Role of N1 in Coupling Angiotensin Receptors to Inhibition of Adenylate Cyclase in Hepatocytes*

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Angiotensin II can inhibit glucagon-stimulated cyclic AMP production in hepatocytes and adenylate cyclase activity in hepatic membranes. Pertussis toxin, an exotoxin produced by Bordetella pertussis, was used to investigate the role of the inhibitory guanine nucleotide-binding regulatory proteins of adenylate cyclase (N1) in coupling angiotensin receptors to the adenylate cyclase system. An assay was developed using [32P] NAD* to quantitate the amount of N1 protein in the membrane and the extent of its ADP-ribosylation catalyzed by toxin. The ability of angiotensin to inhibit adenylate cyclase and interact with its receptor was compared with the degree of modification of N1 in membranes prepared from isolated hepatocytes. In control membranes angiotensin II inhibited basal adenylate cyclase by 35%. When all of the Ni molecules in the membrane were ADP-ribosylated, angiotensin did not inhibit adenylate cyclase. However, the attenuation of angiotensin’s effect on cyclase was not linearly correlated with the degree of modification of N1; ADP-ribosylation of greater than 80% of the N1 was required before a reduction of the angiotensin effect was observed. A possible explanation for this finding is an excess of Ni molecules in the membrane (~3.4 pmol/mg of membrane protein) over angiotensin II receptors (~1.2 pmol/mg of membrane protein). [125I]-angiotensin bound to sites in the membrane with two affinities. Computer fitting of the binding isotherms yielded parameters of N1 = 279 fmol/mg protein, Kd = 0.2 nM; N2 = 904 fmol/mg protein, Kd = 1.4 nM. When all of the Ni molecules in the membrane were ADP-ribosylated, angiotensin bound to only one site with binding parameters of N = 349 fmol/mg protein, Kd = 0.4 nM. GTP-γ-S caused a 7-fold increase in the Kd of this site to 2.7 nM. Overall, the data indicate that the Ni protein mediates the effect of angiotensin on adenylate cyclase. The observation that GTP-γ-S can markedly decrease the affinity of angiotensin receptors when all N1 molecules are ADP-ribosylated suggests that angiotensin receptors may couple to other GTP-binding proteins which may mediate the effects of angiotensin in other signal transduction systems.

The adenylate cyclase complex is comprised of at least 5 proteins located in the plasma membrane (1–7). They include (a) hormone receptors able to initiate stimulation or inhibition of adenylate cyclase, (b) two types of guanine nucleotide-binding regulatory proteins, N, and Ni, and (c) the catalytic unit that is responsible for cyclic AMP production. The guanine nucleotide-binding proteins interact with both the catalytic unit and the receptors, accounting for the GTP requirement for hormonal effects on adenylate cyclase and the ability of GTP to modify the affinity of receptors for agonist ligands.

These guanine nucleotide-binding proteins are also targets of bacterial toxins that are useful probes for defining the interaction of the regulatory proteins with the other components of the cyclase system. Cholera toxin irreversibly activates the protein mediating stimulation of adenylate cyclase (N1), while pertussis toxin acts on the protein regulating inhibition (N1), thus blocking hormonal attenuation of adenylate cyclase (6–8). Interestingly, both types of toxin act by covalently linking the ADP-ribose moiety from NAD* to the α-subunit of their respective substrates, the 45,000-dalton subunit of N1, (8–10) or the 41,000-dalton subunit of N3 (11, 12). ADP-ribosylation of N1 appears to impair its interaction with the cyclase system both by blocking its ability to inhibit the activated catalytic unit (6, 7) and by disrupting its association with receptors for inhibitory ligands (13–20).

Angiotensin II has been demonstrated to inhibit adenylate cyclase in liver (21, 22), adrenal cortex (23, 24), renal cortex (25), and smooth muscle (26). In liver and adrenal, binding experiments using [125I]-angiotensin II have demonstrated two binding sites with differing affinities (27, 28). In all of these tissues guanine nucleotides reduce the affinity of the receptor for angiotensin II (22, 29–31). However, since angiotensin also stimulates phosphatidylinositol breakdown in these cells (32–35), it is possible that GTP-binding proteins may also be involved in the Ca2+-linked response.

In the present study, pertussis toxin was used to investigate the role of the N1 protein in coupling angiotensin receptors to the adenylate cyclase system in isolated hepatocytes. An assay was developed to quantitate the amount of N1 protein in the membrane and its degree of ADP-ribosylation following treatment with toxin. In a series of hepatocyte preparations, the extent of modification of N1 was correlated with the ability of angiotensin to: (a) inhibit glucagon-stimulated cyclic AMP accumulation in intact cells; (b) inhibit adenylate cyclase in membranes prepared from the hepatocytes; and (c) interact with its receptor in the plasma membrane.

The abbreviations used are: N1 and N3, the stimulatory and inhibitory guanine nucleotide-binding regulatory proteins of adenylate cyclase, respectively; Hepes, N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; GTP-γ-S, guanosine 5'-O-(3-thiotriphosphate).

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Angiotensin II Inhibits Adenylate Cyclase via N1

EXPERIMENTAL PROCEDURES

Isolation, Incubation, and Primary Culture of Hepatocytes—Isolated liver cells were prepared from normal or pertussis toxin-treated male Wistar rats according to published methods (36). Hepatocytes were isolated from a density of about 10^6 cells/ml in Krebs-Ringer bicarbonate buffer supplemented with 16 mM L-lactate and 4 mM pyruvate and kept under an atmosphere of 95% O2-5% CO2. When liver cells were prepared from normal dough Bordetella pertussis strain 165 as described (38,39). Cultured hepatocytes were cultured and incubations continued for an additional 20 h. Wistar rats according to published methods (36). Hepatocytes were isolated as described above.

Treatment of Cultured Hepatocytes or Intact Rats with Pertussis Toxin—Pertussis toxin was purified from the culture medium of Bordetella pertussis strain 165 as described (38,39). Cultured hepatocytes were treated with toxin by one of two methods. In the first method, the liver with large doses of toxin, the portal vein was exposed directly into the portal vein. Control animals received the toxin vehicle, and the incisions were closed with wound clips. In later experiments, intraperitoneal injection of toxin was found to be less convenient and to produce a higher degree of ADP-ribosylation of hepatic N1. In these experiments 50 pg of toxin/100 g of body weight was injected directly into the peritoneal cavity. In either case, 10–24 h after the rats were injected with toxin hepatocytes were isolated as described above.

Preparation of Plasma Membranes—Membranes were prepared from cultured or freshly isolated hepatocytes by a combination of the methods described by Pohl (40) and Nakamura et al. (41). All procedures were carried out at 0–4 °C. Cells were centrifuged at 50 × g for 3 min, the supernatant discarded, and the pellet homogenized at a concentration of 3 × 10^6 cells/ml in 5 mM EDTA, 10 mM Hepes, pH 7.4, with 20 strokes of a Dounce homogenizer. The homogenates were centrifuged at 150 × g for 5 min, the pellets discarded, and the supernatants centrifuged at 1500 × g for 15 min. The pellets were resuspended in the initial volume of homogenization buffer and recentrifuged at 15,000 × g for 20 min. These pellets were resuspended with 3 strokes of the homogenizer in a minimal volume of buffer. This suspension was centrifuged on a sucrose density gradient in an SW 50.1 rotor at 150,000 g for 50 min, the pellet discarded, and the solubilized proteins used as the source of Ni. Pilot experiments comparing membranes isolated from hepatocytes with those prepared from intact livers (with which the original conditions were developed) showed the two preparations to be similar in most respects. Most pertinent to the present study is that membranes made from isolated cells do not degrade 125I-angiotensin I1 with the latency and low ligand concentrations that are present in the intact membrane. Taken together these results suggest that both native and ADP-ribosylated N1 are extracted from the membrane with approximately equal efficiency.

ADP-ribosylation of Ni was performed as described (44–46) with minor modifications. The reaction was initiated by adding 4–30 μg of protein in cholate to a tube containing 2 μM N1, 2 μCi of [32P]NAD+, 100 mM Tris, pH 8.0, 10 mM thymidine, 100 μM GTP, 1 mM ATP, 2.5 mM MgCl2, 1 mM EDTA, 10 mM dithiothreitol, 50 mM glycine, pH 8.0, 3 mM 1,2-dimyristoyl phosphatidylcholine, and 0.5 mg/ml ovalbumin in a volume of 100 μl. Reactions were run with and without 25 μg/ml pertussis toxin (an amount determined to be submaximal in pilot experiments). In order to determine that the reactions were going to completion, some extracts from control and toxin-treated membranes were supplemented with 0.1–0.6 pmol of the purified resolved 41,000-dalton α-subunit of Ni (47).

Incubations were continued for 30 min at 30 °C (enough time for 100% ADP-ribosylation to be performed in pilot experiments), stopped by the addition of 1.5 ml of ice-cold 10% trichloroacetic acid, and processed for SDS-polyacrylamide gel electrophoresis and autoradiography as described (48). Based on the experiments demonstrating that the ADP-ribosylation of Ni in extracts is complete (see "Results"), we assume that the purified resolved 41,000-dalton α-subunit of Ni has 1 mol of ADP-ribosylation sites/mole of α-subunit (49), the number of pmol of Ni present was calculated from the specific activity of the [32P]NAD and the number of counts in the 41,000-dalton band cut from the gel.

Bind-picking Experiments—The binding of 125I-angiotensin II to its receptor in membranes from control and toxin-treated hepatocytes was performed essentially as described by Campanile et al. (27). Briefly, binding experiments were performed with 10–20 μg of membrane protein in 100 μl of a buffer containing 20 mM Tris, pH 7.4, 100 mM NaCl, and 10 mM MgCl2. Binding isotherms were performed for membranes isolated from cultured cells. In these experiments 50 pg of toxin/100 g of body weight was injected directly into the portal vein. The binding reaction was allowed to go to completion, some extracts from control and toxin-treated membranes were supplemented with equal efficiency.

Quantitation of N1—The amount of Ni in hepatic membranes and its degree of ADP-ribosylation following treatment with pertussis toxin was measured by using pertussis toxin to catalyze the incorporation of [32P]NAD+ of known specific activity into a solubilized preparation of Ni. Since liver plasma membrane preparations contain substantial amounts of a "NAD-hydrolyzing" activity which degrades the reaction substrate, the Ni molecule was extracted from the membrane with Na+-cholate and diluted into the reaction mixture. This procedure significantly reduced the degradation of NAD+ during the assay (see "Results").

The Ni molecule was extracted from the plasma membranes by incubating 2 mg of membrane protein in 200 μl of a solution containing 20 mM Tris, pH 8.0, 20 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, and 1% (w/v) sodium cholate with constant shaking for 60 min at 4 °C (42). The extracts were centrifuged in a Beckman Airfuge at 110,000 × g for 50 min, the pellet discarded, and the solubilized proteins used as the source of Ni. Pilot experiments using the optimized reaction conditions (see below) demonstrated that this procedure solubilized about 80% of the Ni from the membrane. Almost complete extraction of Ni (95%) could be achieved by subjecting the 110,000 × g pellet to a second 60-min cholate extraction. To determine if the ADP-ribosylated form of Ni was extracted with equal efficiency, plasma membranes were labeled with [32P]NAD+ in the presence of pertussis toxin and divided into equal aliquots. One aliquot was precipitated with trichloroacetic acid and boiled directly in Laemmli sample buffer (43). The second aliquot of [32P]-labeled membranes was extracted with Na–cholate, precipitated with trichloroacetic acid, and the extract boiled in sample buffer. Following electrophoresis on 10% polyacrylamide gels, the [32P]-labeled 41,000-dalton subunits of Ni were cut from the gel and counted. The Ni in the cholate extract contained ~90% of the Ni found in the Na–cholate extract. Taken together these results suggest that both native and ADP-ribosylated Ni are extracted from the membrane with approximately equal efficiency.

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The ability of liver plasma membranes or extracts to degrade NAD* was measured by determining the amount of intact [32P]NAD* left at the end of each ADP-ribosylation assay. One-two μl of the reaction supernatant from the trichloroacetic acid precipitation step was spotted on a polyethyleneimine cellulose thin layer chromatography plate, developed for 2 h in 0.15 M formic acid, 0.1 M LiCl, air dried, and exposed to Kodak XAR-5 X-ray film for 16-24 h. The position of the intact NAD* was determined by comparison with a standard of pure NAD*. The amount of intact [32P]NAD* remaining on the plate was determined by scraping the area into a liquid scintillation vial and counting. Results presented in Fig. 3 are expressed as per cent of the [32P]NAD* originally added to the assay. The amount of [32P]GTP-γ-S bound to N1 was determined as described (46).

Materials—Purified N1 and its resolved 41,000-dalton α-subunit were prepared as described (46, 47) and generously donated by Dr. John K. Northup, University of Calgary, Canada. Chick heart membranes prepared by differential centrifugation were the gift of Dr. Joel Linden, Oklahoma Medical Research Foundation. Transducin was prepared as described (51) and generously provided by Dr. James Miller of the Department of Biochemistry, University of Virginia. Other reagents were obtained from the following suppliers: cholic acid, glucagon, lactate, l-α-dimyristoyl phosphatidylcholine, LiCl, NAD*, sodium salt (Grade VII), and ovalbumin, Sigma; [32P]NAD* and l-15I-angiotensin II, New England Nuclear; fetal calf serum and L-15 medium, Gibco; thymidine, Aldrich; polyethyleneimine cellulose plates, E. Merek and Co. All other reagents were reagent grade or obtained from published sources (22, 27). Molecular weight standards were as described (48).

Calculations and Expression of Results—The data presented in Figs. 1-6 are representative of experiments repeated at least 4 times. Averaged data are presented as the mean ± S.E. with statistical evaluation performed by the one-tailed paired t test.

RESULTS

Effects of Pertussis Toxin in Intact Cells—Since pertussis toxin may require long incubation times to intoxicate intact cells (59), initial experiments were performed with hepatocytes placed into primary culture and treated for 20 h with toxin. The top left panel in Fig. 1 shows that glucagon causes a marked rise in cyclic AMP levels in the cultured cells and that inclusion of a maximal dose of angiotensin II reduces the cyclic AMP levels by about 35%. Although the magnitude and kinetics of the cyclic AMP response in the cultured cells differs from that in fresh hepatocytes (compare top left and bottom left panels), angiotensin II inhibits the glucagon response to approximately the same extent (35-40%) in both hepatocyte preparations.

Treatment of the cultured cells with pertussis toxin for 20 h abolishes the ability of angiotensin to inhibit glucagon-stimulated cyclic AMP accumulation (Fig. 1, top right). Moreover, in the toxin-treated cultures, the glucagon stimulation of cyclic AMP levels was enhanced to about 160% of control levels. These results demonstrate that the intact hepatocyte responsive to the holotoxin and that the consequences of intoxication are similar to those observed in a number of other systems (see Refs. 6 and 7 for a review). Overall, the results presented in the top half of Fig. 1 strongly suggest that N1 mediates the ability of angiotensin to inhibit adenylate cyclase.

While pertussis toxin can clearly modify the hormonal responses of cultured cells, the intent of the present experiments was to examine the relationship between the degree of modification of N1 and the ability of angiotensin to interact with adenylate cyclase. The large quantities of membranes required for these experiments were most conveniently prepared from hepatocytes isolated from intoxicated rats. To this end, rats were injected with 5 μg of toxin/100 g of body weight directly into the portal vein, and hepatocytes were isolated after a 10-24 h incubation period (see "Experimental Procedures" for details). The bottom left panel in Fig. 1 presents the effects of glucagon and angiotensin II on cyclic AMP accumulation in hepatocytes isolated from control rats and the bottom right panel an identical experiment performed with hepatocytes isolated from a rat treated with pertussis toxin. In contrast to the results obtained with cultured cells, angiotensin inhibited glucagon-stimulated cyclic AMP production by about 35-40% in cells prepared from either control or toxin-treated animals. Since similar doses of toxin administered to intact animals have produced biological effects (19, 53-55), the above results suggested that all of the N1 molecules in the hepatocyte membrane had not been modified. Therefore, an assay was developed to measure the degree of ADP-ribosylation of N1 produced by toxin treatment.

Quantitation of the ADP-ribosylation of N1.—The assay for N1 and its extent of ADP-ribosylation was based on the finding that the purified N1 molecule can be ADP-ribosylated by pertussis toxin in solution and that the α-subunit incorporates 1 mol of ADP-ribose/mol of protein (46). Thus it is possible to quantitate the amount of N1 and/or its degree of modification provided the ADP-ribosylation reaction can be made to go to completion with less purified preparations of N1. In the present experiments, the N1 in cholate extracts of plasma membranes was used as the reaction substrate to minimize the hydrolysis of NAD* in the assay (see "Experimental Procedures" and Fig. 3B). The data presented in Figs. 2 and 3 verify that the ADP-ribosylation reaction will go to completion in cholate extracts.

The N1 in a cholate extract of hepatocyte membranes can
be ADP-ribosylated in solution using pertussis toxin and [32P]NAD+ (Fig. 2A, + BPT lane). A band with a M, of 41,000 is extensively labeled, and no labeling is observed in the absence of toxin (−BPT lane). The 32P-labeled protein in the extract migrates identically with the resolved 41,000-dalton α-subunit of Ni, which was ADP-ribosylated with toxin under the same reaction conditions (Fig. 2A, 41K lane). If the extract is supplemented with the α-subunit [32P]ADP-ribose incorporation into the 41,000-dalton band is additive (Fig. 2A, BOTH lane) (see also discussion of Fig. 3, below).

Recently, pertussis toxin has been observed to catalyze the ADP-ribosylation of a substrate other than Ni in brain. This substrate, a 39,000-dalton protein, represents the α-subunit of what currently appears to be a novel GTP-binding protein, termed G. The functional significance of G, is not known, although in brain Ni, and G, appear to be ADP-ribosylated in roughly equal amounts (56, 57). Under the conditions used in these experiments, very little 39,000-dalton substrate is labeled in extracts of liver membranes (Fig. 2B). The two outside lanes (39K lanes) represent standards composed of the 39,000 dalton α-subunit of transducin labeled with [32P]NAD+. The second and third lanes represent ADP-ribosylated extracts of chick heart membranes (HRT) and hepatic membranes (LIV), respectively. The heart membranes clearly demonstrate the presence of the 39,000- and 41,000-dalton substrates whereas very little 39,000 substrate is observed in liver membranes. While this result does not prove that the 39,000-dalton substrate is absent from hepatic membranes, it does suggest that it is not necessary to resolve the two proteins in order to accurately quantitate the amount of Ni, in hepatocyte membranes.

Fig. 3 presents the quantitative aspects of the ADP-ribosylation reactions. The inset in Fig. 3A demonstrates the stoichiometry of the reaction run with the purified resolved α-subunit of Ni. In agreement with the findings of Bokoch et al. (46), there is a 1:1 correlation between the number of pmol of [32P]ADP-ribose incorporated into the α-subunit and the
the reaction is kept within certain limits.

The data in Fig. 3B indicate that the reason for this limit is the increasing rate of NAD+ degradation as greater amounts of protein are added to the assay. Therefore, the amount of extract protein in all reactions mixes was kept within the linear range of the assay (below 10 μg). Much less Ni is labeled if the extracts are prepared from membranes of cells treated with pertussis toxin, suggesting that the ADP-ribosylation site in the Ni molecule is already linked to unla-

beled NAD+ (Fig. 3A).

If the purified Ni protein is added to reaction mixtures containing extracts of control or intoxicated membranes, an appropriately greater amount of [32P]incorporation is obtained. For example, if 0.01 pmol of Ni (an amount near the detection limit) is added to a control extract containing 0.10 pmol of Ni, 0.11 pmol is recovered. If the 0.01 pmol of Ni is added to an extract from completely intoxicated membranes in which all Ni molecules are ADP-ribosylated, only 0.01 pmol is re-

covered.

Taken together, the data presented under "Experimental Procedures" and Figs. 2 and 3 demonstrate that: (a) Ni can be efficiently and consistently extracted from plasma mem-

branes; (b) the ADP-ribosylation of the molecule can be catalyzed by pertussis toxin in the extracts; and (c) complete ADP-ribosylation can be obtained if the amount of extract in the reaction is kept within certain limits.

Using the assay as described, the amount of Ni protein in hepatocyte membranes was determined to be ~3.4 ± 0.3 pmol of Ni/mg of membrane protein (n = 8). The degree of ADP-

ribosylation of the Ni in the membranes prepared from the cultured and fresh hepatocytes used in the experiments pre-

sented in Fig. 1 was also determined (see Fig. 2C). The degree of prior modification of a toxin-treated sample was calculated as a percentage of the [32P]ADP-ribose incorporated into Ni in an extract from the appropriate control membranes run in a parallel reaction. As expected, the Ni in the membranes from cultured hepatocytes treated with toxin (100% lane, far right) was found not to incorporate any [32P]NAD+ and was designated as 100% ADP-ribosylated. This is consistent with the data shown in Fig. 1. Interestingly, the Ni in the mem-

branes from the hepatocytes isolated from the toxin-treated rats were found to be 88% modified (88% lane, middle) as compared to the control membranes (left lane).

During development of the assay, it was discovered that full modification of Ni could be obtained by treating intact rats with an intraperitoneal injection of toxin 72 h prior to sacrifice. Lesser degrees of intoxication were obtained by reducing the incubation time. Thus, membranes from cells in which the Ni was modified to varying degrees were used to investigate the relationship between Ni and the ability of angiotensin to interact with adenylyl cyclase.

Effects of Angiotensin II on Adenylyl Cyclase in Membranes Prepared from TOxin-treated Hepatocytes—The data pre-

sented in Figs. 1 and 2C suggest that full modification of Ni in hepatocytes is necessary to observe an inhibition of cyclic AMP generation. Complete modification of Ni also blocks angiotensin's ability to inhibit adenylyl cyclase (Table 1A). In agreement with previous results obtained with membranes purified from intact livers (21, 22), 10^-6 M angiotensin II lowers basal adenylyl cyclase activity in hepatocyte mem-

branes from control rats by about 35%, from 26.5 to 17.2 pmol of cAMP/min/mg of protein. In membranes in which 100% of the Ni was ADP-ribosylated, basal cyclase was lowered slightly (to ~16 pmol of cyclic AMP/min/mg of protein), but angiotensin did not cause a significant inhibition of enzyme activity.

α2-Adrenergic agonists also inhibit adenylyl cyclase in hepatic membranes (21). Table 1B demonstrates that epinephrine, in the presence of 2 × 10^-5 M l-propranolol, can inhibit adenylyl cyclase about 25% in membranes prepared from hepatocytes. This effect can be antagonized by the α2-

adrenergic antagonist yohimbine. In membranes in which the Ni was 100% modified, epinephrine no longer inhibits ade-

nylyl cyclase. These results suggest that Ni also mediates the effects of α2-adrenergic receptors on adenylyl cyclase in hepatocytes.

The data in Fig. 4 shows that the inhibition of adenylyl cyclase in control hepatocyte membranes has a similar sen-

sitivity to angiotensin II (half-maximal at 10^-8 M) as mem-

branes prepared from intact rat livers (21, 22). Interestingly, note that in membranes in which 89% of the Ni is modified, the effects of angiotensin on cyclase are clearly reduced, but the magnitude of the effect is not commensurate with the degree of modification of Ni. In agreement with Table I, membranes in which Ni is completely modified show no inhibition of adenylyl cyclase at any angiotensin concentra-

tion.
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**TABLE I**
Effect of complete ADP-ribosylation of Ni on the ability of angiotensin II or α2-adrenergic agonists to inhibit adenylate cyclase

Adenylate cyclase activity was measured at 30°C for 10 min as described under "Experimental Procedures." Basal refers to adenylate cyclase activity in the presence of 0.1 mM GTP and 250 mM LiCl. In B, 20 μM 1-propranolol was added to all reactions to block the β-adrenergic effects of epinephrine. β-Blocker was also present under basal conditions. The data represent the mean activity of 3-4 membrane preparations, with each assayed in triplicate.

<table>
<thead>
<tr>
<th>Membrane preparation</th>
<th>Adenylate cyclase activity</th>
<th>Inhibition (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>Basal + 10^{-6} M angiotensin II</td>
</tr>
<tr>
<td></td>
<td>pmol cAMP/min/mg protein</td>
<td>pM angiotensin II</td>
</tr>
<tr>
<td>Control membranes</td>
<td>26.5 ± 2.5</td>
<td>17.2 ± 1.6</td>
</tr>
<tr>
<td>100% modified membranes</td>
<td>15.9 ± 1.8</td>
<td>14.6 ± 1.7</td>
</tr>
</tbody>
</table>

A. Inhibition by angiotensin II

B. Inhibition by α2-adrenergic agonists

<table>
<thead>
<tr>
<th>Membrane preparation</th>
<th>Basal + 20 μM 1-propranolol</th>
<th>Basal + 10^{-4} M epinephrine + 20 μM 1-propranolol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control membranes</td>
<td>27.4 ± 5.9</td>
<td>20.5 ± 4.3</td>
</tr>
<tr>
<td>+30 μM yohimbine</td>
<td>25.5 ± 3.3</td>
<td>7.1 ± 5.9*</td>
</tr>
<tr>
<td>100% modified membranes</td>
<td>16.3 ± 2.9</td>
<td>15.5 ± 2.5</td>
</tr>
<tr>
<td>+30 μM yohimbine</td>
<td>15.8 ± 2.7</td>
<td>3.1 ± 0.6*</td>
</tr>
</tbody>
</table>

* Significant by paired t test (0.02 > p > 0.005).

† Not different by paired t test (p > 0.1).

**FIG. 4.** Inhibition of adenylate cyclase by angiotensin II in membranes prepared from hepatocytes isolated from control or pertussis toxin-treated rats. The magnitude of the angiotensin II effect is expressed as per cent inhibition of basal adenylate cyclase activity measured in the presence of 0.1 mM GTP plus 250 mM LiCl, as described under "Experimental Procedures." The data shown represents averages from 6 control rats (0% modification), 5 rats completely modified by prior treatment with pertussis toxin (100%), and 3 rats partially modified by pertussis toxin treatment (89%). Basal adenylate cyclase activity was about 30-40 pmol of cyclic AMP/min/mg of membrane protein.

**FIG. 5.** Correlation between the extent of ADP-ribosylation of Ni and the ability of angiotensin to inhibit adenylate cyclase activity in membranes or cyclic AMP accumulation in cells. Each point represents data from a single rat using a maximum concentration of angiotensin II. Basal adenylate cyclase activity was measured with 0.1 mM GTP and 250 mM LiCl in the presence and absence of 10^{-6} M angiotensin II. Cyclic AMP accumulation was measured in cells incubated in Krebs-Ringer bicarbonate buffer and stimulated with 10^{-7} M glucagon with or without 10^{-7} M angiotensin II.

The effects of varying degrees of modification of Ni on the binding of 125I-angiotensin II to its receptor are shown in Fig. 6, B and C and Table II. As Ni becomes more highly ADP-ribosylated, the number of high affinity binding sites drops from 279 fmol/mg protein to nearly no detectable sites. The
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**Fig. 6.** Binding of ^125^I-angiotensin II to membranes isolated from hepatocytes prepared from control and pertussis toxin-treated rats. Binding isotherms were performed in the presence (●) and absence (■) of 10^{-4} M GTP-γ-S. The left half of the figure presents binding isotherms corrected for nonsaturable binding. Each point is an average of triplicate determinations. The right half of the figure presents the corresponding Scatchard plots. Membrane preparations were made from hepatocytes with A, no Ni modified by toxin (CONTROL); B, hepatocytes whose Ni was 88% modified by toxin treatment; C, hepatocytes whose Ni was completely modified by toxin treatment.

**Table II**
Effect of ADP-ribosylation of N_i on the binding of angiotensin II

<table>
<thead>
<tr>
<th>Membrane preparation</th>
<th>10^{-4} M GTP-γ-S</th>
<th>N_i (fmol/mg protein)</th>
<th>K_d (nM)</th>
<th>N_i (fmol/mg protein)</th>
<th>K_d (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control membranes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>279 ± 32</td>
<td>0.20 ± 0.06</td>
<td>904 ± 163</td>
<td>1.44 ± 0.62</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>41 ± 12</td>
<td>0.44 ± 0.21</td>
<td>730 ± 157</td>
<td>6.92 ± 1.2</td>
</tr>
<tr>
<td>Membranes with N_i 88% modified</td>
<td></td>
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<td></td>
<td>-</td>
<td>98 ± 9^a</td>
<td>0.12 ± 0.02</td>
<td>570 ± 29^a</td>
<td>0.37 ± 0.09</td>
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<tr>
<td></td>
<td>+</td>
<td>-^b</td>
<td></td>
<td>438 ± 39^a</td>
<td>4.55 ± 1.06</td>
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<td>Membranes with N_i 100% modified</td>
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<td></td>
<td>-</td>
<td>5 ± 1^a</td>
<td>0.05 ± 0.04</td>
<td>349 ± 10^a</td>
<td>0.38 ± 0.11</td>
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<tr>
<td></td>
<td>+</td>
<td>-^b</td>
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<td>184 ± 27^a</td>
<td>2.72 ± 1.05</td>
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</table>

^a Significantly different from control without GTP-γ-S (0.01 > p > 0.005).
^b Significantly different from control with GTP-γ-S (0.025 > p > 0.01).
^c Not different from control with GTP-γ-S (p > 0.1).

The number of low affinity sites is also reduced from 904 fmol/mg protein to 349 fmol/mg protein. When N_i is completely modified, the binding data are best fit to a one-site model (Fig. 6C and Table II). Modification of N_i does not appear to have major effects on the K_d for either binding site (Table II). The apparent decrease in the number of receptors (at least as measured with agonist ligands) as N_i becomes more fully ADP-ribosylated has also been observed in membranes from cultured NG-108 cells (16). Therefore, it seems unlikely that the decrease in receptor number is due to an altered hormonal balance of the intoxicated animal.

Table II contains two other significant findings. First, there are about one-third as many angiotensin II receptors in the membrane (~1.2 pmol/mg protein) as N_i molecules (~3.4 pmol/mg protein). Second, even when N_i is fully ADP-ribosylated by prior intoxication, GTP-γ-S causes a 7-fold increase in the K_d for the lower affinity binding site. This result is in marked contrast to the data obtained in a number of other systems showing that ADP-ribosylation of N_i reduces or abolishes the effects of guanine nucleotides on agonist binding (13–20).

**DISCUSSION**

The data presented clearly support a role for N_i in mediating the effect of angiotensin II on adenylate cyclase in hepatoc-
Angiotensin II Inhibits Adenylate Cyclase via $N_1$

...this is that complete ADP-ribosylation of $N_1$ does not eliminate the effect of GTP-$\gamma$-S on the affinity of angiotensin receptors (Fig. 6 and Table II). This result is in marked contrast to other systems such as NG-108 cells (13, 16), kidney (17), heart (14, 19), pituitary (18), striatum (15), and neutrophils (20) where covalent modification of $N_1$ blocks guanine nucleotide effects on agonist binding. There are two possible explanations for this result. First, in most of the studies cited above, binding experiments were performed by labeling a small percentage of the receptors with a radioactive antagonist and competing for these sites with an unlabeled agonist in the presence and absence of guanine nucleotides. In the present work, all experiments were performed with a labeled agonist ($[^{125}I]$-angiotensin II) which may be more sensitive to the effects of GTP-$\gamma$-S. However, in membranes from both NG-108 cells and neutrophils, prior modification of $N_1$ has been shown to abolish the effects of guanine nucleotides on agonist binding (16, 20). Therefore, it seems unlikely that the use of an agonist ligand alone leads to the results observed in the hepatocyte membranes.

A more interesting possibility is that angiotensin receptors may couple to a separate unidentified guanine nucleotide-binding protein that is a component of another signal-transducing system. A prime candidate is the phospholipase C system that catalyzes inositol lipid breakdown. There are three lines of evidence leading to this conclusion. First, the affinity of certain receptors that are thought to be exclusively coupled to inositol lipid breakdown is decreased by guanine nucleotides. This phenomenon occurs with $\alpha_1$-adrenergic receptors in liver (59) and kidney (17, 60) and muscarinic receptors in 1321N1 human astrocytoma cells (61, 62). Furthermore, if binding experiments are performed with membranes prepared from kidney or 1321N1 cells that have been treated with pertussis toxin, there appears to be no effect of toxin on the binding of $\alpha_1$-adrenergic (17) or muscarinic (62) ligands to receptors in the presence or absence of guanine nucleotides. Second, growing evidence suggests that there is a large family of guanine nucleotide-binding proteins (6, 63). Recently, GTP-binding proteins other than $N_1$ and $N_2$ have been purified from brain (56, 57). The brain protein, termed $G_6$, has been found to reconstitute guanine nucleotide effects on binding of muscarinic ligands to receptors solubilized from brain (64). While there appears to be very little $G_6$ in hepatic membranes (as determined by the present ADP-ribosylation experiments), it is possible that a similar protein, which is not a toxin substrate, can couple to angiotensin receptors. Finally, experiments performed with permeabilized platelets and mast cells have shown that GTP analogues can enhance the secretion of serotonin (65) or histamine (66) from the cells or lower the intracellular Ca$^{2+}$ level required for secretion (66). Furthermore, GTP-$\gamma$-S stimulates the breakdown of phosphorylidyinositol 4,5-biphosphate in membranes isolated from neutrophils (67). Taken together, these results strongly suggest that the transduction system mediating inositol lipid breakdown contains an unidentified guanine nucleotide-binding protein. Angiotensin receptors in the hepatic membranes may couple to such a protein to initiate their effects on phosphorylidyinositol breakdown.
While evidence is accumulating that a novel guanine nucleotide-binding protein may mediate the phosphatidylinositol response in some cells, other experiments demonstrate a role for \(N_i\) in mediating the effects of compound 48/80 on mast cells (68) and fMet-Leu-Phe on neutrophils (20, 69–74). In mast cells, prior treatment with pertussis toxin results in the ADP-ribosylation of a 41,000-dalton protein in the cell membrane and inhibition of the \(\text{Ca}^{2+}\) influx, arachidonic acid release, phosphatidylinositol 4,5-bisphosphate turnover, and histamine release induced by compound 48/80. The amount of ADP-ribosylation of the 41,000-dalton protein correlates with the inhibition of the secretory effects of compound 48/80 (68).

In neutrophils, the peptide \(N\)-formyl-Met-Leu-Phe stimulates chemotaxis and aggregation, granule enzyme release, superoxide generation, inositol lipid breakdown, arachidonic acid release, and a rise in intracellular \(\text{Ca}^{2+}\) concentration. All of these actions of fMet-Leu-Phe are inhibited by prior treatment of the cells with pertussis toxin (20, 69–74). These results are generally interpreted to mean that \(N_i\) (or perhaps \(G_i\)) is involved in mediating the effects of \(\text{Ca}^{2+}\)-mobilizing stimuli at the level of the membrane receptor. Additional support for this hypothesis is provided by experiments demonstrating that reconstitution of partially purified \(N_i\) from brain into the membranes from toxin-treated neutrophils has marked effects on the binding of \([\text{H}]\text{Met-Leu-Phe}\) to its receptor (20).

Taken together, the results obtained with mast cells and neutrophils suggest that \(N_i\) is involved in mediating the transduction of signals causing degranulation in these secretory cells. It is not yet clear whether this result will be applicable to all cells. In fact, in the 1321N1 astrocytoma cells, ADP-ribosylation of \(N_i\) does not abolish the \(\text{Ca}^{2+}\)-linked responses to muscarinic agonists (62, 75). Moreover, pertussis toxin treatment does not abolish the phosphatidylinositol response to angiotensin or vasopressin in hepatocytes.\(^3\) The reason for these differences is not yet apparent, but it seems possible that different receptors may activate the phospholipase C system by coupling with distinct transducing proteins.

Acknowledgments—We would like to thank Dr. John K. Northup, University of Calgary, Canada, for supplying us with purified \(N_i\) and the resolved 41,000-dalton \(\alpha\)-subunit, and many invaluable discussions. We also thank Dr. Joel Linden, Oklahoma Medical Research Foundation, for providing us with chick heart membranes, Dr. James Miller, Department of Biochemistry, University of Virginia, for providing us with the transducin preparation, and Dr. John W. Fleming, University of Indiana, for providing helpful advice about thin layer chromatography of \(\text{NAD}^+\). In addition, we thank Gwendolyn Myers and Vidya Reddy for preparing the pertussis toxin, Dr. George Vandenhoff for synthesizing \((\alpha-\text{[P]}\text{ATP})\), and Hannah Anderson for help with tissue culture techniques.

REFERENCES

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