

# Abnormal Guanine Nucleotide Regulatory Protein in MVP Dysautonomia: Evidence from Reconstitution of G<sub>s</sub>\*

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**ABSTRACT.** We and others have used the term MVP dysautonomia for a particular subset of hyperadrenergic dysautonomia patients. The role of the stimulatory guanine nucleotide regulatory protein (G<sub>s</sub>) in this dysautonomia was studied by cholera toxin-dependent extraction of G<sub>s</sub> from erythrocytes from 11 normal subjects and 14 symptomatic dysautonomia patients and reconstitution into *cyc*<sup>-</sup>S49 lymphoma membranes, which have normal receptor and adenylyl cyclase but lack G<sub>s</sub>. Isoproterenol-stimulated adenylyl cyclase activity in the dysautonomia group was increased compared to that in controls [3.66 ± 0.20 (mean ± SE; n = 14) vs. 2.87 ± 0.14 (n = 11) U *cyc*<sup>-</sup> reconstituted activity/mg erythrocyte protein; P < 0.05]. β-Adrenergic receptor high affinity state

formation was greatest in the severely symptomatic group [K<sub>L</sub>/K<sub>H</sub>: severe symptoms, 130 ± 48 (n = 6); mild symptoms, 33 ± 7 (n = 7); control, 27 ± 6 (n = 11); severe dysautonomia distinct, P < 0.017]. Sodium dodecyl sulfate-polyacrylamide gels of cholera toxin-dependent ADP-ribosylated G-proteins yielded no gross distinction between severely symptomatic and control groups. This subset of hyperadrenergic dysautonomia patients, thus, has supercoupled β<sub>2</sub>-adrenergic receptors (increase in both agonist binding and cyclase activation) conferred by an abnormal G<sub>s</sub>, whose effects on agonist binding reflect the severity of illness. (*J Clin Endocrinol Metab* 72: 867-875, 1991)

**A**MONG the several forms of hyperadrenergic dysautonomias, there is a specific subtype that has been distinguished based upon a distinctive pattern of abnormal cardiovascular autonomic function (1-5). No satisfactory term exists for this subtype, but one of the most commonly applied terms is MVP dysautonomia. This term reflects the historical notion that the dysautonomia is somehow associated with findings of mitral valvular prolapse (MVP), but unfortunately, this term has frequently been misconstrued to imply a more definite and obligatory link to MVP than has been established. Indeed, among patients with this pattern of dysautonomia the coexistence of MVP is inconstant. In this report we retain the term MVP dysautonomia for historical purposes, but wish to focus upon the biochemical defects underlying the dysautonomia rather than upon the putative connection to valvular defects.

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This dysautonomia is characterized subjectively by asthenia, chest pain, syncope or presyncope, orthostatic palpitations or weakness, and symptoms of vasospasm (1, 2, 6-9). We and others have identified in such patients a distinctive objective pattern of dysautonomia upon invasive examination (1-4). Table 1 summarizes the more prominent abnormalities (1, 2). Many symptoms and signs of MVP dysautonomia suggest β-adrenergic hypersensitivity. We have shown that the neutrophil β-adrenergic receptor from these patients is biochemically supersensitive, or supercoupled (1). The supercoupling consists of both increased agonist binding to the receptor (expressed as an increase in the ratio of dissociation constants for the low and high affinity states of the receptor, K<sub>L</sub>/K<sub>H</sub>) and an increase in isoproterenol-stimulated cAMP accumulation (1). As noted in a recent editorial in *Lancet*, this work not only addresses the pathophysiology of this disease process, but also illuminates basic receptor activation processes (10).

The enhanced high affinity state formation could explain many hyperadrenergic features of this dysautonomia, but other features, such as α-adrenergic hypersensitivity, vasospasm, headache, skeletal abnormalities, and gastrointestinal symptoms, are not as well explained

by these  $\beta$ -adrenergic receptor abnormalities. We postulated a unifying explanation that there may be a central defect impacting upon several signal transduction systems, including the  $\beta$ -adrenergic system. Abnormal guanine nucleotide regulatory proteins (G-proteins) could produce such a central defect, given their involvement in numerous systems. The stimulatory G-protein associated with the  $\beta$ -adrenergic receptor is  $G_s$ .  $G_s$  is required for formation of the high affinity, activated state of the  $\beta$ -adrenergic receptor,  $HR_H \cdot G_s^{*GTP}$ , and is, thus, essential to coupling of receptor occupation to enzyme activation (11-13).

$\beta$ -Adrenergic receptor function is actively regulated, frequently via alterations in formation of the high affinity  $HR_H \cdot G_s^{*GTP}$  complex (14, 15). Alterations in high affinity state formation may be reflected by alterations in  $K_L/K_H$  (15-18). We have shown that alterations in  $K_L/K_H$  correspond to alterations in isoproterenol-stimulated cAMP accumulation and in *in vivo* physiological responsiveness of the  $\beta$ -adrenergic system (1). Thus,  $K_L/K_H$  reflects coupling of receptor occupation to enzyme activation, and its determination is one of several ways to assess the state of coupling of the receptor system.

The current study addressed the specific hypothesis that the  $\beta$ -adrenergic receptor supercoupling and supersensitivity observed in this subset of hyperadrenergic dysautonomia (MVP dysautonomia) is a consequence of a functional abnormality in the  $G_s$  of these patients. Our approach involved making a preparation of  $G_s$  from erythrocytes (used because they are an abundant source) from these patients and reconstituting this  $G_s$  into *cyc* S49 murine lymphoma cell membranes. *cyc* S49 murine lymphoma cells possess normal  $\beta$ -adrenergic receptors as well as normal adenylyl cyclase, but lack  $G_s$ . We were, thus, able to specifically assess the functional effects of both normal and putatively abnormal  $G_s$  upon both agonist-binding properties and adenylyl cyclase activation.

## Materials and Methods

### Patients

Fourteen symptomatic women between the ages of 25 and 46 yr were recruited for this study from our larger population of MVP dysautonomic patients within our research clinics of the Section on Hypertension and Clinical Pharmacology of Baylor College of Medicine and The Methodist Hospital. To be included patients had both 1) a compatible history of symptoms

TABLE 1. The typical pattern of abnormalities of physiological studies in MVP dysautonomia (1, 7-9)

- |   |
|---|
| 1. Exaggerated cardioacceleration                                 |
| A. During phase II of the Valsalva maneuver.                      |
| B. Upon standing.   |
| C. Upon administration of isoproterenol.                          |
| 2. Exaggerated vasodilation upon administration of isoproterenol. |

of MVP dysautonomia, as enumerated below, and 2) objective identification of the cardiovascular dysautonomia (see below). The symptoms experienced included palpitations, orthostatic lightheadedness, nonexertional chest pain, headaches, or others (1, 2), and typically occurred as combinations of symptoms. Objective identification of the typical cardiovascular dysautonomia we have reported (1, 2) involved performing quantitative autonomic nervous system function testing. All but one of these patients, therefore, had the dysautonomic features listed in Table 1. The one exception declined an invasive examination, but did manifest exaggerated cardioacceleration in phase II and upon standing, and had postural hypotension; we could not assess the other features adequately without an arterial catheter. Nine of the patients in the current study were indeed part of the group through which we identified the dysautonomia in Ref. 1, and their physiological data were reported therein. The patients were primarily selected for their findings of dysautonomia, but the presence of MVP was documented in all 13 of the patients who underwent M-mode and two-dimensional echocardiographic study, read in a blinded fashion by expert echocardiographers unconnected to this study. In all patients a nonejection systolic click, midsystolic murmur, or both were present.

The combination of historical features and objective evidence for dysautonomia allow these patients to be distinguished from both normal individuals and patients with other forms of dysautonomia. All of these patients have been clinically followed by us for a number of years, and most have undergone several research and clinical evaluations. This long term familiarity with the patients aided in ensuring that a distinct clinical population was chosen.

The range of disease severity was intentionally broad. These patients are known to vary substantially over time as to the nature and severity of their illness. We developed a scale for disease severity that reflected the longitudinal impact of the illness upon their lives. Disease severity was judged according to the historical presence at any time of the following three factors: 1) a history that symptoms had necessitated pharmacotherapy (fludrocortisone and salt,  $\beta$ -adrenergic antagonist, calcium slow channel antagonist, or combinations) or frequent adjustments in either dosage or regimen, 2) the patient's assessment that the symptoms had at some time significantly altered her routine daily activities or work performance, and 3) the history of a serious manifestation or complication of the illness, such as a stroke or pulseless extremity. For each patient a score of either 0 or 1 was given for each factor, A through C and a sum ranging from 0-3 were then determined for each patient. The patient group could be subdivided into two subgroups: mildly symptomatic patients, defined as those with sums of 0 or 1 (mean sum = 0.7), and severely symptomatic patients, defined as those with sums of 2 or 3 (mean sum = 2.6). Each patient was studied biochemically during a clinically stable phase in which symptoms and medications were not undergoing change. Patients remained on their usual medication regimens at the time of the biochemical studies; several patients were receiving fludrocortisone or NaCl, but only one patient was receiving another active drug at the time of study. That patient was found retrospectively to have been using

propranolol intermittently at the time of the biochemical studies. The physiological studies had been performed when all indications had been withheld.

The normal control group consisted of 11 female volunteers, aged 22–39 yr, who had no symptoms suggestive of dysautonomia. These volunteers were free of any substantial chronic illness and were not on medication. Using the scoring system described above, all of the normal volunteers had scores of zero. Both patients and controls were free of recent identifiable infection. Seven of the controls had undergone physiological evaluation as part of a previous study of  $\beta$ -adrenergic receptor function (1). Thus, these controls had undergone extensive invasive assessment of cardiovascular autonomic function and were known in detail to be normal and to be physiologically distinct from the symptomatic patient group. However, the biochemical data presented here are new and have not been presented elsewhere, except as noted in Table 2. The remaining controls did not have identifiable postural tachycardia or hypotension, but did not undergo full invasive studies.

#### Membrane preparations

Erythrocyte membranes were prepared according to the procedure described by Codina *et al.* (19). Ten milliliters of blood

TABLE 2. Binding parameters derived from competition of (-)-isoproterenol with [<sup>125</sup>I]CYP in native neutrophil  $\beta_2$ -adrenergic receptor-adenylyl cyclase systems

	K <sub>L</sub> (nM)	K <sub>H</sub> (nM)	K <sub>L</sub> /K <sub>H</sub>	%R <sub>H</sub>
Patients				
1 <sup>a</sup>	1400	6.3	230	67
2	72	0.069	1100	27
3	1100	9.9	120	72
4	540	4.1	130	76
5	400	1.7	230	56
6 <sup>a</sup>	330	0.40	840	36
7 <sup>a</sup>	1100	2.9	390	73
8	680	2.3	300	62
9 <sup>a</sup>	1200	9.9	120	60
Mean			380	
SEM			120	
Median	680	2.9	230	62
Control subjects				
1	120	1.7	69	34
2	130	1.8	71	43
3	130	1.6	77	41
4	170	3.5	50	58
5	150	8.5	36	58
6	610	8.4	73	58
7	220	4.7	46	58
8	220	4.7	46	47
9	95	2.9	32	48
Mean			56	
SEM			5.7	
Median	150	3.5	50	48
P, control vs. dysautonomia	0.0035	NS	0.012	NS

<sup>a</sup> Patient numbers do not correspond to those of Table 3. Data from Ref. 1.

were drawn into a heparinized syringe and transported on ice. The blood was mixed with equal volumes of buffer (10 mM sodium phosphate at pH 8.0 and 150 mM NaCl at 4 C). The mixture was centrifuged at 5000 rpm at 4 C for 15 min with a Sorvall RC5B in a HB-4 rotor (Sorvall, Norwalk, CT). The supernatant, including the leukocyte layer, was discarded. The erythrocyte pellet was rinsed two additional times with phosphate-saline buffer.

The erythrocytes were lysed by hypotonic shock with 40 vol 10 mM sodium phosphate at pH 8.0, and the membranes were collected by centrifugation in a GSA rotor for 30 min at 10,000 rpm at 4 C. The membrane pellets were washed twice more with 10 mM sodium phosphate, followed by centrifugation in a GSA rotor. The membrane pellets (4–5 mg protein/mL) were stored at -70 C in aliquots of 100  $\mu$ L.

*Cyc* S49 murine lymphoma cells were grown according to the method of Bourne *et al.* (20), and the membranes were prepared according to the procedure of Ross *et al.* (21), except that the membrane purification was stopped after preparation of the 53,000  $\times g$  pellet and Mg<sup>2+</sup>-free buffers were used throughout. These preparations were performed in the Molecular Endocrinology Core Laboratory of the Diabetes Center of Baylor College of Medicine.

A standardized G<sub>s</sub> preparation was required, since the adenylyl cyclase of various *cyc* S49 membrane preparations varies 2-fold in its capacity to be reconstituted to full activity by G<sub>s</sub>-containing cholate extracts. This was performed by extracting G<sub>s</sub> with cholate from erythrocyte membranes pooled from the patient and control samples. The membrane pool was extracted with cholate by the addition of 20 mM  $\beta$ -mercaptoethanol, 10 mM MgCl<sub>2</sub>, and 1% sodium cholate (final concentrations), with incubation on ice with intermittent vortexing for 45 min and centrifugation at 100,000  $\times g$  for 45 min at 4 C. Sodium EDTA (11 mM, final concentration) was added to the supernatant to chelate free magnesium ion. After vortexing, ethylene glycol was added (30%, final concentration), and the preparation was stored in 10- $\mu$ L aliquots at -70 C. Aliquots of G<sub>s</sub> from the pooled cholate-extracted erythrocyte membranes were used with each assay for adenylyl cyclase activity. G<sub>s</sub>-containing cholate extracts were also made individually from each subject and volunteer using the same method.

#### Adenylyl cyclase activity

*Activity with guanosine 5'-0-(3-thiotriphosphate (GTP $\gamma$ S) and NaF.* Crude cholate extract (43–64  $\mu$ g protein/ $\mu$ L) was diluted 1:200 to a final concentration of 0.005% cholate in 10 mM Tris, 1 mM EDTA, 20 mM  $\beta$ -mercaptoethanol, and 0.1% BSA. Then, 10  $\mu$ L of this diluted sample were mixed with 10  $\mu$ L *cyc* S49 membrane (10  $\mu$ g), and 30  $\mu$ L reaction mixture were added to each tube to give a final concentration of 10 mM Tris (pH 8.0), 1 mM EDTA, 1 mM [<sup>3</sup>H]cAMP (~10,000 cpm/50  $\mu$ L sample), 0.1 mM [ $\alpha$ -<sup>32</sup>P]ATP (5–10  $\times 10^6$   $\mu$ L sample), 10 mM MgCl<sub>2</sub>, 20 mM creatine phosphate, 0.2 mg/mL creatine phosphokinase, 0.02 mg/mL myokinase, and 100 nM GTP $\gamma$ S or 10 mM NaF, depending on the assay.

Incubations were performed in triplicate at 32 C for 40 min, and the reactions were stopped by the addition of 100  $\mu$ L adenylyl cyclase stop solution (19). Separation of [<sup>32</sup>P]cAMP

from [ $\alpha$ - $^{32}$ P]ATP was carried out as described by Salomon *et al.* (22) with modifications (23). The activity was a linear function of the quantity of cholate extract used when the concentration of cholate was kept constant (0.001% in the assay).

**Activity with isoproterenol.** In this set of experiments the cholate extract (43–64  $\mu$ g protein/ $\mu$ L) was diluted 1:40 in 10 mM Tris (pH 8.0), 1 mM EDTA, 20 mM  $\beta$ -mercaptoethanol, and 0.1% BSA. Twenty microliters of this diluted cholate extract were mixed with 20  $\mu$ L *cyc*<sup>-</sup> S49 membranes (6–7 mg protein/mL), followed by 40  $\mu$ L 0.6 mM ATP, 10 mM Tris (pH 8.0), 10 mM MgCl<sub>2</sub>, 20 mM creatine phosphate, 0.2 mg/mL creatine phosphokinase, and 0.02 mg/mL myokinase, and G<sub>s</sub> was allowed to integrate into the *cyc*<sup>-</sup> membranes for 20 min at 32 C.

Reconstituted S49 *cyc*<sup>-</sup> adenylyl cyclase activity was then determined by the addition of 10  $\mu$ L of the above mixture to 40  $\mu$ L reagent to give the final standard assay conditions in the presence of 100  $\mu$ M GTP and in the presence or absence of 1  $\mu$ M isoproterenol. These final incubations (three in the presence of GTP and three in the presence of GTP plus isoproterenol) were carried out at 32 C for 20 min and were stopped as described above.

#### G<sub>s</sub> ADP ribosylation and gel electrophoresis

**Cholera toxin (CTX).** Thirty-five micrograms of erythrocyte membrane were incubated in duplicate, as described by Scherer *et al.* (24), at 32 C for 30 min in the presence of 15 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10 mM thymidine, 1 mM ATP, 1 mM GTP, 10 mM MgCl<sub>2</sub>, 2 mM dithiothreitol, 300 mM potassium phosphate, and 10<sup>7</sup> cpm [ $^{32}$ P]NAD in a total volume of 60  $\mu$ L containing 5  $\mu$ g activated CTX (List Biological Laboratories, Inc., Campbell, CA). Activation of CTX was performed as described previously (25).

After the incubation, the samples were processed, as described by Scherer *et al.* (24), and applied either to a standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) 10% acrylamide gel, as described by Laemmli (26), or to a 9% acrylamide gel with a gradient of urea from 4–8 M (24). The gels were stained, destained, and finally autoradiographed using two Lighting Plus intensifier screens (DuPont, Wilmington, DE).

**Pertussis toxin (PTX).** The method used was similar to the one described for ADP ribosylation in the presence of CTX. Thirty-five micrograms of erythrocyte membrane were incubated at 32 C in the presence of 15 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10 mM thymidine, 1 mM ATP, 0.1 mM GDP $\beta$ S, 2 mM dithiothreitol, 0.152% Lubrol PX, 10<sup>7</sup> cpm [ $^{32}$ P]NAD, and 0.5  $\mu$ g activated PTX (given to us by Dr. Ronald Sekura, NICHD) in a final volume of 60  $\mu$ L. Activation of PTX was performed as described previously (26). After 30 min of incubation, the samples were processed as described by Scherer *et al.* (24).

#### Radioligand binding in reconstituted *cyc*<sup>-</sup> membranes

Crude preparations of G<sub>s</sub> were obtained, as described by Codina *et al.* (19), from erythrocyte membranes stored at -70 C. After thawing, 50  $\mu$ L 10% sodium cholate, 10  $\mu$ L 1 M  $\beta$ -

mercaptoethanol, and 25  $\mu$ L 200 mM MgCl<sub>2</sub> were added to a 500- $\mu$ L aliquot of each sample. This preparation was kept on ice for 60 min, with vortexing every 10 min. The mixtures were then centrifuged at 30,000  $\times$  g for 30 min, and G-proteins in the supernatant were concentrated by centrifugation of the samples in a Centricon microconcentrator tube (Amicon Division, W. R. Grace Co., Danvers, MA), using either the Centricon-10 at 5,500 rpm or the Centricon-30 at 4,000 rpm, at 4 C for 60 min. The volume of concentrated protein obtained was diluted 1:5 by the addition of buffer (10 mM Tris at pH 8.0, 1 mM sodium EDTA, 20 mM  $\beta$ -mercaptoethanol, and 0.1% BSA), giving a final cholate concentration of 0.2%.

After thawing, *cyc*<sup>-</sup> membranes (5–10 mg/mL) were diluted to a concentration of 1 mg protein/mL in 10 mM Tris, pH 8.0, and 1 mM EDTA. The *cyc*<sup>-</sup> membranes were then reconstituted with the extracted G<sub>s</sub> by incubating 500  $\mu$ L of the *cyc*<sup>-</sup> membrane preparation with 500  $\mu$ L cholate extract and 50  $\mu$ L 20 mM MgCl<sub>2</sub> and 3% sodium cholate at 32 C for 20 min in a shaking water bath. Thus, during the incubation the concentration of cholate was maintained at 0.25–0.35%, and that of free Mg<sup>2+</sup> ion (excess over EDTA) at less than 1 mM. To make a membrane preparation suitable for radioligand binding assays of agonist interactions with  $\beta$ -adrenergic receptors, the reconstituted *cyc*<sup>-</sup> membrane preparation was diluted with 10 mL 75 mM Tris (pH 7.5) and 7.5 mM MgCl<sub>2</sub> and subjected to Polytron action (8 sec at a setting of 7.5 in the cold; Brinkmann, Westbury, NY). This preparation was then centrifuged at 12,000 rpm in a SS-34 rotor for 10 min at 4 C. The supernatant was centrifuged at 18,000 rpm in a SS-34 rotor for 10 min at 4 C. The final membrane resuspension was in 10 mL of a buffer consisting of 50 mM Tris, pH 8.0, and 10 mM MgCl<sub>2</sub>.

Radioligand binding assays were performed in a total volume of 150  $\mu$ L containing 100  $\mu$ L reconstituted membrane (~40  $\mu$ g protein), 25  $\mu$ L 20 pM [ $^{125}$ I]iodocyanopindolol ([ $^{125}$ I]CYP; Amersham Corp., Arlington Heights, IL), and 25  $\mu$ L of either 6  $\times$  10<sup>-3</sup> M ascorbic acid or a series of dilutions of (-)-isoproterenol (10<sup>-9</sup>–10<sup>-3</sup> M, final) in 6  $\times$  10<sup>-3</sup> M ascorbic acid. The incubation lasted 40 min at 37 C. The binding was terminated by rapid filtration through GFC filters and washing with 25 mL 75 mM Tris, pH 7.4, and 25 mM MgCl<sub>2</sub>. The filters containing the bound radioactivity were then counted.

#### Curve analysis

Binding curves for the competition of isoproterenol for binding of [ $^{125}$ I]CYP to  $\beta$ -adrenergic receptors were analyzed individually. The details of these analyses were previously reported (15). Briefly, an iterative curve-modelling technique was used which creates a set of simultaneous equations expressing the law of mass action using each of the empiric points. The simultaneous equations are solved for parameter estimates, including the affinities of isoproterenol for the low and high affinity states of the receptor, and the proportion of receptors in the high affinity state (%R<sub>H</sub>). The modelling procedure tests successively more complex models (one site, two sites, etc.), comparing fits by F test to determine the best fit and, therefore, the best model for that curve. When two states of the receptor were detected, their dissociation constants were designated K<sub>L</sub> and K<sub>H</sub> for the low (HR<sub>L</sub>) and high affinity (HR<sub>H</sub>·G<sub>s</sub><sup>-GTP</sup>)

states, respectively. It is important to note that the terms  $K_L$  and  $K_H$  refer to the dissociation constants of isoproterenol for interactions with these states of the receptor; the terms  $K_L$  and  $K_H$  do not refer to the states themselves. The methods used for statistical comparison of parameter estimates have previously been described (14). Each curve was fitted individually; the parameter estimates were meaned and compared by unpaired *t* test and Wilcoxon test.

## Results

### Native neutrophils: agonist binding

Agonist binding to  $\beta_2$ -adrenergic receptors was assessed in native neutrophil membranes before either reconstitution or disruption of the natural association of receptor-G-protein-cyclase components. Data for the binding of isoproterenol to native neutrophil  $\beta_2$ -adrenergic receptors were available for nine control volunteers and nine patients (Table 2). We have previously shown that the amount of  $\beta_2$ -adrenergic receptors and their affinity for the antagonist [<sup>125</sup>I]CYP were not different in the MVP dysautonomia patients and normal subjects (1). The values obtained for  $K_L$  and  $K_H/K_H$  in the control group were similar to previous normal values in this laboratory (1, 16–19). In contrast, the  $K_L/K_H$  values for the symptomatic patients were higher than the typical normal values in our laboratory and were statistically significantly distinguishable from those of the control group. As we have previously demonstrated, increasing  $K_L/K_H$  implies greater formation of  $HR_H \cdot G_s^{*GTP}$  (11–19). These observations of enhanced formation of the high affinity state of the  $\beta_2$ -adrenergic receptor are not solely due to medication, since several symptomatic patients in both subgroups were not receiving any medications at the time of the study. The symptomatic patient group in the current study demonstrated biochemical  $\beta_2$ -adrenergic receptor supersensitivity in a manner similar to our previous study group of symptomatic MVP dysautonomic patients, even when patients 1, 6, 7, and 9 are ignored because they were in the previous report (1).

### $G_s$ reconstituted into S49 *cyc*<sup>-</sup> membranes

**Adenylyl cyclase activation.**  $G_s$  was successfully reconstituted into *cyc*<sup>-</sup> S49 lymphoma membranes, as assessed by the effectiveness of (-)-isoproterenol in activating adenylyl cyclase. Table 3 contains a summary of adenylyl cyclase activities of the *cyc*<sup>-</sup> membranes after reconstitution with normal control  $G_s$  and after reconstitution with  $G_s$  from symptomatic patients. Before reconstitution, these membranes were essentially devoid of stimuable adenylyl cyclase activity. However, after reconstitution, stimuable adenylyl cyclase activity was restored. Further, when symptomatic patients were used as the source of  $G_s$ , there was a statistically significant incre-

TABLE 3. Isoproterenol-stimulated adenylyl cyclase activities in reconstituted  $\beta$ -adrenergic receptor-adenylyl cyclase systems for normal volunteers and symptomatic patients

Patient no.	Control volunteer	Symptomatic patient
1	2.9	4.9
2	3.4	4.0
3	3.2	4.2
4	2.8	3.4
5	3.4	2.3
6	3.7	3.0
7	1.7	3.2
8	2.3	5.0
9	3.0	4.3
10	2.0	4.2
11	3.3	3.7
12		3.1
13		2.6
14		3.5
Mean	2.9 ± 0.19	3.7 ± 0.22
Median	3.0	3.6
<i>P</i> value, control vs. patient		0.02

Each value represents the difference between the GTP- plus isoproterenol-stimulated activity and that of GTP alone. Activity is expressed as units *cyc*<sup>-</sup> reconstituted activity per mg erythrocyte protein, when 1 U = nmol cAMP formed/40 min assay by approximately 10  $\mu$ g *cyc*<sup>-</sup> membrane. Patients and controls are not paired, but are simply listed in columns in their enrollment sequences. Each value represents a mean of two experiments, in each of which triplicate determinations were made.

ment in (-)-isoproterenol-stimulated adenylyl cyclase activity compared to the control reconstituted preparation (Table 3). Fluoride-stimulated adenylyl cyclase activity was also increased in the patient group compared to the controls (median, 19; range, 14–25; vs. median, 16; range, 12–23 U *cyc*<sup>-</sup> reconstituted activity/mg erythrocyte protein; *P* < 0.05, by Wilcoxon test). Fluoride stimulates adenylyl cyclase through  $G_s$ , with no known interaction with the  $\beta$ -adrenergic receptor. Similarly, GTP analogs such as GTP $\gamma$ S stimulate adenylyl cyclase through interactions with  $G_s$ , which do not involve  $\beta$ -adrenergic receptors. GTP $\gamma$ S-stimulated adenylyl cyclase activity was 5.5 ± 1.5 (controls) vs. 6.5 ± 1 (patients) U *cyc*<sup>-</sup> reconstituted activity/mg erythrocyte protein (*P* = NS). There was also no difference between mildly and severely symptomatic patients in their activation of adenylyl cyclase by any agonist. Taken together, these data indicate that the  $G_s$  from symptomatic patients stimulates adenylyl cyclase more than that of normal controls, and that this superactivation may be initiated by either adrenergic agonists or NaF.

**$\beta$ -Adrenergic receptor-agonist interactions.** In a manner similar to that of adenylyl cyclase activation described above, reconstitution of the capacity to form the high affinity state of the  $\beta$ -adrenergic receptor was successful.

Figure 1 represents competition curves derived from both control and MVP  $\beta_2$ -adrenergic receptors after reconstitution. Table 4 contains a summary of the binding parameters obtained in the control and symptomatic patient groups. The data for the patients are presented as subgroups of mildly and severely symptomatic patients. This manner of displaying the data highlights the relationship between the severity of illness and the ability to form the high affinity state of the receptor. As a group,  $G_s$  from the symptomatic patients was more active in forming the high affinity state, as assessed by the statistically significant rise in the  $K_L/K_H$  ratio. This rise in  $K_L/K_H$  accompanied by a rise in (-)-isoproterenol-stimulated adenylyl cyclase activity indicates that the reconstituted  $\beta$ -adrenergic receptor-adenylyl cyclase system was supercoupled, as was the native neutrophil  $\beta$ -adrenergic receptor-adenylyl-cyclase system from similar patients (1) (Table 2). When the patient group was subgrouped as to severity of symptoms, there was an even greater distinction between the  $K_L/K_H$  values. The severely symptomatic patients had values much higher than typical for either native normal or reconstituted normal systems. The subgroup with milder symptoms had intermediate values. When the component  $K_L$  and  $K_H$  values were examined, it became clear that the values are the most altered in the patient groups. There was relatively little change in  $K_L$  values. It is important to note that by convention we do not directly relate estimates of affinities of agonists for the two states of the  $\beta$ -adrenergic receptor. Rather, the dissociation constants

$K_L$  and  $K_H$  are given. Thus, the numerical value of  $K_H$  decreases as the affinity of isoproterenol for the high affinity state increases. When expressed as an affinity constant, the affinity of isoproterenol for the high affinity state increases as symptomatic scores increase ( $P < 0.05$ ).

A comparison was made of the binding parameters derived from the reconstituted systems with those derived from native neutrophils. Comparing group data, the elevated  $K_L/K_H$  observed in the native neutrophil preparations from dysautonomic patients corresponded to the elevated  $K_L/K_H$  observed in the reconstituted systems in which the  $G_s$  was derived from the same dysautonomic patients (Tables 2 and 4). However, there was no precise correspondence in  $K_L/K_H$  within the individual patient between the two preparations. The absence of a tight correspondence within the individuals is not entirely surprising, since the observations in native neutrophils were obtained 6–24 months previous to these reconstitution experiments, with substantial changes in medications and symptomatic state occurring in the interim.

#### ADP ribosylation of $\alpha$ -subunits

Taken together the adenylyl cyclase and agonist binding data demonstrate that  $G_s$  derived from the patient group is distinct from normal  $G_s$ , leading to the subhypothesis that such functional distinctions could arise from dysautonomia-associated differences in the amount

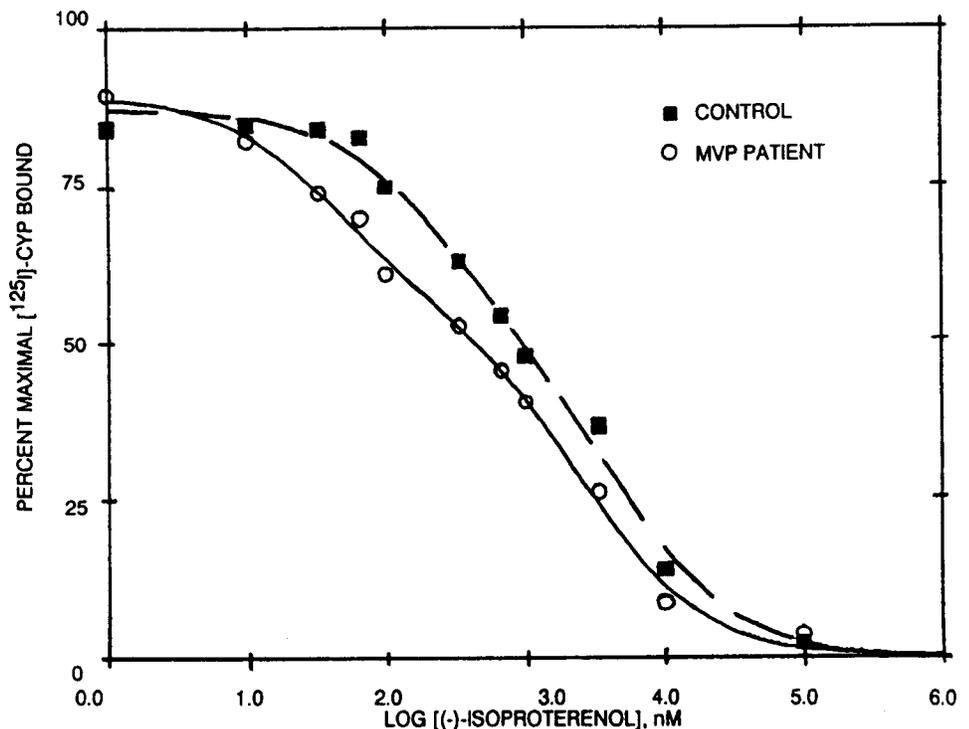


FIG. 1. Competition curves for the competition of (-)-isoproterenol (on abscissa) with [ $^{125}$ I]CYP (on ordinate) for binding to  $\beta$ -adrenergic receptors in reconstituted  $\beta$ -adrenergic receptor-adenylyl cyclase systems. Control curve;  $\square$ , the symptomatic group.

TABLE 4. Binding parameters derived from competition of (-)-isoproterenol with [<sup>125</sup>I]CYP in reconstituted  $\beta$ -adrenergic receptor-adenylyl cyclase systems

Subject no.	K <sub>L</sub> (nM)	K <sub>H</sub> (nM)	K <sub>L</sub> /K <sub>H</sub>	%R <sub>H</sub>
<b>Severely symptomatic</b>				
1	600	5.2	120	17
2	590	11	54	26
3	140	0.39	350	17
4	150	1.0	150	32
5	720	13	55	73
6	660	14	48	55
Mean			130 ± 48 <sup>a</sup>	
Median	600 <sup>b</sup>	11 <sup>c</sup>	85 <sup>d</sup>	32
<b>Mildly symptomatic</b>				
1	350	8.6	40	31
2	450	18	25	15
3	1400	33	42	46
4	1500	30	50	45
5	930	48	20	19
6	*		1	0
7	480	9.1	53	39
Mean			33 ± 6	
Median	710	24	40	31
<b>Control subjects</b>				
1	1000	79	13	56
2	990	18	54	47
3	*		1	0
4	1000	35	29	39
5	1100	19	57	29
6	560	16	34	24
7	1100	56	20	60
8 <sup>f</sup>	1100	61	9.3	45
9 <sup>f</sup>	1500	20	48	31
10	1500	75	20	51
11 <sup>f</sup>	1000	57	9.3	45
Mean			27 ± 19	
Median	1000	46	20	45

The terms K<sub>L</sub>, K<sub>H</sub>, K<sub>L</sub>/K<sub>H</sub>, and %R<sub>H</sub> are defined in the text. Median values are provided, since most parameters were not normally distributed, and medians were compared by Wilcoxon test. Means are provided for K<sub>L</sub>/K<sub>H</sub> values which are normally distributed; the unpaired *t* test was used for comparisons. K<sub>L</sub> and K<sub>H</sub> values do not have normal distributions, so means are not provided and comparisons were non-parametric. In one severely symptomatic patient no interpretable binding curve was obtained.

<sup>a</sup> *P* < 0.017 vs. control.

<sup>b</sup> *P* < 0.003 vs. control.

<sup>c</sup> *P* < 0.001 vs. control.

<sup>d</sup> *P* < 0.005 vs. control and *P* < 0.005 vs. mildly symptomatic patients.

<sup>e</sup> No high affinity state could be detected. The limit of detection by these methods is about 15 for K<sub>L</sub>/K<sub>H</sub> and 10 for %R<sub>H</sub>. When no high affinity state is detected, the affinities for the two states are assumed to approach equality, thus giving a K<sub>L</sub>/K<sub>H</sub> of 1 and %R<sub>H</sub> of 0.

<sup>f</sup> Each value for these controls represents a mean of two determinations.

or structure of G<sub>sa</sub>. Gel electrophoresis of ADP-ribosylated  $\alpha$ -subunits allows an assessment of 1) the property of ADP ribosylation, 2) the approximate mol wt of  $\alpha$ -

subunits, and 3) a very gross estimate of the amount of  $\alpha$ -subunit present. We, thus, examined ADP ribosylation in five controls and five of the most symptomatic patients who had high adenylyl cyclase activation values. ADP ribosylation of the  $\alpha$ -subunit of G<sub>s</sub> is specifically catalyzed by CTX, while ADP ribosylation of the corresponding  $\alpha$ -subunits of the inhibitory protein G<sub>i</sub> is specifically catalyzed by PTX. The former subunit of G<sub>s</sub> is referred to as G<sub>sa</sub>, while there are several  $\alpha$ -subunits of G<sub>i</sub>, referred to as G<sub>ia</sub>. We examined both stimulatory and inhibitory subunits, since conceivably a functional increase in stimulatory activity could arise from a functional diminution of inhibitory activity. The conditions of the gel electrophoresis can be adjusted to optimally distinguish among these subunits (Fig. 2). In the control group the labeled bands thus identified were characteristic for human G<sub>sa</sub> and several species of G<sub>ia</sub> (24). The bands identified in the patient group were indistinguishable from those in the control group. Thus, the property of ADP ribosylation was similar in patient and control groups. The proteins' mobilities on the gels were similar, suggesting that no apparent major difference existed in mol wt.

The careful matching of the preparatory schemes allows a gross assessment of  $\alpha$ -subunit amounts; no difference was detected between control and patient groups by this method. There was, thus, no evidence for either a major deficiency or excess of  $\alpha$ -subunits or a major difference in mol wt. These methods do not allow a

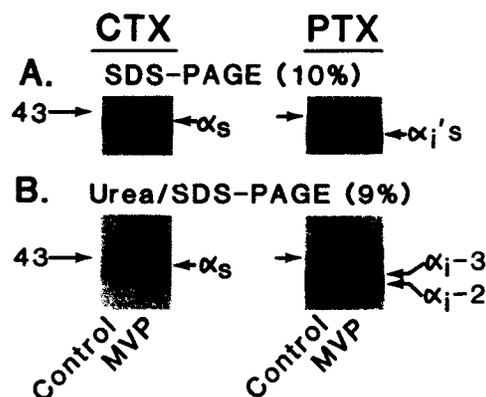


FIG. 2. These are photographs of gels derived from both control and severely symptomatic patients. G<sub>sa</sub> and G<sub>ia</sub> were ribosylated by toxins and analyzed by SDS-PAGE. In A, the separating gel contained 10% polyacrylamide. In B, the separating gel contained 9% polyacrylamide and a 4- to 8-M linear urea gradient (urea gradient/SDS-PAGE), which allowed several G<sub>ia</sub>-subunits to be distinguished. The *lefthand photo* in each pair is labeled CTH, indicating that ADP ribosylation was catalyzed by CTX. The *right hand photo* of each pair is labeled PTX, indicating that the ADP-ribosylation was catalyzed by PTX. CTX-catalyzed ADP-ribosylation acts upon G<sub>sa</sub> subunits, whereas the PTH-catalyzed ADP-ribosylation acts upon G<sub>ia</sub>-subunits, which are observed as at least two distinct entities by urea gradient/SDS-PAGE separation. No major structural difference was seen between control and symptomatic patients for either stimulatory or inhibitory G-proteins.

determination of more selective alterations, such as changes in one or few critical amino acids.

### Discussion

We have previously shown that physiological  $\beta$ -adrenergic hypersensitivity is a component of the cardiovascular dysautonomia of patients symptomatic with MVP<sup>d</sup> dysautonomia (1, 2). Underlying this physiological hypersensitivity is a biochemical hypersensitivity, supercoupling (1). In the current study we demonstrate in patients with this type of dysautonomia that the supercoupling is conferred by a functionally altered  $G_s$ . The functionally altered  $G_s$  confers two properties upon the  $\beta$ -adrenergic-adenylyl cyclase system. First, there is enhanced activation of the adenylyl cyclase enzyme itself, which can be initiated by either  $\beta$ -adrenergic receptor occupation and activation or the nonadrenergic receptor-mediated (but G-protein-dependent) fluoride mechanism. Second, the  $G_s$  from these patients facilitates greater high affinity state formation than does  $G_s$  from normal subjects. The superactive  $G_s$  is sufficient to explain the enhanced formation of  $HR_H \cdot G_s^{*GTP}$ .

$G_s$  is a heterotrimer of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -subunits, the  $\beta$ - and  $\gamma$ -subunits of which are shared with other G-proteins (27). A single functional change in a  $\beta$ -subunit could potentially affect many G-protein systems. Further, large regions of amino acid sequences are shared among the various  $\alpha$ -subunits. Several G-protein systems could be affected if their  $\alpha$ -subunits shared abnormalities of amino acid sequence in a functionally critical region. A unifying explanation for abnormalities of multiple signal transduction systems in MVP dysautonomia could be a commonly held defect in a class of G-proteins (including  $G_s$ ).

Our observation that altered agonist-binding behavior and altered adenylyl cyclase activation were separable properties of the altered  $G_s$  may provide insight into the variable expressivity of the illness. Activation of adenylyl cyclase appears to be altered as a general property of an abnormal  $G_s$  and may, thus, be a marker of the presence of illness. On the other hand, the abnormal agonist-binding properties are related to disease severity and may represent the properties most closely associated with symptomatic expression of the illness.  $\beta$ -Adrenergic receptor-agonist interactions are known to undergo substantial regulation in both upward and downward directions in response to a number of physiological and pharmacological influences. It is understandable, then, that the highly regulatable agonist-binding properties would correspond to symptoms, thus linking the disease-producing potential of an abnormal  $G_s$  to the prevailing physiological and pharmacological conditions in a given patient, resulting in the clinical manifestations of the

illness.

The observed increase in  $K_L/K_H$  in native membranes is primarily due to higher  $K_L$  values, representing lower affinities of isoproterenol for the low affinity state of the dysautonomic  $\beta_2$ -adrenergic receptor. In contrast, the observed increase in  $K_L/K_H$  in reconstituted membranes is primarily due to lower  $K_H$  values, representing higher affinities of isoproterenol for  $HR_H \cdot G_s^{*GTP}$ . In fact, the numerical trend in  $K_H$  is the same in both Table 2 and 4, although statistical significance was not seen for  $K_H$  in Table 2. In Table 4,  $K_L$  does not differ substantially in the three groups, possibly because the same "normal" S49 *cyc*  $\beta_2$ -adrenergic receptor is present in each reconstituted preparation. The unusually high  $K_L$  and  $K_H$  values for severely symptomatic patient 5 are not fully explained, but probably relate to the unusually low total counts in that competition assay. In contrast,  $K_L$  values in Table 2 were dissimilar in patient and control groups. In these competition assays the  $\beta_2$ -adrenergic receptors may not be the same; the native  $\beta_2$ -adrenergic receptor in the dysautonomic patient is exposed to more catecholamines (1) and may be affected by this exposure. We have previously shown in normal  $\beta_2$ -adrenergic receptors that the  $K_L$  value rises upon exposure to  $\beta$ -adrenergic receptor agonists (17, 28), as does that of the  $\beta_2$ -adrenergic receptor from dysautonomic patients (unpublished data from experiments in Ref. 1). Thus, the  $K_L$  values in Table 2 may suggest that native  $\beta_2$ -adrenergic receptors from MVP dysautonomia patients are chronically regulated by augmented endogenous catecholamine concentrations. It is unclear to what extent the  $K_H$  values in Table 2 may reflect chronic exposure to catecholamines.

In the illness pseudohypoparathyroidism type Ia (PHP-Ia) there is a failure of target tissues to respond to PTH. Erythrocytes from patients with this illness have been shown to have a diminution in the quantity of  $G_s$  present (29, 30). It has recently been demonstrated that patients with Albright's hereditary osteodystrophy may have a mutation in the gene encoding  $G_s$  (31, 32). The gene mutation apparently results in structurally abnormal G-proteins, which lack the normal amino-terminus. Although these patients do not have major spontaneous symptoms referable to the  $\beta$ -adrenergic system, erythrocytes from these patients have been shown to have diminished coupling (both  $K_L/K_H$  and  $\%R_H$  were diminished) of their  $\beta$ -adrenergic receptors (33). Patients with PHP-II have no changes in  $K_L/K_H$ , nor do they have abnormal quantities of  $G_s$ . MVP dysautonomia and PHP-Ia appear to be functionally opposite illnesses, at least with respect to  $G_s$ . In both illnesses there may be skeletal abnormalities, which could be manifestations of abnormal  $G_s$  function involving non adrenergic receptor systems.

In summary, the  $\beta$ -adrenergic receptor abnormalities

demonstrated both physiologically and biochemically in patients with MVP dysautonomia are the result of an identifiable functional abnormality in the stimulatory guanine nucleotide regulatory protein,  $G_s$ . This abnormality is not associated with a substantial alteration in the amount of the  $G_{s\alpha}$  subunit or in its apparent mol wt, but more subtle structural changes in  $G_{s\alpha}$  or in the  $\beta\gamma$ -subunits are still possible. A number of nonadrenergic abnormalities in these patients may be explained by shared alterations in the heterotrimeric components of a number of G-proteins.

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