Developmental Changes in Guanine Nucleotide Regulatory Proteins in the Rat Myocardial $\alpha_1$-Adrenergic Receptor Complex

Hyung-Mee Han, Richard B. Robinson, John P. Bilezikian, and Susan F. Steinberg

During development, the cardiac $\alpha_1$-adrenergic chronotropic response changes from positive in the neonate to negative in the adult. The negative chronotropic effect of $\alpha_1$-adrenergic stimulation in the adult depends on maturation of sympathetic innervation and the presence of a pertussis toxin (PT)-sensitive guanine nucleotide–binding (G) protein. To examine the possibility of a developmental change in coupling of a PT-sensitive G protein to the $\alpha_1$-adrenergic receptor, radioligand binding experiments with the iodinated $\beta$-selective radioligand $[^{125}\text{I}]$-labeled $\beta$-(4-hydroxyphenyl)ethylaminomethyl]tetralone ($[^{125}\text{I}]$-IBE 2254) were performed on membranes prepared from control and PT-treated neonatal and adult rat hearts. Scatchard analysis showed fewer $\alpha_1$-adrenergic receptors in the adult than in the neonate (168±10 fmol/mg protein in the neonate vs. 124±13 fmol/mg protein in the adult), but similar affinities (equilibrium dissociation constant 124±29 pM in the neonate vs. 140±34 pM in the adult). PT treatment did not alter the results. In both the neonate and adult, 5'-guanylylimidodiphosphate (Gpp(NH)p, 500 $\mu$M) shifted the 1-epinephrine competition curve to the right and increased the slope factor toward unity. PT had no effect on the 1-epinephrine competition curve in the neonate. However, in the adult PT itself caused a partial shift in the agonist competition curve, reducing but not eliminating the effect of Gpp(NH)p. Consistent with the results from the binding experiments, PT did not have any effect on the $\alpha_1$-adrenergic-mediated positive chronotropic response in the neonate, whereas in the adult the $\alpha_1$-adrenergic-mediated negative chronotropic response was completely converted to a positive one after PT treatment. These results indicate the presence of a PT-insensitive G protein in the neonatal and adult rat heart and the acquisition of a PT-sensitive G protein linked to the negative chronotropic response during development. (Circulation Research 1989;65:1763–1773)

The action of $\alpha_1$-adrenergic agonists to modulate autonomic responsiveness in the heart is dependent on the stage of cardiac development. In the newborn heart $\alpha_1$-agonist stimulation increases automaticity, whereas in the adult heart $\alpha_1$-agonist stimulation reduces automaticity. Sympathetic innervation is implicated in the developmental change of the $\alpha_1$-adrenergic chronotropic response from positive to negative. Primary cultures of neonatal rat ventricular myocytes express an exclusive positive $\alpha_1$-adrenergic chronotropic response, whereas myocytes cocultured with and innervated by sympathetic neurons (nerve-muscle cocultures) express a predominantly negative chronotropic response. Binding of agonist to its receptor promotes the coupling of the receptor to an appropriate guanine nucleotide regulatory (G) protein. The agonist-receptor–G protein complex is characterized by high-affinity agonist binding. Binding of GTP, or a suitable analogue, to the G protein leads to reduction in the agonist affinity, reflecting dissociation of the high-affinity agonist-receptor–G protein complex. These actions of GTP to reduce agonist binding affinity reflect influence of a G protein on agonist-receptor interaction. Certain bacterial toxins are also able to modify the affinity of the receptor for agonists. Specifically, pertussis toxin (PT) ADP-ribosylates certain G proteins and, in doing so, interferes with the ability of these G proteins to couple to receptors. To the extent that a particular PT-sensitive G protein is functionally related to a hormone receptor com-
plex, exposure to PT will reduce agonist binding affinity to the receptor. In addition, the functional linkage of that G protein to the effector response will be inhibited by PT.

Our previous studies have demonstrated that the $\alpha_1$-adrenergic positive chronotropic response of cultured neonatal rat ventricular myocytes is not modified by PT. In contrast, the $\alpha_1$-adrenergic negative chronotropic response of nerve-muscle cocultures is abolished by PT, suggesting changes in the G protein linkage to the cardiac $\alpha_1$-adrenergic receptor induced by innervation. Similar experiments have not been performed in intact rat myocardial tissue. Accordingly, this study was designed in part for assessment of the action of PT in intact neonatal and adult rat hearts after in vivo administration. Additionally, by use of radioligand binding techniques the possibility of developmental changes in G protein linkage of the $\alpha_1$-adrenergic receptor in rat myocardium was examined. Although previous functional studies have implicated the coupling of $\alpha_1$-adrenergic receptors to a PT-sensitive G protein in innervated tissues such as rat nerve-muscle cocultures and adult canine Purkinje fibers, there is a lack of information regarding the mechanism of signal transduction at the $\alpha_1$-adrenergic receptor in noninnervated tissue such as neonatal rat heart. The fact that PT did not alter the $\alpha_1$-adrenergic positive chronotropy in pure muscle cultures does not rule out the possibility of coupling of $\alpha_1$-adrenergic receptors to a PT-sensitive G protein in neonatal rat heart. An effect of guanine nucleotides for modulation of $\alpha_1$-agonist binding affinity would constitute the first direct evidence for an interaction between the $\alpha_1$-adrenergic catecholamine receptor and a G protein in neonatal mammalian cardiac tissue.

Materials and Methods

Materials

1-Epinephrine bitartrate, 1-phenylephrine hydrochloride, and $5^\prime$-guanylylimidodiphosphate [Gpp(NH)p] were obtained from Sigma Chemical, St. Louis, Missouri. Prazosin was generously provided by Pfizer Pharmaceuticals, Groton, Connecticut. 2-[\(\beta-(4\)-Hydroxyphenyl\)]ethylaminomethyl]tetra- 

lone (BE 2254) was iodinated according to Engel and Hoyer,10 and binding was performed as previously described.11 Briefly, [\(\beta^\text{32P}\)]-IBE 2254 (60 pM) was incubated with myocardial membranes (60 $\mu$g) in a final volume of 1 ml for 30 minutes at 30°C. The assay buffer contained Tris HCl 50 mM, MgCl$_2$ 10 mM, ascorbic acid 1 $\mu$M, EDTA 1 mM, and PMSF 0.1 mM (pH 7.5). The assay was terminated by addition of 3% trichloroacetic acid to a final concentration of 5% followed by collection on Whatman paper 3MM. The filter was washed 3 times with 3% trichloroacetic acid and counted by liquid scintillation counting.

Preparation of Membranes From Intact Animals

Membranes from control and PT-treated neonatal and adult rat hearts were prepared according to Colucci et al with modifications. Rats were decapitated, and the hearts were placed in ice-cold phosphate-buffered saline (pH 7.4) containing 1 mM ethylenediaminetetraacetate (EDTA) and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). After removal of the atria and great vessels, the ventricles were transferred to a buffer containing sucrose 0.25 M, histidine 0.03 M, EDTA 1 mM, and PMSF 0.1 mM (pH 7.4) and homogenized twice with a polytron (Brinkman Instruments, Westbury, New York) at speed 8 for 10 seconds. The crude homogenate was centrifuged at 1,500g for 15 minutes for removal of large tissue fragments, nuclear debris, and cellu- 

lar organelles. The supernatant was centrifuged at 43,000g for 15 minutes and resuspended in the assay buffer (50 mM Tris HCl, 10 mM MgCl$_2$, 1 $\mu$M ascorbic acid, 1 mM EDTA, and 0.1 mM PMSF, pH 7.5) at a concentration of 2–3 mg protein/ml. The average membrane yield was approximately 0.5% of the initial wet tissue weight. Membranes prepared in this manner were stored at −80°C and used within 2 weeks.

Preparation of Membranes From Neonatal Myocardial Cell Cultures

Myocardial cultures from the ventricles of 1- to 2-day-old Wistar rats were prepared as described previously.3 Ten milliliters of cell suspension (500,000 cells/ml) was plated in 100-mm dishes, each precoated with protamine sulfate (1 mg/ml) for 45 minutes. The medium was removed and fresh medium added on day 1 and day 4. On day 4, cultures were treated with either 100 ng/ml PT or saline. After 24 hours, the medium was removed and the cell-detaching medium containing (mmol/l) NaCl 130, NaHCO$_3$ 16, KCl 3, NaH$_2$PO$_4$ 0.5, sucrose 0.5, and EDTA 1 mM was added. After 30 minutes, cells were removed by scraping with a rubber policeman and collected in a buffer containing sucrose 0.25 M, histidine 0.03 M, EDTA 1 mM, and PMSF 0.1 mM (pH 7.4). Membranes were prepared as described above.

Binding of [\(\beta^\text{32P}\)]-IBE 2254 to Myocardial Membranes

BE 2254 was iodinated according to Engel and Hoyer,10 and binding was performed as previously described.11 Briefly, [\(\beta^\text{32P}\)]-IBE 2254 (60 pM) was incubated with myocardial membranes (60 $\mu$g) in a final volume of 1 ml for 30 minutes at 30°C. The assay buffer contained Tris HCl 50 mM, MgCl$_2$ 10 mM, ascorbic acid 1 $\mu$M, EDTA 1 mM, and PMSF 0.1 mM (pH 7.5). The assay was terminated by
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[12P]ADP-Ribosylation by Pertussis Toxin

The [12P]ADP-ribosylation assay was performed as described previously. PT was activated by incubation with 10 mM diithiothreitol for 10 minutes at 30° C. Twenty-five microliters (25 µg protein) of membranes was incubated in 65 µl buffer containing 50 mM K2HPO4, 10 units aprotinin, 200 µM GTP, 20 mM thymidine, 5 mM ADP-ribose, 20 mM arginine, 2–6 µM [12P]-NAD (19–58 Ci/mmol), and 2.5 µg PT for 20 minutes at 30° C. The reaction was terminated by the addition of 1 ml ice-cold 7% trichloroacetic acid and centrifugation at 27,000g. The pellet was resuspended in 1% trichloroacetic acid, centrifuged at 27,000g, and solubilized with sodium dodecyl sulfate sample buffer. Electrophoresis was performed on slab gels (main gel 3% acrylamide) at 300 V for 3 hours. The gels were then visualized with Coomassie blue and autoradiography using XRP-5 film (Eastman Kodak, Rochester, New York). The specific protein band on the polyacrylamide gel that coincided with the labeled band on the autoradiogram was excised and counted for determination of the equilibrium dissociation constant (Kd) and the number of binding sites (Bmax). Hill coefficients (slope factors) for nonradioactive agonist competing for the radioligand-binding sites were calculated from the slope of a plot of log [%B/(100-%B)] versus log [nonradioactive agonist], where %B is the percentage of radioligand binding displaced by the competing agonist. Competition binding curves were further analyzed by use of the iterative least-squares curve-fitting program to test the goodness of the fit of the data to a given model. In each case, one- and two-site analyses were compared by use of an F test; the statistically better fit (p<0.05) is reported in Table 3. In comparison of the slope factor, Bmax, and Kd among different groups, one-way analysis of variance (ANOVA) was used. For determination of the difference in means between two groups, multiple comparisons by use of the Bonferroni modified t test were applied, and the differences in mean values at the p<0.05 level were considered significant. In all pharmacological responsiveness curves were analyzed by nested ANOVA, and significant differences were determined at the p<0.05 level. Differences in mean values at the p<0.05 level were determined by use of the Bonferroni critical value for the modified t test.

Results

Binding of [12P]-lBE 2254 to Neonatal and Adult Rat Myocardial Membranes

Characteristics of α1-adrenergic binding were examined in the context of the changing α1-adrenergic...
chronotropic response. In agreement with previous results,13 Scatchard analysis of \[^{125}\text{I}^-\text{IBE 2254}\] binding to adult rat myocardial membranes revealed a single class of binding sites with high affinity (\(K_d=140\pm34\) pM) and limited capacity (\(B_{max}=124\pm13\) fmol/mg protein) (Figure 1, Table 1). In neonatal rat ventricular membranes, \[^{125}\text{I}^-\text{IBE 2254}\] was bound to a single class of \(\alpha\)-adrenergic-binding sites with similarly high affinity (\(K_d=124\pm29\) pM) but higher capacity (\(B_{max}=168\pm10\) fmol/mg protein).

**Exposure of Rats In Vivo to Pertussis Toxin**

The next set of questions required a protocol for in vivo PT administration that would effectively ADP-ribosylate and inactivate the myocardial PT-sensitive G protein. Accordingly, neonatal and adult rats were injected with a range of concentrations of PT. In vitro \[^{32}\text{P}\]ADP-ribosylation assays were performed on cardiac membranes for evaluation of the efficacy of in vivo exposure to PT. As shown in Figure 2, exposure of neonatal and adult rats to increasing concentrations of PT led to progressive reduction in the ability of exogenous \[^{32}\text{P}\]NAD to transfer its \[^{32}\text{P}\]ADP-ribose to the G protein, indicating successful endogenous ADP-ribosylation by PT. Maximal ADP-ribosylation (95%) occurred at 120 \(\mu\)g/kg PT s.c. for neonatal rats and at 30 \(\mu\)g/kg PT i.v. for adult rats. In either case, increasing the dose of PT beyond these concentrations did not further ADP-ribosylate the small amount of residual PT substrate. Therefore, these concentrations were employed in subsequent experiments. The apparent difference in the sensitivity to in vivo PT administration between neonates and adults was at least in part due to differences in the routes of administration (subcutaneous in neonates versus intravenous

**TABLE 1. Lack of Effect of Pertussis Toxin on Number and Affinity of Binding Sites for \[^{125}\text{I}^-\text{IBE 2254}\] in Neonate and Adult Rats**

<table>
<thead>
<tr>
<th></th>
<th>Neonate</th>
<th></th>
<th>Adult</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>PT-treated</td>
<td>Control</td>
<td>PT-treated</td>
</tr>
<tr>
<td>(B_{max}) (fmol/mg protein)</td>
<td>168±10</td>
<td>181±11</td>
<td>124±13*</td>
<td>116±11</td>
</tr>
<tr>
<td>(K_d) (pM)</td>
<td>124±29</td>
<td>151±21</td>
<td>140±34</td>
<td>117±11</td>
</tr>
</tbody>
</table>

\(B_{max}\) and \(K_d\) values were obtained from Scatchard analysis of saturation isotherms of specific \[^{125}\text{I}^-\text{IBE 2254}\] binding. Values are mean±SEM. None of the PT-treated groups were significantly different from their respective control groups. PT, pertussis toxin; \(B_{max}\), number of binding sites; \(K_d\), equilibrium dissociation constant.

*\(p<0.05\) vs. control neonate by one-way analysis of variance followed by Bonferroni modified \(t\) test.
in adults). With subcutaneous injection, more PT may be required for achievement of the same circulating level as with intravenous injection.

Equilibrium binding studies were performed in myocardial membranes prepared from PT- or vehicle-treated neonatal and adult rats. The affinity and maximum binding capacity for $^{[35]I}$-IBE 2254 were identical in these membranes, indicating that PT has no effect on the $\alpha_1$-adrenergic receptor-antagonist interaction (Table 1).

Pharmacological Responsiveness

The functional consequences of in vivo ADP-ribosylation by PT were evaluated by determination of the $\alpha_1$-adrenergic chronotropic response of interventricular septa prepared from neonatal and adult rat hearts. The aim of these experiments was for determination of whether the in vivo PT-treatment protocol inactivated a sufficient quantity of the PT-sensitive G protein in the adult so as to interfere with signal transduction and thereby abolish $\alpha_1$-adrenergic-dependent negative chronotropy. The experiment was also designed for examination of whether in vivo PT administration had any effect on $\alpha_1$-adrenergic-mediated positive chronotropy in the neonatal rat. Control beating rates of interventricular septa from vehicle- or PT-treated animals were not significantly different from each other.

In agreement with previously reported results, the $\alpha_1$-adrenergic agonist phenylephrine ($10^{-8}$ M) induced an increase in beating rate in neonatal interventricular septal preparations from 90±10 to 112±11 beats/min (Figure 3A). The $\alpha_1$-adrenergic-mediated positive chronotropic response was similar in vehicle- and PT-treated preparations. In contrast, as shown in Figure 3B, $10^{-8}$ M phenylephrine decreased the automatic beating rate in the adult septum from 90±12 to 69±10 beats/min (Figure 3A). The $\alpha_1$-adrenergic-dependent negative chronotropic response was abolished by prior exposure of adult rats to 30 μg/kg PT in vivo; after PT administration, $10^{-8}$ M phenylephrine significantly increased the beating rate from 84±17 to 108±17 beats/min (n=10, p<0.05). Thus, a similar degree of in vivo ADP-ribosylation by PT has no effect on the $\alpha_1$-adrenergic positive chronotropic response of the neonatal heart but totally eliminates the $\alpha_1$-adrenergic negative chronotropic response of the adult heart.

For further examination of the degree of inactivation of PT substrates required for the conversion of the $\alpha_1$-adrenergic-mediated chronotropic response from negative to positive in the adult, experiments were performed on interventricular septal preparations from adult rats treated with an intermediate dose of PT (5 μg/kg body wt). At this dose of PT, only 26±5% of the PT substrate remained intact based on data obtained from matching ADP-ribosylation experiments on the same tissue samples. However, as shown in Figure 3B, $10^{-8}$ M phenylephrine still induced the negative chronotropic response (from 95±12 to 75±11 beats/min, p<0.05). The dose-response curves of the control adult and 5-μg/kg PT–treated adult were not significantly different, as tested by nested ANOVA (p>0.05). Therefore, approximately one quarter of the potentially available PT substrate appears to sufficiently couple $\alpha_1$-adrenergic receptors to the effector system so as to achieve the full negative chronotropic response in the adult rat heart.

Effect of Guanine Nucleotides on Agonist Binding to $\alpha_1$-Adrenergic Receptors in Neonatal and Adult Rat Myocardium

One means by which coupling of G proteins to their hormone receptors can be assessed is deter-
Figure 4. Effect of Gpp(NH)p on competition of l-epinephrine for [\(^{125}\)I]-IBE 2254 binding sites in membranes prepared from control and PT-treated neonatal and adult rat hearts. Heart membranes were incubated with 60 pM [\(^{125}\)I]-IBE 2254 and increasing concentrations of l-epinephrine in absence or presence of 500 μM Gpp(NH)p. Data points are mean of duplicate determinations. Results shown are representative of several such experiments (n=5 for control neonate, n=4 for PT-treated neonate, n=6 for control adult, n=6 for PT-treated adult). Curves are based on computer analysis of data. PT, pertussis toxin; Gpp(NH)p, 5'-guanylylimidodiphosphate.

Table 2. Effect of Pertussis Toxin on Slope Factor in Neonatal and Adult Rats

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Without Gpp(NH)p</th>
<th>With Gpp(NH)p (500 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neonate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>0.73±0.01</td>
<td>0.99±0.05†</td>
</tr>
<tr>
<td>PT-treated</td>
<td>4</td>
<td>0.72±0.01</td>
<td>0.96±0.03†</td>
</tr>
<tr>
<td>Adult</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>0.68±0.03</td>
<td>0.96±0.01†</td>
</tr>
<tr>
<td>PT-treated</td>
<td>6</td>
<td>0.84±0.03*</td>
<td>0.96±0.04‡</td>
</tr>
</tbody>
</table>

Slope factor was calculated by method of Hill. Values are mean±SEM of four to six separate experiments. Gpp(NH)p, 5'-guanylylimidodiphosphate; PT, pertussis toxin.

*p<0.05 compared with adult control without Gpp(NH)p.
‡p<0.05 compared with value without Gpp(NH)p.


TABLE 3. Effect of Guanyl Nucleotides on Competition by \( \alpha \)-Epinephrine for \([^{125}I] \)-IBE 2254 Binding Sites in Neonate and Adult Rats

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PT-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( K_d ) (nM)</td>
<td>( K_i ) (nM)</td>
</tr>
<tr>
<td>Neonate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without Gpp(NH)p</td>
<td>76 (19%)</td>
<td>1,080 (81%)</td>
</tr>
<tr>
<td>With Gpp(NH)p (500 ( \mu )M)</td>
<td>2,380 (100%)</td>
<td>2,000 (100%)</td>
</tr>
<tr>
<td>Adult</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without Gpp(NH)p</td>
<td>111 (24%)</td>
<td>1,920 (76%)</td>
</tr>
<tr>
<td>With Gpp(NH)p (500 ( \mu )M)</td>
<td>2,020 (100%)</td>
<td></td>
</tr>
</tbody>
</table>

\( \alpha \)-Epinephrine competition binding data were analyzed by nonlinear curve-fitting program LIGAND.\(^{14-15}\) Data shown represent values obtained from simultaneous coanalysis of four to six experiments. \( K_d \) and \( K_i \) are equilibrium dissociation constants of high- and low-affinity states. Percentage of high- and low-affinity states is shown in parentheses. PT, pertussis toxin; Gpp(NH)p, 5'-guanylylimidodiphosphate.

squares curve-fitting analysis indicated that the \( \alpha \)-epinephrine competition curve was significantly better fitted to a two-site model. Approximately 24% of the \( \alpha \)-epinephrine binding sites were in the high-affinity state \( \left( K_d = 111 \text{ nM}\right) \), and 76% were in the low-affinity state \( \left( K_i = 1,920 \text{ nM}\right) \), Table 3). Addition of Gpp(NH)p caused a rightward shift and increased the slope factor close to unity (Figure 4B, Table 2). In the presence of Gpp(NH)p, the binding of \( \alpha \)-epinephrine was better fitted to a one-site model of a homogeneous population of low affinity \( \left( K_i = 2,020 \text{ nM}\right) \), Table 3). Thus, these data suggest that the \( \alpha \)-adrenergic receptor is coupled to a G protein in both neonatal and adult rat hearts.

Effect of Pertussis Toxin on Agonist Binding to \( \alpha \)-Adrenergic Receptors in Neonatal and Adult Myocardium

One consequence of ADP-ribosylation associated with PT is uncoupling of its G protein substrate from the hormone receptor. The functional consequences of this effect have been demonstrated in adult hearts (Figure 3B). The counterpart of this effect of PT for uncoupling of the G protein from the hormone receptor is reduced affinity of the agonist-receptor interaction and loss of the effect of guanine nucleotides on agonist binding. Therefore, binding studies were performed on cardiac membranes prepared from PT-treated neonatal and adult rats and compared with untreated control preparations.

In neonates, PT had no effect on the affinity of the \( \alpha \)-adrenergic receptor for \( \alpha \)-epinephrine (Figure 4C): even after PT treatment, \( \alpha \)-epinephrine competition of \([^{125}I] \)-IBE 2254 binding gave a shallow curve, similar to that observed in control neonatal membranes (Table 2). The results from nonlinear least-squares curve-fitting analysis again indicated a significantly better fit of the binding data to a two-site model \( \left( K_d = 38 \text{ nM}, 22\%\right) ; K_i = 1,060 \text{ nM}, 78\%\); Table 3). As in control neonatal membranes, addition of Gpp(NH)p resulted in a rightward shift and steepening of the agonist competition curve (Figure 4C, Table 2). In the presence of Gpp(NH)p, the binding of \( \alpha \)-epinephrine was better fitted to a one-site model \( \left( K_i = 2,000 \text{ nM}\right) \), Table 3). Persistence of high-affinity \( \alpha \)-adrenergic agonist binding that remains sensitive to the regulatory effects of Gpp(NH)p after exposure to PT indicates that the G protein linked to the neonatal \( \alpha \)-adrenergic receptor is not a substrate for PT.

On the other hand, as shown in Figure 4D, the displacement curve describing \( \alpha \)-epinephrine binding to adult myocardial \( \alpha \)-adrenergic receptors shifted rightward and steepened in membrane preparations obtained from animals exposed in vivo to PT (Table 2). Although PT significantly increased the slope factor for \( \alpha \)-epinephrine binding to \( \alpha \)-adrenergic receptors in adult myocardium, it still remained less than 1. Therefore, an effect of Gpp(NH)p for further modulation of agonist binding was explored. Gpp(NH)p increased the slope factor even closer toward unity (Table 2), suggesting the existence of a residual component of guanine nucleotide modulation of \( \alpha \)-adrenergic agonist binding affinity in PT-treated adult cardiac membranes. The presence of a residual shift by Gpp(NH)p after PT treatment in the adult rat heart is more clearly illustrated in the Hill plots of the combined data from several experiments (Figure 5). In the absence of Gpp(NH)p, the Hill plot of the \( \alpha \)-epinephrine competition binding curves gave a line with shallow slope in the control neonate \( \left( 0.73 \pm 0.01\right) \), control adult \( \left( 0.68 \pm 0.03\right) \), and PT-treated neonate \( \left( 0.72 \pm 0.01\right) \). Addition of 500 \( \mu \)M Gpp(NH)p increased the steepness of the line to near unity in all three cases. On the other hand, as shown in Figure 5D, in the adult, PT itself partially increased the steepness of the line from that of the control adult \( \left( 0.84 \pm 0.03\right) \) in PT-treated group vs. \( 0.68 \pm 0.03\) in control group, \( p < 0.05\). Upon addition of 500 \( \mu \)M Gpp(NH)p, the steepness increased further toward unity \( \left( 0.96 \pm 0.04, p < 0.05\right) \) compared with \( 0.84 \pm 0.03\) for control). Nonlinear least-squares curve-fitting analysis was unable to resolve two affinities in the PT-treated adult. However, the estimated affinity of the single resolved site \( \left( 1,440 \text{ nM}\right) \), which lay intermediate between the high and low affinities of the control adult, and the apparent decrease in this affinity in the presence of Gpp(NH)p \( \left( 1,910 \text{ nM}\right) \) were both consistent with the persistence of a
Figure 5. Hill plot of competition studies with and without 500 µM Gpp(NH)p. Data are mean±SEM of several experiments (n=5 for control neonate, n=4 for PT-treated neonate, n=6 for control adult, n=6 for PT-treated adult). Lines were drawn by least-squares linear regression analysis. %B, percentage of radioligand binding displaced by competing agonist; PT, pertussis toxin; Gpp(NH)p, 5'-guanylylimidodiphosphate.

Figure 6. Binding of $[^{125}I]^{-} \text{IBE 2254}$ to membranes prepared from neonatal myocardial cell cultures. Neonatal rat myocardial cell cultures and membranes from them were prepared as described. As shown in inset in Panel B, incubation of cultures with 100 ng/ml PT for 24 hours completely inactivated PT substrate. Complete inactivation also was observed in Lubrol-treated preparations. Binding experiments were performed with and without 500 µM Gpp(NH)p at a single concentration (10^{-6} M) of L-epinephrine (Epi). Guanine nucleotide-induced decrease in receptor affinity was reflected as an increase in binding of $[^{125}I]^{-} \text{IBE 2254}$ due to nucleotides. Data shown are mean±SEM of four experiments. Administration of 500 µM Gpp(NH)p significantly increased $[^{125}I]^{-} \text{IBE 2254}$ binding in both control and PT-treated cultures (*p<0.05, paired t test). PT, pertussis toxin; Gpp(NH)p, 5'-guanylylimidodiphosphate.

Discussion

The experiments presented in this paper have employed in vivo PT administration for examination...
of the properties of the α₁-adrenergic receptor complex in the neonatal and adult rat heart. The results support and extend our previous observations,7 and together they indicate that the change in chronotropic responsiveness from positive in the neonate to negative in the adult requires increased coupling of a PT-sensitive G protein to the cardiac α₁-adrenergic receptor during development. In addition, the results demonstrate, in both neonatal and adult rat heart, a G protein linked to the α₁-adrenergic receptor that does not serve as a substrate for PT.

Guanine nucleotides have been shown to exert regulatory effects on receptors that mediate adenylate cyclase activity and other biochemical effectors. It is now well established that guanine nucleotides are required for both hormonal activation and hormonal inhibition of adenylate cyclase. They are also required for receptor-mediated transduction of activities mediated by cyclic GMP phosphodiesterase, phosphatidylinositol hydrolysis, and ion channels. When modulate receptor function, in part, by decreasing the affinity of agonist, but not of antagonist, for the receptors to which they are linked. This action of guanine nucleotides is considered as evidence for a molecular interaction between a G protein and a receptor.4,5

Several groups of investigators have provided evidence that the α₁-adrenergic receptor is coupled to a G protein in various tissues,4,5,20-22 although other investigators have been unable to show such regulation.23-25 The inconsistent reports concerning guanine nucleotide modulation of the α₁-adrenergic agonist binding affinity by other laboratories may reflect the relatively small effect of guanine nucleotides on the α₁-agonist binding affinity (compared with that seen at the β-adrenergic receptor) and its requirement for specific assay conditions. In particular, inclusion of EDTA and PMSF during the preparation of membranes and in the binding assay may be crucial.25 Our present data clearly demonstrate that guanine nucleotides can modulate α₁-agonist binding affinity in both neonatal and adult rat myocardium, indicating that the α₁-adrenergic receptor is coupled to a G protein in both neonatal and adult rat myocardium. This is the first direct demonstration of a linkage function of a G protein to the α₁-adrenergic receptor in the neonatal rat myocardium.

The identity of the G protein involved in signal transduction at the α₁-adrenergic receptor has been investigated by use of PT, which is known to ADP-ribosylate and functionally inactivate certain G proteins. The results of these studies are conflicting. Several investigators reported that a wide variety of α₁-adrenergic-mediated responses are sensitive to PT treatment. These responses include an α₁-adrenergic-mediated increase in [32P] incorporation into phosphatidic acid and phosphatidylinositol,26 activation of guanylate cyclase,27 inhibition of β-adrenergic-induced cyclic AMP generation,28 stimulation of Na⁺,K⁺-ATPase and inhibition of an outward K⁺ current,29 negative chronotropic response,7,30 and an increase in myocardial contractility.31 In contrast, Schmitz et al23 and Steinberg et al32 reported that PT does not affect α₁-adrenergic-mediated inositol phosphate production in the heart. Similarly, several investigators using radioligand binding techniques have reported that PT does not affect guanine nucleotide modulation of agonist binding affinity to the α₁-adrenergic receptor.25,26,33 Differences in the PT sensitivity of the α₁-adrenergic response observed could be due to tissue differences employed in each study or incomplete inactivation of the endogenous PT substrate.

Our results indicate that the myocardial α₁-adrenergic receptor can couple to both a PT-sensitive and a PT-insensitive G protein. Persistent modulation of agonist binding by guanine nucleotides after PT treatment in the neonate indicates that the G protein to which the α₁-receptor is coupled in the neonatal rat heart is not a PT substrate. On the other hand, in the adult rat heart, the α₁-agonist-induced negative chronotropic response, as well as the effect of guanine nucleotides on agonist binding affinity, are sensitive to PT, indicating that the α₁-adrenergic receptor in the adult rat myocardium becomes coupled to a PT-sensitive G protein. It is noteworthy that although PT significantly reduced the ability of guanine nucleotides to modulate agonist binding in the adult, a residual shift by guanine nucleotides remained after PT treatment. There are several possible explanations for this residual shift by guanine nucleotides in adult myocardium exposed to PT. The in vivo PT treatment protocol effectively ADP-ribosylated and inactivated up to 95% of the PT-sensitive G protein. However, a small amount (approximately 5%) persisted in the PT-treated myocardium and could be responsible for the residual shift by guanine nucleotides after PT treatment. A more likely interpretation of the data is that the α₁-adrenergic receptor in the adult rat heart can couple to at least two G proteins, one of which is PT-sensitive and the other PT-insensitive. PT treatment would abolish the coupling of α₁-receptors to a PT-sensitive G protein but would have no effect on the coupling of α₁-receptors to a PT-insensitive G protein. Thus, the component of guanine nucleotide modulation of the agonist competition binding curve influenced by the PT-insensitive G protein would remain intact after exposure to PT.

Results of this study emphasize the importance of maximal modification of the PT substrate when implicating its involvement in the biological response. In adult rats injected with an intermediate dose of PT (5 μg/kg), an α₁-adrenergic–mediated negative chronotropic response comparable with the response seen in the control myocardium persisted, despite considerable inactivation of the myocardial PT substrate. This would suggest that the PT-sensitive G protein that is coupled to the α₁-adrenergic receptor in the adult is present in excess.
Alternatively, there may be several different PT-sensitive G proteins that have different sensitivity to in vivo exposure to PT. The PT-sensitive G protein to which the α₁-readrenergic receptor is coupled may be less sensitive to inactivation than the others. Thus, if this particular PT-sensitive G protein is to be inactivated, most of the others may have to be inactivated first.

The identity of the PT-sensitive G protein that is acquired during development is not clear at present. Probable candidates are two known G protein classes that are substrates for PT-dependent ADP-ribosylation, referred to as G_i and G_o. Using a clone from rat olfactory neuroepithelium, Jones and Reed reported the expression of three different G_s in the rat heart. Recently, Luetje et al demonstrated that the level of expression of the messages for the individual G_i subspecies is regulated differentially with development in the rat heart. Obviously, much more work needs to be done to determine which PT-sensitive G protein becomes coupled to the α₁-readrenergic receptor during development. In addition, other developmentally regulated mechanisms distal to the G protein also may be required for the expression of the mature α₁-readrenergic negative chronotropic response.

The coincidence in the neonate between the presence of a PT-insensitive G protein linked to the α₁-receptor and the α₁-readrenergic-mediated positive chronotropic response raises the possibility that this response is mediated by a PT-insensitive G protein. Recent studies have demonstrated a role for a PT-insensitive G protein in the linkage of the α₁-receptor to inositol trisphosphate accumulation in myocardial cells (Steinberg et al, unpublished observation). This finding may suggest that α₁-readrenergic activation of inositol phospholipid metabolism via a PT-insensitive G protein underlies α₁-readrenergic positive chronotropy. In addition, in the PT-treated adult, there is an α₁-readrenergic-mediated positive chronotropic response and a residual shift by guanine nucleotides. Thus, it is possible that in both the adult and the neonate, a PT-insensitive G protein links the α₁-readrenergic receptor to a positive chronotropic response. Whether the PT-insensitive G proteins that are coupled to the α₁-readrenergic receptor in the adult and the neonate are the same remains to be resolved.

Acknowledgments

The authors express their gratitude to Dr. Lena Sun for her assistance in some of the experiments and to Ema Stasko for preparing the myocardial cell cultures.

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**KEY WORDS** • pertussis toxin • \(\alpha\)-adrenergic receptor • development • myocardium • G protein
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Circ Res. 1989;65:1763-1773
doi: 10.1161/01.RES.65.6.1763

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