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Adenylyl Cyclase and Guanine Nucleotide-Binding Proteins in Supersensitive Guinea Pig Ventricles

M. I. ROBERTS, P. S. BISER, J. M. STADEL, D. A. TAYLOR, and W. W. FLEMING

Department of Pharmacology and Toxicology, West Virginia University Health Sciences Center, Morgantown, West Virginia 26505 (M.I.R., P.S.B., D.A.T., W.W.F.), and Department of Pharmacology, SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania 19406-0939 (J.M.S.)

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SUMMARY

Chronic treatment with reserpine (0.1 mg/kg/day × 7 days) leads to the development of adaptive supersensitivity of ventricular myocardium of guinea pigs. The compensatory increase in sensitivity is associated with a small increase in β-adrenoreceptor number. However, sensitivity is increased to a number of agonists that do not interact with β-adrenoceptors. An evaluation of the role of both adenylyl cyclase and guanine nucleotide-binding regulatory proteins in the development of adaptive supersensitivity was carried out using crude membrane fragments from untreated control and chronically reserpine-treated guinea pigs. Quantitative analysis of $G_{s\alpha}$ and G_i protein concentrations was accomplished using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting. Chronic treatment with reserpine reduced basal levels of adenylyl cyclase activity by nearly 60%. The reduced activity was not the result of a loss of endogenous norepinephrine, because incubation of tissues in the presence of propranolol did not alter the basal level of adenylyl cyclase activity. Incubation in the presence of guanylylimido diphosphate (10^{-5} M) also significantly reduced basal adenylyl cyclase activity, by nearly 70%. Chronic treatment with reserpine failed to significantly alter the activation of adenylyl cyclase by isoproterenol, impromidine, NaF, or forskolin. These data suggest that chronic treatment with reserpine does not alter agonist-induced activation of adenylyl cyclase. Furthermore, analysis of $G_{s\alpha}$ and G_i indicated that chronic treatment with reserpine did not affect the levels of these regulatory proteins in ventricular myocardial membranes. The data indicate that the enhanced sensitivity of guinea pig ventricular myocardium is not the result of an alteration in adenylyl cyclase activity or in the concentration of guanine nucleotide regulatory proteins. Therefore, the enhanced responsiveness to widely diverse agonists must be due to an alteration in cellular function beyond the level of adenylyl cyclase.

A number of tissues (e.g., neurons, exocrine glands, skeletal muscle, smooth muscle, and cardiac muscle) express the phenomenon of adaptive supersensitivity as a compensatory adjustment to the chronic interruption of neural input to that tissue (1, 2). The enhanced sensitivity of a tissue is usually illustrated as a left-ward shift of the dose- or concentration-response curve for an agonist. The specific characteristics and mechanisms underlying the development of the phenomenon differ from tissue to tissue within an animal and from species to species for a given tissue. In addition, multiple mechanisms may underlie the development of the phenomenon in a single tissue (2). Although a number of potential mechanisms exist, the only mechanisms that have been clearly established to mediate the observed increase in sensitivity are 1) an increase in receptor number, 2) an alteration in the intracellular transduction mechanisms responsible for the expression of receptor occupation, and 3) a partial depolarization of cell membranes (see Ref. 2). The particular characteristics of the supersensitivity that evolves can assist in distinguishing which mechanism is the most likely candidate to explain the increased sensitivity of a given tissue to agonists. A partial depolarization, for example, would affect a number of different agonists to a similar degree. In contrast, increased sensitivity that is highly specific in nature would point to a change in receptor number, a change in intracellular transduction mechanisms, or a combination of the two as the underlying mechanism(s).

The development of adaptive supersensitivity in cardiac muscle has been the subject of considerable research for a number of years (for review, see Ref. 3), but the mechanism underlying the development of the phenomenon has been elusive. Several factors contribute to this lack of success in identifying the cellular mechanism. First, the myocardium responds to agonists in several ways [e.g., an increase in rate of contraction (chronotropism) and/or an increase in force of contraction (inotropism)], which may not utilize the same intracellular transduction pathways. Second, the phenomenon has been studied in several different species using different sources of myocardial tissue

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ABBREVIATIONS: G protein, guanine nucleotide-binding protein; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*, *N*,*N*′,*N*′-tetraacetic acid; Gpp(NH)p, guanylylimido diphosphate.

(e.g., atria, ventricles, or papillary muscles). Third, different methods and treatment regimens have been used to produce the phenomenon.

Much of the effort has focused on the guinea pig myocardium, where chronic interruption of neurotransmission to the myocardium has been accomplished by treatment with reserpine (4–6), as well as surgical sympathectomy (7). Chronic reductions in neural activity lead to the time-dependent development of enhanced sensitivity in all parts of the myocardium. However, the characteristics of that enhanced sensitivity are slightly different, depending upon the particular area of the heart being studied. In the right atrium of the guinea pig, for example, the enhanced sensitivity is highly specific for the effects of isoproterenol and other β-adrenoceptor agonists (4), with no change in sensitivity to forskolin (8). In the left atrium, however, enhanced sensitivity develops to the inotropic effects of both isoproterenol (and other β-adrenoceptor agonists) (5, 9) and forskolin (5). In contrast, chronic treatment with reserpine leads to the development of enhanced sensitivity of ventricular muscle to isoproterenol, forskolin, and impromidine, a histamine H_2 receptor agonist (6). The changes in sensitivity occur in the absence of alterations in receptor number or affinity in right atria (4) or papillary muscles (9) or with only modest increases in β-adrenoceptor number in ventricular muscle (6, 10). The agonists used in studies utilizing left atria and ventricular muscle have a common site of action at the level of adenylyl cyclase. These data would suggest that, as proposed by Tenner *et al.* (6), changes in β-adrenoceptor number as well as alterations in the biochemical sequelae to receptor occupation may contribute to the development of adaptive supersensitivity in guinea pig ventricular muscle.

The experiments presented here were undertaken to test the hypothesis proposed by Tenner *et al.* (6) by directly examining the adenylyl cyclase system in the ventricular myocardium after chronic treatment with a regimen of reserpine known to produce enhanced sensitivity of the ventricular myocardium to several different agonists. Furthermore, previous studies had indicated a difference in cAMP production in supersensitive left atria (8), and studies evaluating the activity of the enzyme responsible for cAMP generation would be valuable for identifying the cellular locus for adaptive sensitivity changes in the myocardium. Because the change in sensitivity is least specific in the ventricular myocardium, these studies would also serve as a valuable step in characterizing the phenomenon in other myocardial tissues (e.g., atria). In addition, adenylyl cyclase is coupled to β-adrenoceptors and histamine receptors via G proteins (11). The current availability of highly specific methods to assess the concentration of these proteins will provide another level of assessment of the coupling between receptor occupation and the cellular response. A preliminary account of these studies has been presented (12).

Materials and Methods

Pretreatment schedule. Adult male albino guinea pigs (300–500 g) were randomly assigned to either a control (untreated) or reserpine-treated group (animals received reserpine at 0.1 mg/kg/day for 7 days). Reserpine used for injection was prepared from reserpine (Serpasil) as previously described (13), which involved dissolution of reserpine 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 (14) and to lead to the development of supersensitivity (4–6, 15). Guinea pigs were treated daily for 7 days before sacrifice and weight was monitored daily to ensure that no substantial weight loss occurred in any animal due to reserpine treatment. Guinea pigs treated with reserpine did not lose weight during the treatment.

Preparation of ventricular membranes. After pretreatment, guinea pigs were stunned and sacrificed by exsanguination via incision of the carotid arteries. After thoracotomy and exposure of the myocardium, the entire heart was removed and the ventricles were separated from the atria, rapidly blotted, and immediately wrapped in aluminum foil and submerged in liquid nitrogen. The frozen ventricles were stored at −70° until used for preparation of membranes. Membranes were prepared from the frozen ventricles according to the method described by McMurchie *et al.* (16). Briefly, individual ventricles were removed from storage, thawed, rapidly minced, and homogenized in 40 ml of ice-cold STEM buffer composed of the following: 250 mM sucrose, 20 mM Tris, 1 mM EDTA, and 1 mM $MgCl_2$, with pH = 7.4 at 30°. Homogenization was accomplished with three consecutive 30-sec bursts of a Polytron homogenizer (Brinkman Instruments) at a setting of 5. Each homogenate was then filtered through four layers of cheesecloth into a 50-ml culture tube. Homogenates were then centrifuged at 500 × *g* for 15 min at 4°, the supernatant was discarded, and the resultant pellet was resuspended in 20 ml of STEM buffer. After a second centrifugation at 500 × *g* for 15 min, the supernatants were discarded and the resultant pellet was resuspended in 1 ml of STEM buffer to yield a crude membrane fraction (P0-500 × *g*, low speed membrane pellet). Each homogenate was then divided into 10 aliquots of approximately 200 μl each before refreezing at −70° until use in the adenylyl cyclase assay. During both the preparation of the membrane fraction and subsequent use in the adenylyl cyclase assay, homogenates were kept on ice at all times. Protein concentration was determined using the method of Bradford (17).

Adenylyl cyclase assay. Adenylyl cyclase [ATP pyrophosphate-lyase (cyclizing); EC 4.6.1.1] activity was assayed according to the method of Salomon (18). Membrane fraction preparations from four different ventricles from each treatment group were incubated in a shaking water bath for 10 min at 30°, 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% bovine serum albumin, 1 mM Na_2 EDTA, 0.5 mM EGTA, 1 mM dithiothreitol, 1 mM cAMP, 1 mM isobutylmethylxanthine, 10 mM phosphocreatine, 21.2 units/ml creatine phosphokinase, 0.5 μCi of [α-^{32}P]ATP, and an appropriate concentration of drug. When forskolin was used as an agonist, the reaction mixture contained 0.7% ethanol in which the drug was dissolved. Gpp(NH)p (10^{-5} M) replaced GTP in the incubation medium when either isoproterenol or impromidine was used as an agonist. Reactions were initiated by the addition of 10 μl (20 μg) of protein to the reaction tube, to yield a final volume of reaction of 60 μl.

The reaction was terminated after 10 min by the addition of 100 μl of a stop solution containing 2% SDS, 40 mM Na_2ATP, 1.4 mM cAMP, and approximately 20,000 cpm of [^{3}H]cAMP (specific activity 36.1 Ci/mmol). The [^{3}H]cAMP served as an internal standard during the chromatographic separation of ATP and cAMP and was used to determine recovery. After 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 (18). Appropriate eluates were collected, and aliquots were added to scintillation cocktail and quantitated by liquid scintillation counting for ^{32}P and ^{3}H, using a Packard liquid scintillation counter. [^{32}P]cAMP generated was calculated as [^{32}P]cAMP recovered times the percentage of recovery, which was determined based on the recovery of [^{3}H]cAMP. Percentage of recovery under the conditions described generally ranged from 75 to 93%. Under the conditions of the assay, production of [^{32}P]cAMP was

linear over at least 20 min and with crude membrane protein concentrations up to 100 μg/tube.

SDS-PAGE and immunoblot analysis of G protein α subunits. Electrophoresis of membrane proteins through polyacrylamide was accomplished using the discontinuous system described by Laemmli (19). Proteins to be studied were isolated using the supernatant from the initial centrifugation step for preparation of the crude membrane fraction. The supernatant was centrifuged at 40,000 × *g* for 30 min at 4°. The resulting pellet was resuspended in STEM buffer and frozen until the day of protein analysis. Fifty micrograms of protein prepared as described were solubilized in sample buffer, and the analysis of molecular weight was performed on a 10% acrylamide gel at 200 V for 5 hr at 4°. The protein concentrations used were optimal for separation and within the linear range to ensure accurate comparative quantitation within each gel.

Immunoblot analysis was performed essentially as described previously (20, 21). Briefly, proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane 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, 0.1% SDS), and blocked with 3% bovine serum albumin 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 anti-$G_{s\alpha}$ rabbit antiserum, as described by O'Donnell *et al.* (21). SDS-PAGE analysis of G_i was accomplished using a 1/500 dilution of anti-G_i antiserum (rabbit antiserum 8730), which recognizes the carboxyl terminus of $G_{1\text{-}3\text{-}\alpha}$ and was kindly provided by Dr. David Manning, University of Pennsylvania. Attempts to quantify G_o in ventricular membranes using anti-G_o antiserum revealed extremely low levels of this protein that were near the lower limit of detection and could not be accurately quantitatively analyzed.

The nitrocellulose was washed three times over a 15-min period in buffer A, and the membrane was 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 over a 15-min period in buffer A. Dried nitrocellulose membranes were 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. As with protein concentrations, exposure times were controlled for optimal quantitation and comparison between tissues from the different treatment groups. Several exposures of the film were made and quantified to ensure that exposure times were optimal. In addition, a submaximal exposure was selected for use in the figures provided in Results and for comparative quantitation. Previous experience with this antibody and quantification system included the quantitation of increases in $G_{s\alpha}$ in S49 lymphoma cells (21). To be certain that levels of protein and exposure levels were below saturation, proteins extracted from S49 cells were included as standards.

Statistical analysis. Data are presented as the mean and standard error 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 analysis of variance, 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 ventricle and conducted at the same time. Mean values of adenylyl cyclase activity were considered to be significantly different if p was <0.05. Comparisons of regulatory G protein levels were made by comparing the values quantified from the image analysis system for samples from control and reserpine-treated tissues.

Chemicals. [α-^{32}-P]ATP (specific activity, 30 Ci/mmol) and [^{3}H]cAMP (specific activity 44.5 Ci/mmol) were purchased from DuPont NEN Research Products (Boston, MA). Gpp(NH)p was purchased from Boehringer Mannheim (Indianapolis, IN). Dowex AG W50-X4 (200–400 mesh) was purchased from Bio-Rad (Richmond, CA). Impromidine was generously supplied by SmithKline Beecham Pharmaceuticals (King of Prussia, PA). All other chemicals and drugs were purchased from Sigma Chemical Co. (St. Louis, MI). Stock solutions of forskolin (10^{-2} M) were made in 70% ethanol, and appropriate volume were added to incubations to achieve an appropriate final concentration of forskolin in 0.7% ethanol. Stock solutions of ATP, GTP, [^{32}P]ATP, Gpp(NH)p, creatine phosphokinase, impromidine, and isoproterenol were made in 2 mM Tris, pH 7.4 at 30°. Ascorbic acid was added to isoproterenol stock solutions to a final concentration of 1 mM.

Results

Effects of reserpine treatment on basal and maximal adenylyl cyclase activity. Basal levels of adenylyl cyclase activity of crude ventricular membranes were consistent among several different control groups incubated in normal reaction mixture (e.g., groups 1 and 4, Table 1). The addition of ethanol (0.7%), as a solvent for forskolin, to the incubation medium reduced basal activity by about 30% but the difference was not significant. In contrast, incubation in the presence of Gpp(NH)p (10^{-5} M), which was included in the reaction mixture when either isoproterenol or impromidine was used as agonist, significantly reduced basal activity, by 67% (Table 1). Chronic treatment with reserpine (0.1 mg/kg/day) for 7 days tended to reduce basal levels of adenylyl cyclase activity in all cases (Table 1). The effect of reserpine treatment was most dramatic in tissues from groups 1 and 4, in which tissues were incubated in reaction mixture with no solvent or other additions. Under these conditions, basal adenylyl cyclase activity was significantly reduced in preparations from animals pretreated with reserpine, by about 43% in each case (Table 1). The reduction in basal activity by reserpine was not due to the loss of stimulatory catecholamines, because incubation in the presence of propranolol (10^{-7} M) did not significantly alter basal enzyme activity in control tissues or tissues from reserpine-pretreated animals (Table 1, groups 1 and 4). In the presence of ethanol, the effect of reserpine treatment was less marked, leading to a level of activity that was only 30% less than control (Table 1, group 3). The smallest effect of reserpine treatment was observed under conditions where Gpp(NH)p was included in the incubation medium (Table 1, group 3). Under these conditions, pretreatment with reserpine reduced basal values by only about 10%, which was not significantly different from corresponding untreated basal values. However, incubation in the presence of Gpp(NH)p (10^{-5} M) still significantly reduced basal values by nearly 50%, in comparison with basal levels of enzymatic activity under incubation conditions of no drug, ethanol, or propranolol (Table 1).

Chronic treatment with reserpine reduced the maximum effect achieved by any of the agonists used (Table 2). In no

TABLE 1

Influence of reserpine treatment and incubation medium on basal adenylyl cyclase activity

Group	Incubation condition	*n*[a]	Adenylyl cyclase activity	
			Untreated	Reserpine-treated
			pmol of [^{32}P]cAMP/mg of protein/min	
1	Control	8	440 ± 29	249 ± 19[b]
2	+Ethanol (0.7%)	11	319 ± 46	215 ± 29
3	+Gpp(NH)p (10^{-5} M)	7	152 ± 11[c]	135 ± 11[c]
4	+Propranolol (10^{-7} M)	4	476 ± 39	273 ± 23[b]

[a] *n*, number of experiments.
[b] Significantly different from corresponding untreated basal values ($p < 0.01$).
[c] Significantly different from control incubation conditions ($p < 0.05$).

TABLE 2

Influence of reserpine treatment on maximal effects of agonists on adenylyl cyclase activity

Percentage of basal activity was calculated using basal levels from Table 1, corresponding to appropriate incubation conditions (i.e., isoproterenol and improdine basal levels, incubation with Gpp(NH)p; forskolin basal levels, incubation with 0.7% ethanol; and NaF basal levels, control).

Agonist	n[a]	Adenylyl cyclase activity			
		Untreated		Reserpine-treated	
		pmol of [^{32}P]cAMP/mg of protein/min	% of basal activity	pmol of [^{32}P]cAMP/mg of protein/min	% of basal activity
Isoproterenol	7	392 ± 27	258	340 ± 34	252
Impromidine	4	373 ± 18	245	364 ± 15	270
NaF	4	877 ± 112	200	641 ± 64	257
Forskolin	11	2144 ± 245	672	1825 ± 208	849

[a] *n*, number of experiments.

instances, however, did the reduction in mean maximum response reach statistical significance between control tissues and tissues from animals pretreated with reserpine. The tendency toward a reduction in maximal effects of the activating agonists by reserpine treatment merely reflects the depression of basal adenylyl cyclase activity (Table 1), because the maximum effects of the agents in the tissues from pretreated groups were not depressed when expressed as a percentage of elevation of activity by each agonist over basal levels (Table 2). The percentage of elevation above basal was calculated using basal levels for the appropriate reaction mixture from Table 1 [e.g., isoproterenol and impromidine basal levels were those in the presence of Gpp(NH)p, whereas basal levels for forskolin were those with 0.7% ethanol added to the reaction mixture].

Effect of reserpine on agonist-stimulated adenylyl cyclase activity. All of the agonists used produced concentration-dependent elevations in cAMP production by adenylyl cyclase. As indicated in Table 2, the agonists varied considerably in their ability to maximally stimulate the enzyme, with the most dramatic effect being produced by forskolin. Maximal activation of adenylyl cyclase by forskolin led to a nearly 7-fold increase in activity (Table 2). As illustrated in Fig. 1, however, chronic treatment with reserpine did not alter the concentration-response relationship for forskolin-induced activation of the enzyme. The least effective activators of adenylyl cyclase were the receptor-coupled agonists isoproterenol (Fig. 2) and impromidine (Fig. 3). Both of these agonists produced concentration-related elevations in cAMP production (Figs. 2 and 3) with maximal effects that were approximately 3-fold above basal levels (Table 2). Chronic treatment with reserpine did not alter the concentration-response relationship for either of these agonists (Figs. 2 and 3). In contrast to other agonists, chronic treatment with reserpine reduced the responsiveness of ventricular membrane fragments to sodium fluoride (Fig. 4). This effect was significantly different ($p < 0.05$) at the lower

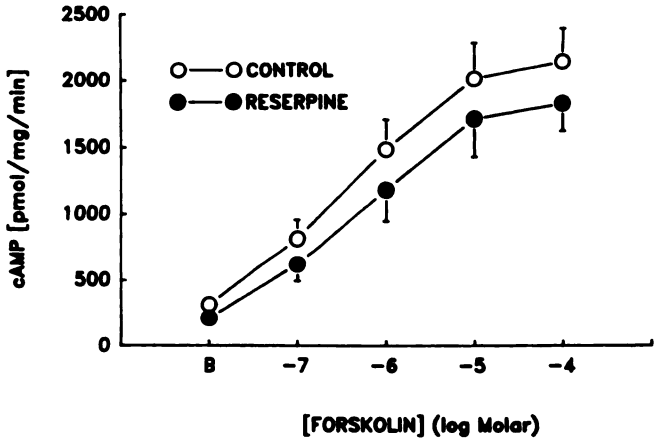

Fig. 1. Forskolin-stimulated adenylyl cyclase activity in crude membrane preparations of guinea pig ventricular tissues. No differences were observed between control and reserpine-treated tissues, (11 experiments). Note that basal enzyme activities correspond to those indicated for group 2 in Table 1 and are not significantly different from one another.

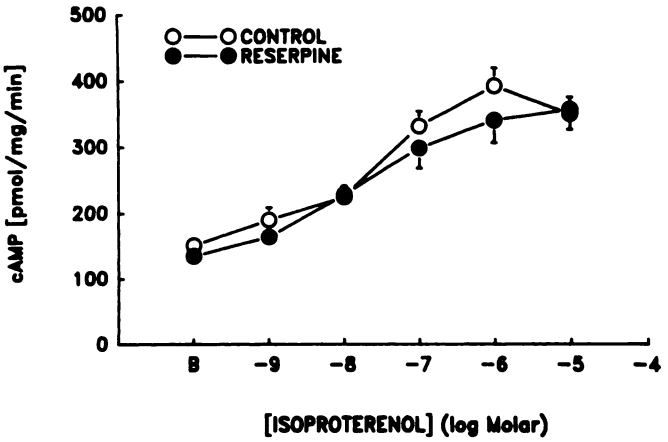

Fig. 2. Isoproterenol-stimulated adenylyl cyclase activity in crude membrane preparations of guinea pig ventricular tissues. No differences were observed between control and reserpine-treated tissues (seven experiments). Note that basal levels of enzyme correspond to those values presented in Table 1 (group 3) and are not significantly different from each other.

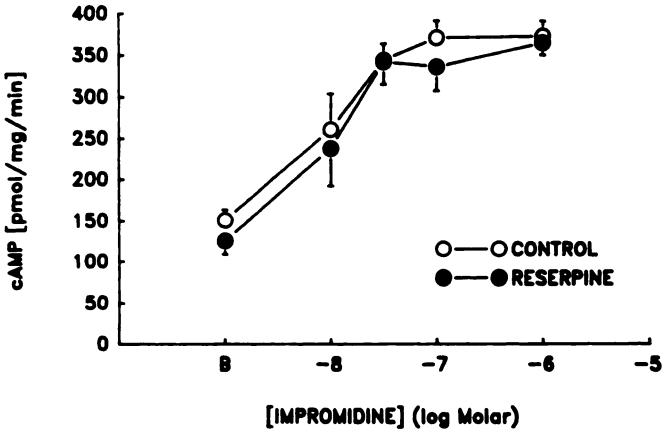

Fig. 3. Impromidine-stimulated adenylyl cyclase activity in crude membrane preparations of guinea pig ventricular tissues. No differences were observed between control and reserpine-treated tissues (four experiments). Basal enzyme activity corresponds to that reported in Table 1 (group 3) and is not significantly different between control and reserpine-treated groups.

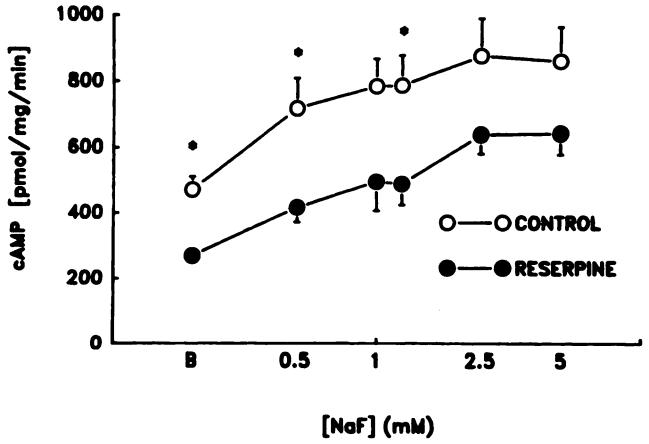

Fig. 4. Fluoride-stimulated adenylyl cyclase activity in crude membrane preparations of guinea pig ventricular tissues. Notice depression of basal levels of activity in reserpine-treated tissues and also the parallel nature of the dose-response curves. Basal levels correspond to those presented in Table 1 (group 1). *, Significantly different from control, $p < 0.05$ (four experiments).

doses used. Maximal effects of sodium fluoride were reduced by chronic treatment with reserpine, but the difference was not statistically significant (Table 2). Any effects of reserpine pretreatment on responses to sodium fluoride may be a function of reduced basal levels.

Effect of chronic reserpine treatment on G protein α levels in ventricular myocardium. Analysis of $G_{s\alpha}$, G_o, and G_i was accomplished in proteins extracted from ventricles of untreated animals and animals treated with reserpine (0.1 mg/kg/day) for 7 days. The concentration of protein loaded onto each gel was equal between samples, well below saturating concentrations, and in the linear range in order to permit accurate quantitative comparison by densitometry. As illustrated in Fig. 5, no differences in content of $G_{s\alpha}$ were detected between the two groups of tissues. Positive control preparations obtained from S49 mouse lymphoma cells indicated the presence of two bands, which correspond to the two isoforms of $G_{s\alpha}$. The most intense reaction product developed toward the heavier isoform of the protein (Fig. 5). In contrast, in ventricular membranes the predominant isoform corresponded to the shorter isoform of $G_{s\alpha}$. Similar results were obtained in two additional ventricular preparations from each group. Image analysis of the immunoblots failed to detect any quantitative differences in the amount of $G_{s\alpha}$ between the two groups. Levels of G_o, as evidenced by immunoblot analysis, were very low and sufficiently close to background levels to make quantitative detection difficult (data not shown). However, no substantial differences in signal were observed between control and reserpine-treated groups (data not shown). Quantitative analysis of G_i proteins also revealed no significant differences in the content of G_i in ventricular membranes after treatment with reserpine (Fig. 6). As illustrated in Fig. 6, the protein concentration used for quantitative analysis was at equal but substantially lower levels in both tissues. Positive control preparations obtained from rat cerebral cortex are also illustrated. Note that the reaction product for the positive control was denser than those obtained with the samples, indicating that the concentrations used were below saturation. Similar results were obtained in two other preparations from each group.

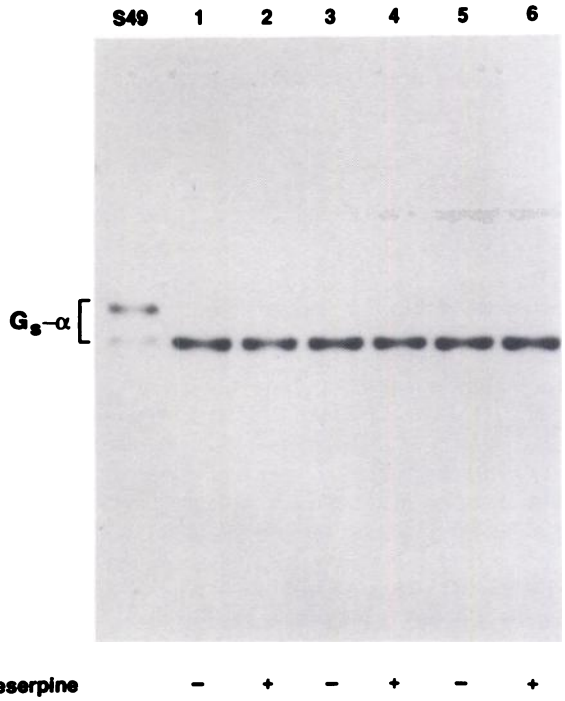

Fig. 5. Immunoblot of $G_{s\alpha}$ in membrane fractions of guinea pig cardiac tissue. Membranes were prepared, in the presence of 1 mM Mg^{2+}, from control animals (–) or guinea pigs pretreated for 7 days with reserpine (0.1 mg/kg/day) (+). Fifty micrograms of membrane protein were resolved on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was probed with anti-$G_{s\alpha}$ rabbit antiserum (1/500), and the antibodies that cross-reacted with anti-$G_{s\alpha}$ were detected by ^{125}I-Protein A. *Lanes 1*, *3*, and *5*, preparations from control tissues; *lanes 2*, *4*, and *6*, ventricular membranes prepared from animals pretreated with reserpine. Fifty micrograms of S49 mouse lymphoma cell membranes were included as a $G_{s\alpha}$ standard. This experiment was repeated twice with comparable results.

Discussion

Previous work by Tenner *et al.* (6) demonstrated that ventricular muscle from guinea pigs treated chronically (7 days) with reserpine (0.1 mg/kg/day) was supersensitive to the inotropic effects of isoproterenol, impromidine, and forskolin. A similar pretreatment schedule has been used in this laboratory to evoke enhanced pharmacological responsiveness in right atrium (4, 8, 15) and in left atrium (5). In spite of a modest increase in the number of β-adrenoceptors, the nonspecific nature of enhanced responsiveness in ventricular preparations would suggest that the cellular change responsible for the enhanced sensitivity must develop, at least in part, at a site beyond the receptor. As suggested by Tenner *et al.* (6), a likely site for such an alteration in cellular function is the adenylyl cyclase system. Cros and McNeill (22), using a different treatment regimen (2.5 mg/kg of reserpine per day × 2 days), did observe a small enhancement in adenylyl cyclase activation that was selective for epinephrine. The data provided from the present study would suggest that the observed enhanced responsiveness is not due to a change in either the activity of adenylyl cyclase or the amount of regulatory G proteins associated with receptor-mediated activation or inhibition of that enzyme.

Chronic treatment with reserpine leads to a 43% reduction in basal adenylyl cyclase activity (Table 1). The reduction in activity of the enzyme is not a function of the absence, in the

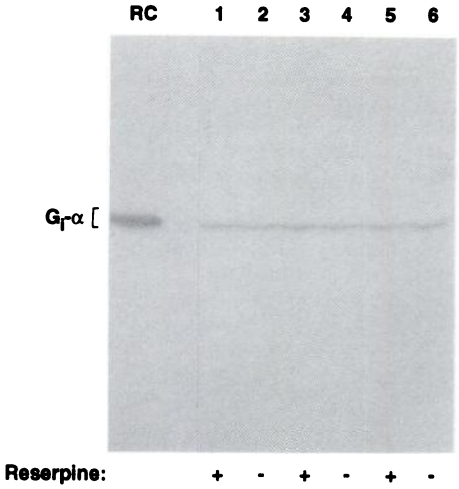

Fig. 6. Immunoblot of G_i in membrane fractions of guinea pig cardiac tissue. Membranes were prepared, in the presence of 1 mM Mg^{2+}, from guinea pigs pretreated for 7 days with reserpine (0.1 mg/kg/day). Fifty micrograms of membrane protein were resolved on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was probed with anti-$G_{i\alpha}$ rabbit antiserum (1/500), and the antibodies that cross-reacted with G_i were detected by ^{125}I-Protein A. *Lanes 1*, *3*, and *5*, preparations of tissues from animals pretreated with reserpine (+); *lanes 2*, *4*, and *6*, membranes isolated from control tissues (−). Fifty micrograms of membranes from rat cerebral cortex (*RC*) were included as a G_i standard. This experiment was repeated twice with comparable results.

crude membrane fractions, of the endogenous transmitter substance norepinephrine, because ventricular membranes from untreated animals incubated in the presence of propranolol (10^{-7} M) possess enzyme activity equivalent to that obtained in its absence (Table 1). Furthermore, membrane fractions obtained from animals chronically treated with reserpine exhibit the same reduction in activity of the enzyme in the presence or absence of propranolol (Table 1). Thus, chronic treatment with reserpine leads to a marked reduction in basal adenylyl cyclase activity that is not the result of a difference in the levels of endogenous norepinephrine present in the membrane fractions.

An intriguing observation is the inhibitory effect of Gpp(NH)p on ventricular myocardial membrane adenylyl cyclase (Table 1). The addition of this concentration of the less hydrolyzable analogue of GTP to the incubation mixture is essential for isoproterenol- or impromidine-induced activation of the enzyme in these membrane fractions (data not shown). However, addition of Gpp(NH)p to ventricular membranes obtained from untreated animals leads to a 65% reduction in basal enzyme activity. In membranes obtained from animals pretreated with reserpine, the addition of Gpp(NH)p led to approximately 50% inhibition of basal activity (Table 1). Such a marked inhibitory action of Gpp(NH)p on adenylyl cyclase activity is not readily explicable, because Gpp(NH)p enhances basal activity in papillary muscles (23) and in crude membrane fractions of left and right atria.[1] Hudson and Fain (24) and Insel *et al.* (25) observed inhibition of adenylyl cyclase activity by Gpp(NH)p or GTP that was particularly pronounced in preparations in which cyclase activity was augmented through the addition of forskolin. In addition, Kelly *et al.* (26) using NG-108-15 neuroblastoma cells observed inhibitory effects of Gpp(NH)p on activated adenylyl cyclase that were likely mediated through an interaction of the nucleotide with G_i. Clearly, however, explanation of these data requires additional experimentation and is beyond the scope of these studies.

The capacity of receptor-mediated agonists to activate adenylyl cyclase is not altered by chronic reserpine treatment. Isoproterenol and impromidine activate the enzyme to the same degree (Table 2) and in the same manner (Figs. 2 and 3) in membrane fractions obtained from tissues of control or reserpine-treated animals. The maximal effect obtained for both of these agonists (i.e., nearly 2.5-fold increase) is similar to that previously reported by others (27, 28). Similarly, the ability of forskolin to activate adenylyl cyclase is unaltered by pretreatment with reserpine (Fig. 1; Table 2). These data would suggest that receptor-coupled activation of adenylyl cyclase and activation of the catalytic subunit of the enzyme are not altered by chronic treatment with reserpine. The fact that basal levels of enzyme activity are reduced by nearly 50% and maximal effects of all agonists are not significantly different might suggest that subtle differences in the efficiency of coupling of the cyclase to intracellular mediators might develop after treatment with reserpine. In any event, the data suggest that no marked changes in the catalytic subunit or receptor-gated activation of that subunit develop in tissues obtained from animals treated with reserpine.

Cros and McNeill (22) reported a similar lack of alteration in sensitivity to histamine-induced activation of adenylate cyclase. These investigators did, however, observe a significant enhancement of activation of adenylyl cyclase by epinephrine in membrane fractions obtained from animals pretreated with reserpine. It should be noted that Cros and McNeill (22) used a much larger dose of reserpine (2.5 mg/kg) for a shorter time period (2 days) than in the present study. Previous studies (14) have indicated that a dose of 0.1 mg/kg of reserpine is sufficient to produce 95% depletion of cardiac norepinephrine within 24 hr. In addition, Iwayama *et al.* (29) have shown that doses of reserpine of 5.0 mg/kg produce marked cellular damage within 12 hr. The doses used in the present study are those previously used to induce supersensitivity in the right atrium (4, 8, 15), left atrium (5, 30), and ventricle (6), and do not induce electron microscopic evidence of myocardial cellular damage (29).

An additional factor that may contribute to the differences between the results of the present study and those of Cros and McNeill (22) may be the agonist used. The presence of a significant number of α-adrenoceptors that mediate a positive inotropic effect in guinea pig ventricle (31) may complicate the use of epinephrine, which was the agonist used by Cros and McNeill (22). The conclusion drawn by these authors, however, that chronic treatment with reserpine does not alter the adenylyl cyclase system in ventricle, is consistent with the observations reported in the present study.

The reduced response of ventricular membranes from animals treated with reserpine to activation of adenylyl cyclase by NaF (Fig. 3) appears to reflect the reduced levels of basal enzymatic activity (Table 1). The activation of the enzyme by NaF appears to be parallel throughout the concentration range used. Thus, the ability of NaF to activate the enzyme seems to be unchanged in spite of the lower levels of basal enzymatic activity. Such lower levels of activity are not due to reduced amounts of $G_{s\alpha}$ because immunoblot analysis indicates that the levels of these proteins are unchanged (Fig. 5). Analysis of the concentration of $G_{s\alpha}$ revealed that ventricular muscle possesses

[1] Unpublished observations.

a predominance of the longer splice variant of $G_{s\alpha}$ (32), in contrast to the S49 mouse lymphoma cells in which the shorter form predominates. Such a difference in isoform may produce differences in the interaction of the isoform with GTP (32). Another possibility is that such an effect is related to differences in the amount of G_i or G_o regulatory G proteins in tissues from animals treated with reserpine. This seems less likely, however, because the basal levels of adenylyl cyclase from animals treated with reserpine are significantly reduced (Table 1) and, as illustrated in Fig. 5, the levels of $G_{s\alpha}$ remain unchanged. A reduction in basal adenylyl cyclase activity without a change in $G_{s\alpha}$ can occur only if levels of G_i or G_o are elevated. Under such conditions, the effect of agents that utilize these regulatory proteins (e.g., NaF) should be affected by chronic treatment with reserpine. This is clearly not the case. Furthermore, quantitative analysis of G_i and G_o content revealed no significant differences in tissues from animals treated with reserpine, compared with those from control animals. Analysis of G_o content suggests that the levels of this regulatory protein in guinea pig ventricular membranes are very low and not apparently elevated to a detectable level after treatment with reserpine.

In summary, these studies have directly measured the activation of adenylyl cyclase in crude membrane fragments of ventricular myocardium from untreated and chronically reserpine-treated guinea pigs. Agonists tested were selected on the basis of previous demonstrations that enhanced sensitivity developed to the inotropic effects of these agents (6). The data indicate that the enhanced responsiveness is not due to an alteration in adenylyl cyclase, to an increased amount of $G_{s\alpha}$, or to a change in the levels of G_i. The activation of the enzyme by any agonist was not enhanced by reserpine pretreatment and, in fact, the basal levels of adenylyl cyclase activity appear to be reduced by such pretreatment. One potential explanation for the change in responsiveness of the ventricular myocardium is an alteration in cellular function beyond the activation of adenylyl cyclase (e.g., cAMP-dependent protein kinase). An evaluation of such an action is beyond the scope of this study. The data suggest that enhanced inotropic responses in ventricles to several different agonists (6) are not the result of a quantitative difference in $G_{s\alpha}$ or G_i or in the level of adenylyl cyclase. The reduced basal levels of enzymatic activity and the lack of significant differences in agonist-induced activation of the system suggest that an amplification in the system must occur beyond the level of adenylyl cyclase or in the efficiency with which the system operates at the cellular level.

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Send reprint requests to: Dr. David A. Taylor, Department of Pharmacology/Toxicology, WVU Health Sciences Center, PO Box 9223, Morgantown, WV 26506-9223.