Myocardial Adenylate Cyclase Activity in Acute Murine Chagas' Disease

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We have studied the influence of myocardial infection with *Trypanosoma cruzi* on the β-adrenergic adenylate cyclase complex in mouse myocardial membranes. The maximal rate of cAMP generation (V_max) and the concentration of agonist associated with 50% of the maximal activity (apparent K_μ) were determined for a series of agents. Six days after infection, the V_max for isoproterenol significantly declines without a change in the apparent K_μ. After 21 days of infection, both the V_max and apparent K_μ for isoproterenol are reduced. At 6 and 21 days of infection, the affinity of the β-receptor for [125I]iodocyanopindolol declines from 0.84 to 3.6 and 3 nM, respectively, while the receptor density increases with the duration of infection from 33 to 57 and 82 fmol/mg protein, respectively. The V_max (but not the apparent K_μ) for forskolin and Mg^{2+}- and Mn^{2+}-associated activities declines also after 21 days. Another adenylate cyclase activity, which was stimulated by the nonhydrolyzable guanine nucleotide Gpp(NH)p, declines in relation to the duration of infection. Inhibitors of adenylate cyclase activity were also studied. Inhibition of adenylate cyclase activity by adenosine and by Gpp(NH)p (in the presence of forskolin) declines after 21 days of infection. The results suggested that the coupling proteins Ns and Ni, which mediate stimulatory or inhibitory control of receptors to adenylate cyclase activity, might be altered by infection. As monitored by cholera toxin- and pertussis toxin–dependent ADP ribosylation of their respective substrates, which include Ns and Ni proteins, respectively, there are declines in the availability of both substrates as a result of *T. cruzi* infection. For infected membranes, the addition of NADP enhances the magnitude of cholera toxin–dependent ADP ribosylation and renders the magnitude of pertussis toxin–dependent ADP ribosylation equal to that observed in uninfected membranes. The results support the hypothesis that infection with *T. cruzi* results in profound generalized alterations of the adenylate cyclase complex at several different sites.

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Chagas' disease, caused by the hemoflagellate *Trypanosoma cruzi*, is a major cause of heart disease in South America. Pathological and biochemical studies in humans and experimental animals clearly indicate that the autonomic nervous system is affected by this disease.1–9 Machado and his coworkers4–6 have shown depletion of acetylcholine and norepinephrine in the hearts of infected rats. In addition, our laboratory has demonstrated significant alterations in levels of choline acetyltransferase, nicotine acetylcholine receptors, and acetylcholinesterase in tissues obtained from susceptible mice acutely infected with *T. cruzi*. Of particular interest is the observation that cardiac choline acetyltransferase activity is depleted before morphological alterations are evident. Recently, our laboratory has shown that the response of the β-adrenergic receptor adenylate cyclase complex to prolonged stimulation by isoproterenol (desensitization) differs markedly in *T. cruzi*-infected L6E9 myoblasts in culture when compared with uninfected cells.

Presently, five known components constitute the adenylate cyclase complex: stimulatory and inhibitory receptors coupled to the stimulatory and inhibitory guanine nucleotide–binding proteins Ns and Ni, respectively, and the final common component, the catalytic unit.10 In myoblasts, our data suggest that the effects of *T. cruzi* infection appear to be restricted to postreceptor components in that after infection, the actual density of the stimulatory β-adrenergic receptors is unchanged.10 To ascertain whether similar changes may be expected as a consequence of infection in an animal model of Chagas' disease, we now have extended these studies to an examination of the myocardial β-adrenergic adenylate cyclase complex in acute Chagas' disease of mice. Our observations support the hypothesis that infection with *T. cruzi* leads to alterations in the adenylate cyclase complex at postreceptor sites common to the actions of several regulators of the cardiac response.

Materials and Methods

Materials

[^32P]NAD and [3H]cAMP were obtained from New England Nuclear-Dupont, Boston, Massachusetts. Gpp(NH)p was obtained from Sigma Chemical, St. Louis, Missouri. Pertussis toxin was obtained from List...
Adenylate Cyclase Assay was accomplished by sequential Dowex and alumina "stopping solution." Isolation of [MP]cAMP was obtained from Schwartz Mann Laboratories, New York at 4°C. The resulting crude homogenate was centrifuged at 11,000g for 20 minutes at 4°C. Supernatant was immediately centrifuged at 43,000g for 30 minutes at 4°C. This final pellet was resuspended in homogenizing buffer without EDTA and stored at -60°C. Membranes retained adenylate cyclase activity without change for up to 3 months. Preparation was used directly in the adenylate cyclase assay but was modified before its use in the ADP-ribosylation assays (see below). For pathological studies, hearts were excised as described above and immediately fixed in 10% neutral buffered formalin. The hearts were bisected in the apex-to-base plane perpendicular to the ventricular septum to facilitate examination of atria, ventricles, and conduction tissue. Both halves of the heart were embedded in paraffin and sectioned at 5 μm. Three levels were prepared from each heart and were stained with hematoxylin and eosin. Inflammatory infiltrates, myocellular necrosis, myocardial fibrosis, and frequency of parasitic pseudocysts were the variables evaluated. Stains were used when necessary.

Adenylate Cyclase Assay

The reaction mixture contained 50 mM Tris-HCl (pH 7.4), 2.5 mM Mg unless otherwise noted, 0.143 mM ATP, an ATP regenerating system (creatine phosphate/creatine phosphokinase), ATP (l-[α-32P]ATP, 1-2 × 10⁶ cpm/assay tube), and 6 mM theophylline in a final volume of 75 μl. Agents dissolved in 50 mM Tris-HCl (pH 7.5) were added in concentrations noted in individual experiments. Forskolin was added from a 0.3% ethanol stock solution. The adenylate cyclase reaction was terminated by the addition of an ATP-cAMP "stopping solution." Isolation of [32P]cAMP was accomplished by sequential Dowex and alumina chromatography with [H]cAMP as a recovery marker.

Analysis of Data

Determination of the apparent K_m and V_max for adenylate cyclase activation by agents were made with a linear regression analysis of the double reciprocal (Lineweaver Burke Analysis) of 1/(V - V_o) versus 1/S, where V_o is adenylate cyclase activity in the absence of the agent under consideration, and V is the adenylate cyclase activity in the presence of the agent at concentration S. In the figures, dropped arrows represent the apparent K_m as determined by the Lineweaver Burke Analysis for the experiment shown. Significance was determined with Student's t test.

ADP-Ribosylation Studies

ADP ribosylation was performed according to the method of Kaslow et al with modifications. Membranes prepared as previously described were subject to an additional centrifugation at 15,000g for 10 minutes at 4°C. The supernatant was saved and subsequently centrifuged at 43,000g for 30 minutes at 4°C. This final pellet was resuspended in 50 mM Tris buffer (pH 7.5) in a volume equal to one tenth the volume of the original membrane suspension, and this resulted in a final membrane protein concentration of 5–10 mg/ml from which 25 μl were incubated in 65 μl buffer (10 U trasytol, 20 mM thymidine, 5 mM ADP-ribose, 20 mM arginine, 23–60 Ci/mmol [32P]NAD, 50 mM KPO4 at pH 7.5) and other agents as noted. Before addition to the reaction mixture, cholera toxin (5 μg) or pertussis toxin (2.5 μg) was activated by incubation with dithiothreitol (20 mM) for 10 minutes at 30°C. For incubations with cholera toxin, 100 μM Gpp(NH)p was added; for incubations with pertussis toxin, 100 μM each of GTP and ATP was added. The complete reaction mixture with or without the appropriate toxin was then incubated for 20 minutes at 3°C and terminated by the addition of 1 ml ice-cold 7% trichloroacetic acid (TCA) and centrifuged at 12,000g. The pellet was resuspended in 1% TCA, recentrifuged at the same speed, and solubilized in sample buffer (10 mM Tris-HCl, 0.1% sodium dodecyl sulfate, 0.1% 2-mercaptoethanol, pH 6.8). Samples were boiled for 2 minutes. Electrophoresis was performed on vertical slab gels (main gel 10%; stacking gel 1.25% acrylamide) at 150 V for 1.5 hours. Gels were stained subsequently with Coomassie blue and analyzed by autoradiography.

Intensity of bands corresponding to a particular molecular weight was determined spectrophotometrically as the optical density (OD/pixel) with QUANTIMET 970 (Cambridge Instruments, Cambridge, Massachusetts).

Determination of β-Receptor Sites

The binding assay used [3H]iodocyanopindolol (ICYP). Cyanopindolol was iodinated and purified to a specific activity of 2,200 Ci/mmol according to methods previously published. Aliquots of myocar-
dial membranes (100–300 μg) were distributed to tubes containing 
[^3H]ICYP in a final volume of 1 ml whole-cell buffer (0.15 M NaCl, 0.01 M Tris at pH 7.5), 0.01 M KCl, and 1 mg/ml bovine serum albumin with 2 mg/ml dextrose. The binding assay was carried out in a shaking water bath for 20 minutes at 37° C. The reaction was terminated by filtering the suspension over Gelman A/E glass fiber filters. The filters were washed with 10 ml 0.01 M Tris at room temperature, and radioactivity was determined in a Packard Auto-
Gamma scintillation counter. Specific binding was defined as the difference between total binding and binding inhibited by propranolol (0.1 μM). In general, specific binding ranged from 40% to 70% of total binding. Maximal binding capacity was determined by Scatchard analysis.15–18

Results

The time course of parasitemia in mice infected with T. cruzi is shown in Figure 1. No significant parasitemia was detected 6 days after infection. By 12 days, however, more than 90,000 trypomastigotes/ml were present; by 21 days after infection, parasitemia increased almost 10-fold and reached a plateau thereafter. Despite the logarithmic increase in parasitemia, no statistical difference in either heart rate or body weight was apparent for up to 21 days after infection when compared to matched, uninfected controls (data not shown). For this study, we routinely examined mice at 6 and 21 days after infection, corresponding to times associated with no significant parasitemia and maximal parasitemia.

The extent of cardiac pathology 6 and 21 days after infection was determined. At 6 days, there were no organisms or evidence of pathology, except for an occasional, small inflammatory focus. In contrast, at 21 days (Figure 2), there was diffuse acute inflammation throughout the myocardium with foci of myocardial edema and degeneration. Additional findings at 21 days included numerous scattered pseudocysts without an associated inflammatory reaction. There were no fresh fibrin-platelet vegetations on the tricuspid valve, and chorda tendineae or adventitial inflammation at the root of the aorta.

We next determined the extent to which infection with T. cruzi influenced the β-adrenergic receptor and related components of the adenylate cyclase complex of the myocardium. To this end, adenylate cyclase activity was determined in response to isoproterenol in myocardial membranes prepared from control mice and mice infected for 6 and 21 days. The experiments shown here are the results of one of three separate analyses of experimental animals, all showing qualitatively similar results. In control mice, increasing concentrations of isoproterenol alone or in the presence of Gpp(NH)p resulted in increased adenylate cyclase activity (Figure 3A and summarized in Table 1). The apparent K_s for this reaction was 0.6 μM, and it was unaffected by the presence of Gpp(NH)p (Table 1). In animals infected for 6 days (Figure 3B), isoproterenol alone or in combination with Gpp(NH)p also stimulated adenylate cyclase activity in a dose-dependent manner. However, the V_max (maximal rate of conversion of ATP to cAMP) was reduced from 24 to 16 pmol/mg/min at day 6. The copresence of Gpp(NH)p along with isoproterenol did not restore maximal isoproterenol-dependent adenylate cyclase activity to control levels. Despite the decline in the V_max in 6-day-infected mice, the apparent K_s for isoproterenol was equal to control uninfected animals. While the shallow dose-response curves for isoproterenol-dependent adenylate cyclase activation may mask more subtle changes associated with 6 days of infection, the absence of significant alterations was a consistent observation in each of three separate determinations.

At 21 days after infection, the V_max decreased further to 10 pmol/mg/min (Figure 3C). In addition, the apparent K_s in 21-day-infected animals was shifted significantly to the right (1.3 μM, Table 1) and was unaffected by Gpp(NH)p. It is important to note that while the values for V_max reflect the increment in adenylate cyclase activity due to isoproterenol when compared with adenylate cyclase activity measured in the absence of the catecholamine (basal activity), the actual basal adenylate cyclase values varied from 15 to 35 pmol/mg/min, and they showed no consistent relation with the duration of infection.

A possible influence of infection on the β-receptor per se was evaluated next. As identified by [^3H]ICYP (Figure 4), in membranes prepared from uninfected mice, β-adrenergic receptor density was 33.5 fmol/mg protein with an associated K_s of 0.84 nM. In membranes prepared from 6- and 21-day-infected mice, β-adrenergic affinity decreases from control values (0.84 nM) to 3.6 and 3 nM, respectively; β-receptor

Figure 1. Time course of parasitemia in mice infected with Trypanosoma cruzi. Mice were infected with T. cruzi as described in "Materials and Methods." On the days of infection noted, samples of blood were drawn, and parasitemia was determined as described in "Materials and Methods." Each point represents the mean ± SD of at least five animals. Note that measurements of trypomastigotes less than 0.1 x 10^9/ml were nonsignificant. This is indicated on the figure by the dotted line connecting day 0 to day 13.
density increases with duration of infection from control values (33.5 fmol/mg protein) to 57.5 and 82 fmol/mg protein, respectively. The striking decrease in [125I]ICYP binding affinity accompanying infection is in marked contrast to the negligible change in affinity for isoproterenol-dependent adenylate cyclase activity observed with infection.

The relative importance of these changes in the β-receptor could be understood better after evaluating other agonists of adenylate cyclase activity such as Gpp(NH)p, forskolin, and Mg2+ and Mn2+, which are agents that act at sites distinguishable from the classic hormone receptor.11 As shown in Figure 5 and summarized in Table 2, increasing concentrations of Gpp(NH)p increased adenylate cyclase activity in myocardial membranes prepared from uninfected mice and mice infected for 6 and 21 days. Similar to observations for isoproterenol and Gpp(NH)p, the V_max for forskolin substantially declined at day 21 of infection (Figure 6; Table 2). In contrast to isoproterenol and Gpp(NH)p, the V_max for forskolin was not significantly different between uninfected and infected animals at 6 days. For both uninfected and infected animals, there was no significant change in the K_m for forskolin.

Activation of adenylate cyclase activity by divalent cations occurs by yet another mechanism, different from that associated with isoproterenol, Gpp(NH)p, or forskolin,20 and may involve mechanisms more directly related to the catalytic unit. As shown in Figure 7A, Mg2+ stimulated adenylate cyclase activity in a dose-dependent manner. For membranes prepared from control animals, the V_max for Mg2+ was 18 pmol/mg/min and increased slightly (but not significantly) to 23 pmol/mg/min at 6 days. At 21 days of infection, the V_max for Mg2+ declined to 10 pmol/mg/min. There was little or no associated change in the K_m for the guanine nucleotide at either time point after infection.

The stimulation of adenylate cyclase activity by forskolin involves an action at the catalytic unit of adenylate cyclase as well as a direct action at Ns.19 Similar to observations for isoproterenol and Gpp(NH)p, the V_max for forskolin substantially declined at day 21 of infection (Figure 6; Table 2). In contrast to isoproterenol and Gpp(NH)p, the V_max for forskolin was not significantly different between uninfected and infected animals at 6 days. For both uninfected and infected animals, there was no significant change in the K_m for forskolin.

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TABLE 1. Isoproterenol-Dependent Adenylate Cyclase Activity in Control and Infected Mice

<table>
<thead>
<tr>
<th></th>
<th>Isoproterenol</th>
<th>Isoproterenol and Gpp(NH)p (1 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>Kᵦ (μM)</td>
<td>Vₘₐₓ (pmol/mg/min)</td>
</tr>
<tr>
<td>Control</td>
<td>0.7 ±0.02</td>
<td>24 ±3</td>
</tr>
<tr>
<td>Day 6</td>
<td>0.7 ±0.03</td>
<td>16 ±2</td>
</tr>
<tr>
<td>Day 21</td>
<td>1.3 ±0.12</td>
<td>10 ±2</td>
</tr>
</tbody>
</table>

Shown here are values for Kᵦ and Vₘₐₓ expressed as the mean ± SD from three experiments performed as described in legend to Figure 3. Determination of Kᵦ and Vₘₐₓ is as described in "Materials and Methods."

*Significantly different from control (p<0.01); †significantly different from control and day 6 (p<0.05).

Gpp(NH)p inhibits the adenylate cyclase activity in uninfected mouse heart membranes by 30%. At day 6, the ability of Gpp(NH)p to inhibit forskolin-dependent adenylate cyclase activity does not significantly change. However, at 21 days of infection, there is a decline in both forskolin-dependent adenylate cyclase activity per se (Figure 6) as well as the ability of Gpp(NH)p to inhibit this activity. These results are essentially identical to those obtained for GTP when used under conditions as a stimulant of forskolin-dependent adenylate cyclase activity (Figure 8). Thus, the magnitude of GTP-dependent stimulation of forskolin-dependent adenylate cyclase activity is significantly diminished at 21 days of infection. To examine further the compromised inhibitory actions on adenylate cyclase activity as a result of infection with T. cruzi, adenosine, an inhibitor of adenylate cyclase activity, was studied. As shown in Figure 9, increasing concentrations of adenosine alone, or in the presence of GTP or Gpp(NH)p, resulted in an increased magnitude of inhibition of forskolin-dependent adenylate cyclase activity. There did not appear to be a significant difference between the patterns of adenosine-dependen-

Figure 3. Isoproterenol-dependent adenylate cyclase activity in membranes prepared from control and infected mice. Isoproterenol-dependent adenylate cyclase activity was determined as described in "Materials and Methods" for control mice (Panel A; filled symbols) and mice infected for 6 days (Panel B; half-filled symbols) and 21 days (Panel C; open symbols). Circles represent adenylate cyclase activity in response to isoproterenol alone, and triangles represent adenylate cyclase activity in response to isoproterenol in the presence of 1 mM Gpp(NH)p. Arrows are concentrations of agonist associated with 50% increase in adenylate cyclase activity. The apparent Kᵦ and Vₘₐₓ for this experiment, determined as described in "Materials and Methods," is one of three determinations averaged in Table 2. Values shown here are means of three separate determinations; standard deviations are less than 14% of the mean and are not included for the sake of clarity.

branes (as has been reported in other systems) independent of the duration of infection. The concentration of Mn²⁺ associated with a 50% maximal adenylate cyclase activity (0.23 ± 0.04 mM) is uninfluenced by the duration of infection. Similar to the results obtained with Mg²⁺, the Vₘₐₓ for Mn²⁺ (94 and 89 pmol/mg/min for uninfected and 6-day-infected mice, respectively) declines at 21 days of infection (20 pmol/mg/min).

We next performed studies to determine whether inhibition of adenylate cyclase activity was compromised in a manner similar to its stimulation. To this end, we examined the ability of Gpp(NH)p to inhibit forskolin-dependent adenylate cyclase activity, and we contrasted this with the ability of GTP to stimulate forskolin-dependent adenylate cyclase activity. As shown in Figure 8, when expressed as the percent change in forskolin-dependent adenylate cyclase activity determined under otherwise identical reaction conditions but in the absence of the guanine nucleotide,
ent inhibition of forskolin-dependent adenylate cyclase activity in membranes prepared from controls or animals infected for 6 days (Figures 9A and 9B). However, as expected, in animals infected for 21 days, the magnitude of inhibition by adenosine was much less than that observed in controls or 6-day-infected animals.

Our results indicate that receptor-dependent and independent stimulation and/or inhibition of the adenylate cyclase complex change during the course of infection with T. cruzi, and they suggest that the function of Ns and Ni may both be directly impaired by infection with T. cruzi. To test this possibility directly, we examined choleragen toxin- and pertussis toxin-associated ADP ribosylation of their membrane substrates, which include Ns and Ni, respectively. By itself, the magnitude of choleragen toxin-dependent ADP ribosylation decreased slightly in membranes prepared from 6- and 21-day-infected mice (Figure 10) by 16% and 20%, respectively, when compared with the magnitude of cholera toxin-dependent ADP ribosylation in uninfected mice (Figure 11). To examine whether these changes reflect alterations in the substrate requirements for, or the stability of NAD, we determined choleragen toxin-dependent ADP ribosylation in the presence of the NADase inhibitor NADP. In uninfected membranes, NADP increased cholera toxin-dependent ADP ribosylation. NADP also increased cholera toxin-dependent ADP ribosylation in membranes prepared from 6-day-infected mice but the magnitude of this effect was considerably less than that observed in uninfected membranes. These effects of NADP are small and are better appreciated by reference to Figure 11, where the increment in cholera toxin-dependent ADP ribosylation due to NADP is 60% and 25% for control and 6-day-infected mice. In contrast, NADP increased the cholera toxin-dependent ADP ribosylation in membranes prepared from 21-day-infected mice almost 200% (Figure 11).

In the presence of pertussis toxin, ADP ribosylation occurs in a 39-kDa band in infected and uninfected membranes (Figure 10, Lanes 4). The magnitude of pertussis toxin-dependent ADP ribosylation decreased by 40% and 57% in membranes prepared from 6- and 21-day-infected mice, respectively, when compared with the magnitude of pertussis toxin-dependent ADP ribosylation in uninfected control mice (Figure 11, but not shown in Figure 10). In contrast, in the presence of NADP, pertussis toxin-dependent ADP ribosylation (Figure 10, Lanes 4) occurs with almost the same magnitude as that observed in infected membranes, independent of the duration of infection. This lack of

### Table 2. Gpp(NH)p- and Forskolin-Dependent Adenylate Cyclase Activity in Control and Infected Mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>Gpp(NH)p</th>
<th>Forskolin</th>
<th>Gpp(NH)p</th>
<th>Forskolin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>98 ± 7</td>
<td>200 ± 30</td>
<td>2.5 ± 0.4</td>
<td>3 ± 0.4</td>
</tr>
<tr>
<td>Day 6</td>
<td>51 ± 4*</td>
<td>220 ± 40</td>
<td>3.2 ± 0.5</td>
<td>2 ± 0.5</td>
</tr>
<tr>
<td>Day 21</td>
<td>38 ± 6†</td>
<td>130 ± 20</td>
<td>1.6 ± 0.6</td>
<td>3.3 ± 0.2</td>
</tr>
</tbody>
</table>

Values shown are mean ± SD from three separate experiments performed as described in legend to Figure 4 for Gpp(NH)p and Figure 5 for forskolin. K_m and V_max were determined as described in "Materials and Methods." For all values of K_m, differences were not statistically significant. *Significantly different from control (p<0.01); †significantly different from control and day 6 (p<0.05).
FIGURE 7. Divalent cation–dependent adenylate cyclase activation in mouse heart membranes prepared from mice infected with T. cruzi. Magnesium– (Panel A) and manganese–dependent (Panel B) adenylate cyclase activities in membranes prepared from control mice (filled circles) and mice infected for 6 days (half-filled circles) and 21 days (open circles) were determined as described in “Materials and Methods,” except that the incubation temperature was 30° C. Points represent means of three separate determinations for this one of three experiments, all qualitatively similar. Standard deviations are less than 13% of the mean and are omitted for the sake of clarity.

FIGURE 8. Stimulation and inhibition of forskolin-dependent adenylate cyclase activity in mouse heart membranes as a function of time of infection with T. cruzi. Membranes were prepared as described in “Materials and Methods.” Adenylate cyclase assays were carried out in the presence of 100 µM forskolin and 1 mM Mg2+ for 10 minutes at 30° C in the presence or absence of 100 µM GTP (open bars) or 1 µM Gpp(NH)p (hatched bars). Results represent mean of four determinations. Standard deviations are less than 15% of the mean. Activity due to forskolin alone was 260 ± 32, 290 ± 48, and 160 ± 22 pmol/mg/min for control and 6- and 21-day-infected mice, respectively.

Discussion

In this report, we describe the consequences of early T. cruzi infection on the integrity of the murine myocardial β-adrenergic receptor complex and its components. The experimental design suggested that alterations in the β-adrenergic adenylate cyclase complex occurs at a time characterized by minimal pathology and clinical signs. The present results are consistent with our previous studies demonstrating that biochemical abnormalities antedate gross pathology, and these may represent early events in the pathogenesis of chronic Chagasic heart disease.

Unlike our studies in myoblasts, infection alters both receptor and postreceptor elements of the β-adrenergic receptor complex. The infection-associated increase in β-receptor density (but not the decline in affinity) is similar to our observations of acetylcholine receptors in mice reported previously. Postreceptor modulation of adenylate cyclase activity is altered by infection with T. cruzi, most likely at the level of the regulatory proteins Ns and Ni. Several lines of evidence support this conclusion. First, infection diminishes the magnitude of the guanine nucleotide–dependent synergistic contribution to adenylate cyclase activation, an event requiring Ns. It is interesting to note that the actual ratio V_{max} isoproterenol over V_{max} Gpp(NH)p varies between 3.2 and 4, independent of the duration of infection, which is again consistent with a generalized infection-associated alteration in adenylate cyclase activity. In infected membranes, the contribution of Gpp(NH)p and GTP to isoproterenol- and forskolin-dependent adenylate cyclase activities significantly declines. Additional evidence consistent with a compromise in the function of Ns includes the general decline in adenylate cyclase activity due to several agents after 6 days, and all agents after 21 days, of infection. While we have tested adenylate cyclase activity due to Mn2+ and forskolin, agonists that stimulate adenylate cyclase activity in the absence of Ns, they are by no means specific stimulants of catalytic adenylate cyclase activity in the membrane.\textsuperscript{21,22} Hence, our data do not permit us to determine to what extent infection affects the integrity of the catalytic unit per se. In addition to Ns, the compromised ability of Gpp(NH)p and adenosine to inhibit forskolin-dependent adenylate cyclase activity in myocardial membranes of 21-day-infected mice implicates an involvement of the guanine nucleotide–binding protein Ni, as well, inasmuch as a functional Ni is necessary to demonstrate these phenomena.

While by no means established, the infection-associated decline in the ability to both stimulate and inhibit adenylate cyclase activity with a coincident...
The pertussis toxin substrate declines by greater than 40% at 6 days of infection, while the compromise in agent-dependent inhibition of adenylate cyclase activity is at best 5%. In the event that Ns and Ni constitute only a portion of the total cholera toxin and pertussis toxin substrates in these myocardial membranes, the infection-associated changes in toxin-dependent ADP ribosylation would bear little relation to the real changes in Ns or Ni in situ. Furthermore, even if the precise alterations in Ns and Ni due to infection could be determined, such information would not in itself be definitive since the stoichiometric relations between Ns and Ni and adenylate cyclase activity in situ are not certain at this time. With regard to Ni, it has been reported that this relation is clearly not linear.

Several possibilities can account for the apparent decline in the substrates for cholera and pertussis toxins associated with infection. First, infection could result in an actual physical loss of the guanine nucleotide-binding proteins (the holoproteins or components thereof). Alternatively, infection could alter the accessibility of these proteins to the toxins. In either case, by actual loss or sequestration, participation by these proteins in the regulation of adenylate cyclase activity would be impaired by infection. Experiments to resolve these issues are currently in progress. Alternatively, infection may have altered the enzyme kinetics and cofactor requirements for the toxin-dependent ADP ribosylation reaction. One possible candidate could be NADase activity, which competes with the toxins for the substrate NAD. To explore this possibility, we included NADP, an inhibitor of NADase activity, in our assay. Our results were clearly different for cholera and pertussis toxins. In the former case, only for membranes prepared from 21-day-infected membranes did the inhibitor of NADase activity increase the magnitude of cholera toxin–dependent ADP ribosyla-

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**Figure 9.** Adenosine-dependent inhibition of forskolin-dependent adenylate cyclase activity in mouse heart membranes prepared from control and infected mice. Adenosine-dependent inhibition of forskolin-stimulated adenylate cyclase activity was determined in an adenylate cyclase assay containing 100 μM forskolin at 30°C for 10 minutes in 1 mM Mg²⁺ for control mice (Panel A), mice infected for 6 days (Panel B), and mice infected for 21 days (Panel C). Results are means of three separate determinations. Standard deviations are less than 15% of the mean and for the sake of clarity are not included.

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**Figure 10.** Cholera toxin– and pertussis toxin–dependent ADP ribosylation of control and infected mouse heart membranes. Cholera toxin– and pertussis toxin–dependent ADP ribosylation was performed as described in "Materials and Methods." The gel shown here is one of three, all qualitatively similar. Molecular-mass markers are as noted in the figure. Lanes 1, no added toxin. Lanes 2, cholera toxin and Gpp(NH)p (100 μM). Lanes 3, cholera toxin, Gpp(NH)p (100 μM), and NADP (100 μM). Lanes 4, pertussis toxin and 100 μM each of ATP, NADP, and GTP. Spectrophotometric analysis of band intensity is summarized in Figure 11.
Alterations in adenylate cyclase activity have been studied in other experimental systems, and it may be useful here to compare several of these models with aspects of our observations. For example, desensitization of the β-adrenergic complex is associated with a decline in β-adrenergic adenylate cyclase activity. The loss of isoproterenol-dependent adenylate cyclase activity seen as a result of β-adrenergic desensitization occurs through uncoupling of the β-receptor from Ns followed in time by a physical loss of the β-receptor from the membrane. Uncoupling is thought to be a consequence of phosphorylation of the receptor component. However, in homologous β-adrenergic desensitization, there is little or no change in adenylate cyclase activity in response to stimulatory agents that operate independently of both the β-adrenergic receptor and the coupling units. In this regard, infection with T. cruzi is different in that declines in adenylate cyclase activities in response to agents that operate at postreceptor sites occur as well, and paradoxically, β-receptor density increases. Alternatively, in heterologous desensitization, refractoriness to stimulation by several agents that activate adenylate cyclase activity can be achieved by increasing intracellular cAMP independently of the receptor cyclase complex. However, heterologous desensitization is not associated with an increase in β-adrenergic receptor density, change in affinity, or the kinds of alterations observed with the choler toxin and pertussis toxin subunits. Nonetheless, infection may share with heterologous desensitization some common biochemical mechanisms that are as yet unknown.

An additional consideration to account for the infection-associated decline in toxin substrates includes the possibility that available N protein–acceptor sites for ADP ribose may be altered as a consequence of infection. In this latter instance, an enzymatic reaction, perhaps an infection-related ADP ribosylation itself, could effectively eliminate Ns or Ni as available in vitro substrates for cholera and pertussis toxins, and at the same time, perhaps it could alter their respective functions so as to produce the changes observed in this study. We are attracted to this possibility because ADP ribosyltransferase activity has been found in T. cruzi. This putative consequence of infection-associated ADP ribosylation activity contrasts with the observation that cholera toxin–dependent ADP ribosylation of membranes or intact cells decreases potential substrate for subsequent in vitro cholera toxin–dependent ADP ribosylation, but at the same time, it leads to an increase in both basal- and agent-stimulated adenylate cyclase activity. In the present study, infection was associated not only with a decrease in substrate for cholera toxin–dependent ADP ribosylation but a decrease in adenylate cyclase activity. If it is ultimately shown that endogenous ADP ribosyltransferase activity is increased in infected hearts, one will have to consider the interesting possibility that infection-associated ADP-ribosyltransferase activity has different functional consequences (e.g., adenylate cyclase activity) than the consequences of cholera toxin– and pertussis toxin–associated ADP-ribosylation activities.

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An additional consideration to account for the infection-associated decline in toxin substrates in-
Another model to compare with our study is the decline in both the magnitude and sensitivity of adenylate cyclase activation by isoproterenol after the homogenization of tissues. Homogenization may permit endogenous proteolytic activity to digest components of the receptor cyclase complex, thereby altering their activity. Adenylate cyclase activity in response to other noncatecholamine agonists, such as forskolin, declines as a result of homogenization per se. It may be that infection renders components of the adenylate cyclase complex more susceptible to the consequences of homogenization-associated proteolytic activity. Such a formulation is viewed by us as unlikely in view of the fact that EDTA, a powerful inhibitor of many proteases, is included in the homogenization buffers, and we have shown previously that EDTA prevents the apparent activation of these proteases.

The functional consequences of the infection-associated changes in adenylate cyclase activity are difficult to assess. By several criteria, throughout 21 days of infection with T. cruzi, we failed to observe any significant compromise in the viability of these animals. It has been reported that between 3 and 7 months after infection in mice, the isometric-developed tension of right ventricles was lower than in the ventricles of uninfected mice. Therefore, it may be possible that the changes reported here are not distinguishable by such gross measurements of myocardial function. That more subtle changes in contractility are associated with infection and apparent only in individual cells remains to be determined. It appears more likely that the changes reported here may initiate events the culmination of which manifest themselves as the kinds of alterations in myocardial function observed in the chronic model of the disease.

The possibility that the presence of inflammatory cells in the myocardium may contribute to the results observed here appears to be unlikely. Thus, even at 6 days of infection, examples of inflammatory cell infiltration are almost impossible to find, despite the profound changes in the properties of the adenylate cyclase complex. Furthermore, the patterns of alterations in the properties of the adenylate cyclase complex at 21 days of infection, that is, increased β-receptor density at a time of reduced β-adrenergic responsive adenylate cyclase activity and a decline not only in the total adenylate cyclase activity but in the magnitude of the modulation of that activity, are inconsistent with simply a contribution of the inflammatory cell adenylate cyclase complex.

Alterations in receptor-mediated activation and inhibition of adenylate cyclase activity in the cardiomyopathy associated with atherosclerotic heart disease, diabetes, and hypertension have been reported. However, these data were obtained only in chronic, well-established disease characterized by severe functional cardiac compromise and extensive pathological myocardial involvement. In contrast, in the present studies, there was no evidence of extensive pathological functional abnormalities. In studies of chronic cardiomyopathies, the most frequent and impressive finding has been a reduction in catecholamine stores. Similar to results reported here, in dogs with hypertrophic hearts secondary to long-term aortic banding, Vatner et al found a 44% increase in β-adrenergic receptors associated with a twofold decrease in receptor affinity. Isoproterenol-dependent adenylate cyclase activity was decreased. In addition, muscarinic receptor density fell. The consequences of muscarinic-dependent inhibition of adenylate cyclase activity were not reported. Divergent results have been obtained in other systems. In a later report, heart failure in dogs was found to be associated with a decreased level of Na. Of particular interest would be the determination of whether alterations reported for adrenergic or muscarinic receptors in chronic cardiomyopathies had already occurred before the gross physiological or pathological manifestations. It may well be that independent of etiology, early molecular alterations in either the stimulatory or inhibitory arms of the adenylate cyclase complex may be common to many forms of cardiomyopathy.

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