presented herein was supported, in part, by a grant from NIH (GM29840).

References

1. Fleming WW and Westfall DP, Adaptive supersensitivity. In: *Catecholamines, Handbook of Pharmacology* (Eds. Trendelenburg U and Weiner N), Vol. 90/I, pp. 509–559. Springer, Heidelberg, 1988.
2. Fleming WW and Taylor DA, Cyclic AMP and adaptive supersensitivity in guinea-pig atria. *J Neural Transm* **34** (Suppl): 179–185, 1991.
3. Fleming, WW, McPhillips JJ and Westfall DP, Postjunctional supersensitivity and subsensitivity of excitable tissues to drugs. *Ergeb Physiol* **68:** 55–119, 1973.
4. Hawthorn MH, Taylor DA and Fleming WW, Characteristics of adaptive supersensitivity in the left atrium of the guinea-pig. *J Pharmacol Exp Ther* **241:** 453–457, 1987.
5. Tenner TE, Young J, Riker BJ and Ramanadham S, Non-specific supersensitivity induced by reserpine in guinea-pig cardiac ventricle tissue. *J Pharmacol Exp Ther* **246:** 1–6, 1988.
6. Hawthorn MH and Broadley KJ, β-Adrenoceptor ligand binding and supersensitivity to isoprenaline of ventricular muscle after chronic reserpine pretreatment. *Naunyn Schmiedebergs Arch Pharmacol* **320:** 240–245, 1982.

7. Roberts MI, Biser PS, Stadel JM, Taylor DA and Fleming WW, Adenylyl cyclase and G-proteins in supersensitive guinea pig ventricles. *Mol Pharmacol* **42:** 784–791, 1992.
8. Torphy TJ, Westfall DP and Fleming WW, Effect of reserpine pretreatment on mechanical responsiveness and [^{125}I]iodohydroxybenzylpindolol binding sites in the guinea-pig right atrium. *J Pharmacol Exp Ther* **223:** 352–362, 1982.
9. Kelly E, Keen M, Nobbs P and MacDermot J, NaF and guanine nucleotides modulate adenylate cyclase activity in NG108-15 cells by interacting with both G_s and G_i. *Br J Pharmacol* **100:** 223–230, 1990.
10. Holmer SR and Homcy CJ, G proteins in the heart: A redundant and diverse transmembrane signaling network. *Circulation* **84:** 1891–1902, 1991.
11. Seamon KB and Daly JW, Forskolin: Its biological and chemical properties. In: *Advances in Cyclic Nucleotide and Protein Phosphorylation Research* (Eds. Greengard P and Robison GA), Vol. 20, pp. 1–150. Raven Press, New York, 1986.
12. Laurenza A, McHugh-Sutkowski E and Seamon KB, Forskolin: A specific stimulator of adenylyl cyclase or a diterpine with multiple sites of action. *Trends Pharmacol Sci* **10:** 442–447, 1989.
13. Levitski A, From epinephrine to cAMP. *Science* **241:** 800–806, 1988.
14. Roberts MI, Stadel JM, Torphy TJ, Taylor DA and Fleming WW, Alterations in adenylyl cyclase activity may account for reserpine induced supersensitivity in guinea-pig left atria. *Pharmacologist* **33:** 173, 1991.
15. Leyden AF, Pomerantz E and Bouchard EF, Pharmaceutical aspects of reserpine. *J Am Pharm Assoc* **45:** 771–775, 1956.
16. Crout JR, Muskus AE and Trendelenberg U, Effect of tyramine on isolated guinea-pig right atria in relation to their noradrenaline stores. *Br J Pharmacol* **18:** 600–611, 1962.
17. Taylor DA, Westfall DP, DeMoraes S and Fleming WW, The effect of pretreatment with reserpine on the diastolic potential of guinea-pig atrial cells. *Naunyn Schmiedebergs Arch Pharmacol* **293:** 81–87, 1976.
18. McMurchie EJ, Patten GS, Charnock JS and McLennan PL, The interaction of dietary fatty acid and cholesterol on catecholamine-stimulated adenylate cyclase activity in the rat heart. *Biochim Biophys Acta* **898:** 137–153, 1987.
19. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254, 1976.
20. Salomon Y, Adenylate cyclase assay. *Adv Cyclic Nucleotide Res* **10:** 35–55, 1979.
21. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685, 1970.
22. O'Donnell JK, Sweet RW and Stadel JM, Expression and characterization of the long and short splice variants of $G_{s\alpha}$ in S49 *cyc*$^-$ cells. *Mol Pharmacol* **39:** 702–710, 1991.
23. Luetje CW, Tieje KM, Christian JL and Nathanson NM, Differential tissue expression and developmental regulation of guanine nucleotide-binding regulatory proteins and their messenger RNAs in rat heart. *J Biol Chem* **263:** 13357–13365, 1988.
24. Lynch CJ, Blackmore PF, Johnson EH, Wange RL, Krone P and Exton JH, Guanine nucleotide binding regulatory proteins and adenylate cyclase in livers of streptozotocin- and BB/Wor-diabetic rats: Immunodetection of G_s and G_i with antisera prepared against synthetic peptides. *J Clin Invest* **83:** 2050–2062, 1989.
25. Clapham DE, Direct G protein activation of ion channels? *Annu Rev Neurosci* **17:** 441–464, 1994.
26. Tang W-J and Gilman AG, Adenylyl cyclases. *Cell* **70:** 869–872, 1992.