SUMMARY The development of experimental deoxycorticosterone-salt (DOCA-salt) and renal artery clip hypertension in rats is associated with alterations in the sensitivity of the myocardium to adrenergic stimulation. We studied β-adrenergic receptors and isoproterenol-stimulated adenylate cyclase in myocardial membranes from hypertensive rats to determine whether this altered sensitivity is associated with any change in β-adrenergic receptors. The specific binding of the β-adrenergic antagonist, 131I-iodohydroxybenzylpindolol, was used to measure numbers and affinities of receptors in myocardial membrane preparations. Cardiac membranes from both DOCA-salt and renal hypertensive rats showed significantly fewer β-receptors than did membranes from control, normotensive rats. Receptor affinity remained unchanged. This decrease was from 110 ± 19 to 49 ± 5 fmol/mg protein for DOCA-salt hypertension and from 110 ± 18 to 75 ± 16 fmol/mg protein for renal artery clip hypertension. Isoproterenol-stimulated adenylate cyclase activity also was lower in membranes from hypertensive rats, whereas basal and fluoride-stimulated activities were unchanged.

THE quantitative importance of the sympathetic nervous system in relation to normal blood pressure control and to the etiology of various types of hypertension still is uncertain. However, several observations have suggested changes in sympathetic activity in essential hypertension and in various experimental hypertensive models. Elevated plasma catecholamine levels are found in a proportion of hypertensive patients (de Champlain, 1977), and many commonly used antihypertensive drugs interfere with the function of some part of the sympathetic nervous system. In experimental deoxycorticosterone-salt (DOCA-salt) and in renal hypertension, plasma catecholamines also are elevated (de Champlain, 1977; Reid et al., 1977). Moreover, destruction of the sympathetic nervous system with 6-hydroxydopamine and adrenalectomy prevents the establishment of hypertension in these animal models (Chalmers, 1975). Changes in sympathetic activity in experimental hypertension also have been shown by alterations in the turnover of norepinephrine, a decreased turnover being reported in the brain stem and an increased turnover in the heart (de Champlain, 1977). Presumably, this implies increased cardiac sympathetic drive in these models. This increased sympathetic drive, together with the reduced ability for norepinephrine uptake reported for DOCA-salt hypertension (de Champlain and Van Ameringen, 1972), should mean greater concentrations of transmitter at the neuromuscular junction.

Decreasing the activity of the sympathetic nervous system by surgical denervation or by treatment with guanethidine or 6-hydroxydopamine leads to an increase in the postsynaptic response to catecholamines (Glaubiger et al., 1978; Pik and Wolleman, 1977). The increased responsiveness is due partially to increases in the density of β-adrenergic receptors (Glaubiger et al., 1978; Sporn et al., 1976). Physiological changes in sympathetic stimulation also can lead to changes in tissue responsiveness. In the rat pineal gland, increased intensity of sympathetic stimulation leads to reduced responsiveness to catecholamines, and this desensitization is associated with a reduced concentration of β-adrenergic receptors (Kebabian et al., 1975).

Hearts of animals with experimentally induced hypertension have a decreased sensitivity to catecholamines (Cohen and Berkowitz, 1976; Fuyiwara et al., 1972; Kunos et al., 1978). Moreover, in vitro experiments have shown that membrane fractions prepared from hypertensive hearts have a decreased catecholamine-stimulated adenylate cyclase activity (Amer, 1973; Amer et al., 1975). In many tissues, a decrease in isoproterenol-stimulated adenylate cyclase activity appears to be due largely to a decreased concentration of β-adrenergic receptors (Kebabian et al., 1975; Mukerjee et al., 1975). However, factors other than receptors control adrenergic responsiveness, and in many instances, alterations in adenylate cyclase or cAMP response are far greater than any alteration in receptor concentration (Sporn et al., 1976; Malbion et al., 1978; Johnson et al., 1978). This work has been...
undertaken to determine whether the reduced catecholamine sensitivity of the hypertensive heart is associated with a change in β-receptor concentration.

Methods

Materials

[125I]Sodium iodide (carrier-free, 2 mCi, in about 0.02 ml of dilute NaOH) and [2-3H]adenosine 5'-triphosphate were obtained from the Radiochemical Centre. The following compounds were obtained from the Sigma Chemical Company; adenosine 5'-triphosphate (ATP), phospho(enol)pyruvate (Na salt), guanosine 5'-triphosphate (GTP), pyruvate kinase, caffeine, adenosine 3',5'-cyclic monophosphate (cAMP), and Dowex 50W resin.

Hydroxybenzylpindolol and pindolol were provided by Sandoz. All other chemicals were A.R. grade.

Animal Models

Renal Artery Clip Hypertension (One-Kidney Goldblatt Model)

Sprague-Dawley rats of either sex, weighing 120-150 g, were anesthetized with ether. The right kidney was removed. The left renal artery was dissected free and a silver clip, 2 mm wide with a 0.22-mm gap, was used to constrict the artery (Hutchinson et al., 1975).

Control rats were nephrectomized unilaterally, and the left renal artery was dissected free but not clipped.

DOCA-Salt Hypertension

Adult Sprague-Dawley rats of either sex, weighing 120-150 g, were nephrectomized unilaterally under ether anesthesia and subsequently injected weekly with 12.5 mg deoxycorticosterone pivalate (Percorten M, Ciba-Geigy) and given 1% saline drinking water.

Control rats were nephrectomized unilaterally and given saline drinking water.

Systolic blood pressure of conscious rats was measured twice weekly from the tail by a plethysmographic method.

Cardiac Membrane Preparations

Rats were killed by cervical dislocation. The hearts were removed immediately and placed in 0.9% saline at 0°C. The hearts were washed free of blood, and the blood vessels, atria, and epicardial fat were removed. The ventricles were weighed, minced with scissors, and homogenized in 3-5 volumes of buffer (0.25 M sucrose; 10 mM Hepes, pH 7.5) using a “Polytron” homogenizer (10 seconds, speed setting 4). The ventricular homogenate was diluted with an equal volume of cold 0.25 M sucrose buffer and filtered through gauze.

30,000 g Pelleted Membranes

The filtered homogenate was centrifuged twice at 3,000 g for 5 minutes in the SS34 rotor of a Sorvall RC-2B centrifuge at 5°C. The final supernatant was layered over 5 ml of 0.5 M sucrose, 10 mM Hepes, pH 7.5, and centrifuged at 30,000 g for 15 minutes. The pellet was suspended in 10 mM Hepes, pH 7.5, to a final protein concentration of 2-5 mg/ml.

Sucrose Step Gradient Purification

All sucrose solutions were prepared in 10 mM sodium phosphate at pH 7.5. The filtered homogenate was centrifuged at 30,000 g for 15 minutes. The pellet was resuspended in 1.9 M sucrose, 10 mM phosphate, pH 7.5 (10 ml per heart). A sucrose step gradient was prepared in 35-ml tubes of the Sorvall TV-850 rotor as follows: 5 ml of 2.2 M sucrose; 10 ml of 1.9 M sucrose (containing the heart homogenate); 10 ml of 1.5 M sucrose. Tubes were filled with 0.25 M sucrose and centrifuged at 40,000 rpm for 1 hour in a TB-850 Sorvall OTD-2 ultracentrifuge at 2°C. Membranes floating on the 1.9 M sucrose band and on the 1.5 M sucrose band were diluted with 10 mM Hepes, pH 7.5, and centrifuged at 30,000 g for 15 minutes. The final pellet was resuspended in 10 mM Hepes, pH 7.5, to a final protein concentration of 2-5 mg/ml.

Sucrose Floating Method

The filtered homogenate was centrifuged at 30,000 g for 15 minutes. The pellet was resuspended in 1.7 M sucrose, 10 mM sodium phosphate, pH 7.5 (15 ml/g of heart). Eight milliliters of 1.7 M sucrose containing heart homogenate were overlayed with 0.25 M sucrose, 10 mM sodium phosphate, pH 7.5, in 15-ml tubes of the Beckman SW 27.1 rotor. Tubes were centrifuged at 27,000 rpm in a SW 27.1 rotor in the Sorvall OTD-2 ultracentrifuge at 2°C. Membranes containing 1.7 M sucrose and 0.25 M sucrose were diluted with 10 mM Hepes, pH 7.5, and centrifuged at 30,000 g for 15 minutes. The final pellet was resuspended in 10 mM Hepes, pH 7.5, to a final protein concentration of 2-5 mg/ml.

Enzyme and Protein Measurement

5'-Nucleotidase and ouabain-inhibited p-nitrophenyl-phosphatase were measured according to Kidwai et al. (1971). Ouabain-inhibited activity was taken as the difference between incubations containing 10-5M ouabain and those containing no inhibitor.

Adenylate cyclase was measured as described by Harden et al. (1976) using [3H]labeled ATP as substrate. All assay tubes contained 0.13 mM GTP. Separation of cAMP was achieved by the method of Krishna et al. (1968) with recovery of cAMP determined by absorbance measurements.

Protein concentration was measured by the
method of Lowry et al. (1951) using bovine serum albumin as standard.

**β-Receptor Analysis**

**Preparation of Iodohydroxybenzylpindolol (IHYP)**

The preparation of $^{125}$I-labeled IHYP (2.2 Ci/μmol) was as described by Maguire et al. (1976).

Preparation of "radioinert" IHYP was similar, except that purification was carried out by paper chromatography and thin layer chromatography, and trace amounts (<5 mCi/μmol) of $^{125}$I were used for tracing purposes during purification. Following iodination and extraction, the preparation was chromatographed on Whatman 3 MM chromatography paper in 0.1 M ammonium formate buffer, pH 8.5. Fractions with an Rf between 0.05 and 0.1 were eluted, concentrated under a nitrogen stream, and rechromatographed on Merck thin layer chromatography plates (0.25-mm-thick silica gel) in toluene: diethylamine 6:5. $^{125}$I-labeled material, traveling with an Rf of 0.9/13, was eluted with 0.01% phenol in ethylacetate and stored at —20°C until required.

**Binding of IHYP to Rat Myocardial Membranes**

The method used was similar to that described by Harden et al. (1976). Criteria of β-receptor binding were as described by Woodcock et al. (1978a). Binding was saturable, stereospecific with L-propranolol and completed with 10-20 times more potency for binding sites than D-propranolol. The order of potency of agonist binding was DL-isoproterenol $>$ L-epinephrine $>$ D-norepinephrine $>$ D-epinephrine. Binding was rapid, equilibrium being achieved within 15 minutes at 37°C, and reversible either by dilution or by addition of β-adrenergic ligands. Half-time of the dissociation ($t_{1/2}$) was 12-15 minutes in either case. Duplicate incubations of 0.5 ml contained freshly prepared myocardial membranes (200-500 μg protein); 50 mM sodium phosphate buffer, pH 7.4; 4 mM MgSO$_4$; 30,000-40,000 counts/min $^{125}$IHYP (final concentration 20-30 pm); and varying concentrations of "radioinert" IHYP over the concentration range of $10^{-10}$ to $10^{-6}$M.

Samples were incubated for 40 minutes at 37°C, and binding was terminated by adding 1 ml of $10^{-4}$ M pindolol, 20 mM sodium phosphate, pH 7.4, and 4 mM MgSO$_4$ at 37°C. The samples were filtered immediately through Whatman GF/C glass fiber filters and immediately washed with 20 ml of 20 mM sodium phosphate, pH 7.4, 4 mM MgSO$_4$ at 37°C. Radioactivity on the filters was counted in a Packard Autogamma scintillation spectrometer. In the absence of "radioinert" IHYP, an average of 1000-2000 counts/min of $^{125}$IHYP were bound per tube, representing 2-3% of the total added counts. Non-specific binding was taken as binding in the presence of $10^{-6}$ pindolol. This value was generally 10-20% of the total binding and was subtracted to give the "specific" binding value. Binding data were assessed by the method of Scatchard (1949). Values are expressed as means ± SEM. Statistical significance was evaluated using the Student's t-test.

## Results

**Blood Pressure and Cardiac Hypertrophy of Hypertensive Rats**

Table 1 shows the blood pressure and cardiac hypertrophy of DOCA-salt-treated rats and renal artery clip rats, together with their appropriate controls. Measurements were made 4-6 weeks after commencement of treatment. Treated rats developed consistent and comparable increases in blood pressure and ventricular weight.

**Correlation of β-Receptors with Plasma Membrane Marker Enzymes**

Extrapolation from receptor concentration in membrane preparations to the situation in intact heart in vivo assumes that membrane recovery is constant, even when the membranes are prepared from hearts of different sizes.

To investigate this problem, membranes were prepared by a number of different methods (see Methods), and the plasma membrane marker enzymes (5'-nucleotidase and ouabain-inhibited p-nitrophenylphosphatase) were measured, as well as IHYP binding. The ratio of receptors to 5'-nucleotidase activity was determined in the different membrane preparations, as well as receptor concentration per milligram of protein.

Whereas IHYP binding per milligram of protein varied in the different membrane preparations from 47 to 145 fmol/mg in normotensive rats, binding per

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Systolic blood pressure (mm Hg)</th>
<th>Heart wt (g)</th>
<th>Heart/body wt (%)</th>
<th>No. of experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOCA-salt treated</td>
<td>173 ± 6*</td>
<td>1.09 ± 0.04*</td>
<td>0.42 ± 0.01*</td>
<td>12</td>
</tr>
<tr>
<td>Salt-treated controls</td>
<td>134 ± 4</td>
<td>0.8 ± 0.02</td>
<td>0.32 ± 0.01</td>
<td>12</td>
</tr>
<tr>
<td>One-kidney clip</td>
<td>210 ± 6*</td>
<td>1.25 ± 0.1*</td>
<td>0.44 ± 0.02*</td>
<td>14</td>
</tr>
<tr>
<td>One-kidney control</td>
<td>127 ± 5</td>
<td>0.73 ± 0.02</td>
<td>0.30 ± 0.01</td>
<td>14</td>
</tr>
</tbody>
</table>

*Values are means ± 1 SEM.

* $P < 0.01$ compared to controls.
TABLE 2  IHYP Binding to Rat Myocardial Membranes Prepared by Different Centrifugation Methods and Expressed as Units of Protein or Plasma Membrane Marker Enzymes

<table>
<thead>
<tr>
<th>Membrane preparation</th>
<th>Protein yield (mg/g)</th>
<th>IHYP binding</th>
<th>No. of experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(fmol/mg protein)</td>
<td>(fmol/5'-nucleotidase)</td>
</tr>
<tr>
<td>Normotensive rats</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30,000 g pellet</td>
<td>20 ± 4</td>
<td>89 ± 16</td>
<td>7.0 ± 0.4</td>
</tr>
<tr>
<td>1.5 M sucrose band</td>
<td>19.5 ± 4</td>
<td>110 ± 20</td>
<td>7.2 ± 0.2</td>
</tr>
<tr>
<td>1.9 M sucrose band</td>
<td>45</td>
<td>47</td>
<td>6.4</td>
</tr>
<tr>
<td>1.7 M sucrose band</td>
<td>22 ± 2</td>
<td>145 ± 18</td>
<td>6.8 ± 0.7</td>
</tr>
<tr>
<td>Renal hypertensive rats</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30,000 g pellet</td>
<td>18.5 ± 3</td>
<td>41.5 ± 9.5</td>
<td>3.57 ± 0.34</td>
</tr>
<tr>
<td>1.7 M sucrose band</td>
<td>23 ± 5</td>
<td>51 ± 1</td>
<td>4.07 ± 5</td>
</tr>
</tbody>
</table>

Values are mean ± 1 SEM.

5'-nucleotidase unit was remarkably constant (Table 2). The β-receptor concentration in whole heart was calculated, using this ratio and the enzyme activity in the total heart homogenate. The derived values, so obtained, were also very constant in normotensive rats. A similar value of IHYP binding per gram of heart was obtained when receptor concentration was measured per unit of ouabain-inhibited β-nitrophenylphosphatase, instead of 5'-nucleotidase (28.4 ± 1.3 pmol/g compared with 31 ± 1 pmol/g). Thus, although IHYP binding per milligram of protein varied widely in different membrane preparations, binding expressed on a basis of enzyme activity was constant.

IHYP binding per 5'-nucleotidase unit also was constant for hypertensive heart using two different membrane preparations but was consistently lower than for control heart (Table 2).

β-Receptor Concentration in Cardiac Membranes from Hypertensive Rats and Controls

Myocardial membranes prepared from DOCA-salt rats and renal artery clip rats were analyzed for β-receptor concentration and affinity, using 125I-IHYP binding. The membranes used for these experiments were prepared by upwards floatation in 1.7 M sucrose. Figure 1 shows the results of typical experiments. As shown by the Scatchard plots, receptor concentration was lower in membranes from hypertensive hearts, but the affinity for 125I-IHYP was unchanged. Table 3 shows the concentrations of β-receptors relative to protein and relative to 5'-nucleotidase activity. Receptor concentration was lower in membranes prepared from hypertensive heart, whether IHYP binding was expressed per milligram of protein or per 5'-nucleotidase unit.

Therefore, the observed differences in IHYP binding between membranes prepared from control and hypertensive hearts do not appear to be caused by differences in the recovery of receptors and probably reflect real differences in the available receptor concentration in vivo.

Isoproterenol-Stimulated Adenylate Cyclase Activity in Membranes from Hypertensive Hearts

If adenylate cyclase activity is not limiting, a decrease in β-receptor concentration should lead to a decreased maximum activity of isoproterenol-stimulated adenylate cyclase. Both isoproterenol- and sodium fluoride-stimulated adenylate cyclase were measured in the same membrane preparations used in the binding experiments. The results are summarized in Table 3.

Whereas basal and NaF-stimulated activities were similar in normotensive and hypertensive hearts, isoproterenol-stimulated adenylate cyclase activity was consistently and significantly lower in membranes from hypertensive hearts.

Discussion

In recent years, evidence has accumulated for changes in adrenergic receptors under different pathological and physiological conditions. Some of

Figure 1  125I-IHYP binding to myocardial membranes from DOCA-salt hypertensive rats, renal artery clip hypertensive rats (1K Clip), and their appropriate controls. Scatchard analysis of the binding data is expressed as the ratio of IHYP bound (mol/mg protein) to IHYP free (mol/liter) plotted against increasing IHYP in fmol/mg protein.
these changes are due to alterations in the concentration of norepinephrine available to bind the receptor, increases in concentration leading to a decreased number of receptors, and conversely, decreases in norepinephrine concentration leading to an increase in receptor number (Sporn et al., 1976; Kebabian et al., 1975; Mukerjee et al., 1975). In some instances, changes in receptors are due to regulation by other factors such as thyroid status, cortisol levels, or the estrogen-progesterone balance (Williams et al., 1977; Wolfe et al., 1976; Roberts et al., 1977).

Myocardial membrane preparations from rats made hypertensive by either renal artery clipping or DOCA-salt treatment contained a lower concentration of β-adrenergic receptors than membranes from control rats. We found no evidence for any change in receptor affinity for IHYP, suggesting that the receptor itself is unchanged.

In studies such as these, where changes in receptor concentrations are detected in membrane preparations, it is important to ensure that observed differences are a true reflection of changes in the intact tissue in vivo. This is particularly important when comparing receptors in control hearts with those in hypertrophied, hypertensive hearts for which membrane recovery may be different. Our approach to this problem has been to compare results obtained with different membrane preparations and to relate receptor concentrations to activity of the plasma membrane marker enzyme, 5'-nucleotidase. The concentration of receptors based on enzyme activity was constant in membranes prepared by different methods. Receptor concentration based on 5'-nucleotidase activity also was constant for hypertensive hearts but was significantly lower than for control hearts. Thus, receptor concentration was lower in membranes from hypertensive heart, whether this was based on the protein content or the enzyme content of the membranes, and most likely reflects a true reduction in β-receptors in the intact heart in vivo.

If β-receptor concentration limits maximal isoproterenol-stimulated adenylate cyclase activity, reduction in receptor number would be expected to reduce maximal cyclase activation. Conversely, if cyclase activity is limiting, a receptor decrease would be expressed as a reduced apparent affinity of the enzyme for isoproterenol. Decreased isoproterenol-stimulated adenylate cyclase was found in membranes from hypertensive heart, in agreement with the reduced β-receptor concentration estimated by IHYP binding. Reduced isoproterenol-stimulated adenylate cyclase activity in hypertensive rat heart has been reported previously (Amer, 1973; Amer et al., 1975; Triner et al., 1975). However, although there was a greater reduction in β-receptors measured by IHYP binding in DOCA-salt rats than in renal artery clip rats, the decrease in isoproterenol-stimulated adenylate cyclase activity was similar. This may mean that additional postreceptor factors are involved in the reduced adrenergic responsiveness of renal artery clip rats. Alternatively, it may be a reflection of the limited coupling of receptors to adenylate cyclase in rat heart membranes (Maguire et al., 1977) so that differences between the two models are obscured.

Hypertensive, hypertrophied rat hearts contain fewer β-adrenergic receptors than controls. The decrease could be related to the hypertensive process, to some factor common to both models, or may be a function of the cardiac hypertrophy. It is of interest that these results are quantitatively very similar to those reported recently by Limas and Limas (1978), who studied β-adrenergic receptors in spontaneously hypertensive rats using [3H]dihydroalprenolol to measure concentration and affinity of β-receptors. We also have reported decreased cardiac β-receptors in spontaneously hypertensive rats (Woodcock et al., 1978b). The similarity of the results obtained in three different models of experimental hypertension suggests that a decrease in cardiac β-receptors is a common feature of three different hypertension models. Moreover, Limas

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>1H-IHYP binding</th>
<th>Adenylate cyclase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>fmol/mg protein</td>
<td>K&lt;sub&gt;H&lt;/sub&gt; (nM)</td>
</tr>
<tr>
<td>Salt controls</td>
<td>110 ± 19</td>
<td>5.98 ± 0.4</td>
</tr>
<tr>
<td>DOCA-salt hypertension</td>
<td>49* ± 5</td>
<td>3.23* ± 0.5</td>
</tr>
<tr>
<td>One-kidney control</td>
<td>110 ± 18</td>
<td>4.82 ± 0.8</td>
</tr>
<tr>
<td>One-kidney hypertension</td>
<td>75* ± 16</td>
<td>2.43* ± 0.3</td>
</tr>
</tbody>
</table>

Each experiment consisted of two rats from experimental and control groups matched for body weight and blood pressure.

* P < 0.01, experimental vs. control.

† P < 0.005.
and Limas (1978) found decreased β-receptors in hearts from spontaneously hypertensive rats prior to the establishment of hypertension and hypertrophy. An increase in heart weight of 30–35% also was observed in the hyperthyroid rats studied by Williams et al. (1977), but this was associated with an increase in β-receptors. This makes it unlikely that a decrease in β-receptors is a feature of cardiac hypertrophy in general. The concentration of β-receptors can be decreased by exposure of tissues to catecholamines in vivo or in vitro (Mukerjee et al., 1975). In the piaimed gland, increased sympathetic stimulation leads to a decrease in β-receptor concentration and isoproterenol-stimulated adenylate cyclase (Kebabian et al., 1975). The reduction in receptors observed in hypertensive hearts may be a reflection of increased cardiac sympathetic drive in these experimental models.

Acknowledgments

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