nominally Ca^{2+}-free solution for 45 to 60 minutes to remove surrounding cells. After injection of RNA or water the oocytes were incubated in 70% L-15 medium (Gibco) containing penicillin (100 μg/ml) and streptomycin (100 U/ml) at room temperature (20° to 25°C). The oocytes were voltage-clamped at 20° to 23°C by the use of conventional two-microelectrode techniques.
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Coexistence of Guanylate Cyclase and Atrial Natriuretic Factor Receptor in a 180-kD Protein

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Atrial natriuretic factor (ANF) is a peptide hormone that is released from atria and regulates a number of physiological processes, including steroidogenesis in adrenal cortex and testes. The parallel stimulation of membrane guanylate cyclase and corticosterone production in isolated fasciculata cells of rat adrenal cortex has supported the hypothesis of a mediatory role for cyclic guanosine monophosphate (cyclic GMP) in signal transduction. A novel particulate guanylate cyclase tightly coupled with ANF receptor was purified approximately 273,000-fold by two-step affinity chromatography. The enzyme had a molecular size of 180 kilodaltons and was acidic in nature with a *p*I of 4.7. Its specific activity was 1800 nanomoles of cyclic GMP formed per minute per milligram of protein. The purified enzyme bound ANF with a specific binding activity of 4.01 nanomoles per milligram of protein, a value that is close to the theoretical binding activity of 5.55 nanomoles per milligram of protein for 1 mole of the ligand binding 1 mole of the receptor protein. These results indicate that the guanylate cyclase–coupled ANF receptor exists in a 180-kilodalton protein of rat adrenocortical carcinoma and represent a step toward the elucidation of the basic mechanism of cyclic GMP–mediated transmembrane signal transduction in response to a hormone.

STUDIES WITH ISOLATED FASCICULata cells of rat adrenal cortex and rat adrenocortical carcinoma indicated a physiological mediatory role for cyclic guanosine monophosphate (cyclic GMP) in steroidogenic signal transduction and led to the proposal of a hypothetical working model in which membrane guanylate cyclase was the key enzyme in receptor-mediated cyclic GMP signal pathway [reviewed in (*1*)]. Until recently a strong bias existed against the presence of a hormone-dependent membrane guanylate cyclase in any endocrine or nonendocrine tissue. The belief was that there was only one guanylate cyclase, a soluble enzyme, which was documented to be hormone-independent and nonspecifically activated by a variety of nitrite-generating compounds and agents that affect the oxidation-reduction potential of biological reactions (*2, 3*). These reservations were overcome by the demonstration of two distinct types of guanylate cyclase—membrane and soluble—in rat adrenal and rat adrenocortical carcinoma cells; only the membrane enzyme is hormone-specific (*4–6*).

More recently, the above results were corroborated in various rat tissues by demonstrating that atrial natriuretic factor (ANF) selectively stimulates particulate guanylate cyclase (*7*); these tissues included the rat adrenal gland (*8*). In vivo infusion studies with rat adrenal venous blood (*9*) and in situ studies with isolated fasciculata cells of rat adrenal cortex showed that ANF stimulates the production of corticosteroids (*10*). Similarly, in mouse interstitial (*11*) and Leydig cells (*12, 13*), testosterone production is increased by ANF. The mechanism of the ANF-dependent stimulation of steroidogenesis is not known, but the stimulation of membrane guanylate cyclase in parallel with the generation of an ANF-dependent steroidogenic signal suggested that this enzyme may have a role in mediating signal transduction (*10*).

Elucidating the biochemical mechanism of the mediatory role of cyclic GMP in receptor-mediated transmembrane signal transduction requires purification of the membrane guanylate cyclase. Only partial purification of any mammalian particulate guanylate cyclase has been achieved to date (*14*). We now describe purification of the membrane guanylate cylase and demonstrate that this enzyme is tightly coupled with the ANF receptor.

Membranes isolated from rat adrenocortical carcinoma cells were solubilized as in (*15*), adjusted to a final concentration of 5 m*M* $MnCl_2$, and adsorbed onto a guanosine triphosphate (GTP)–agarose affinity resin, which was suspended in and extensively washed with buffer A [25 m*M* triethanolamine hydrochloride (*p*H 7.6), 5 m*M* $MnCl_2$, and 1 m*M* 3-[(3-cholamidopropyl) - dimethylammonio] - 1 - propanesulfonate (CHAPS)] until there was no detectable protein (absorbance at 280 nm). The guanylate cyclase was eluted at room temperature with 25 m*M* triethanolamine (*p*H 7.6) 1 m*M* CHAPS, and 2 m*M* EDTA. The pooled enzymic fractions were adjusted to 5 m*M* Mn^{2+} and adsorbed to the cyclic GMP–Sepharose, which had been equilibrated with buffer A. The resin was loaded onto a small column (1.6 by 8 cm); flow-through was cycled back on the column once; and the column was washed extensively with buffer A. The enzyme was eluted with buffer A containing 2 m*M* EDTA (crossed affinity purification step) (lane 5 in Fig. 1A). The enzyme was thus purified approximately 273,000-fold.

The homogeneity and authenticity of the membrane guanylate cyclase is shown by the following criteria. (i) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of the purified protein shows a single stained band with a molecular mass of 180 kD (Fig. 1A); (ii) isoelectric focusing of the native and iodinated protein indicates a single symmetrical activity peak with a *p*I of 4.7 ± 0.10 (mean ± SEM) (Fig. 1, B and C); and (iii) Western blot analysis of the GTP affinity-purified enzyme shows a single 180-kD band although the SDS-PAGE of the GTP affinity-purified protein shows multiple Coomassie-stained bands.

The specific activity of the purified particulate guanylate cyclase is 1800 nmol of

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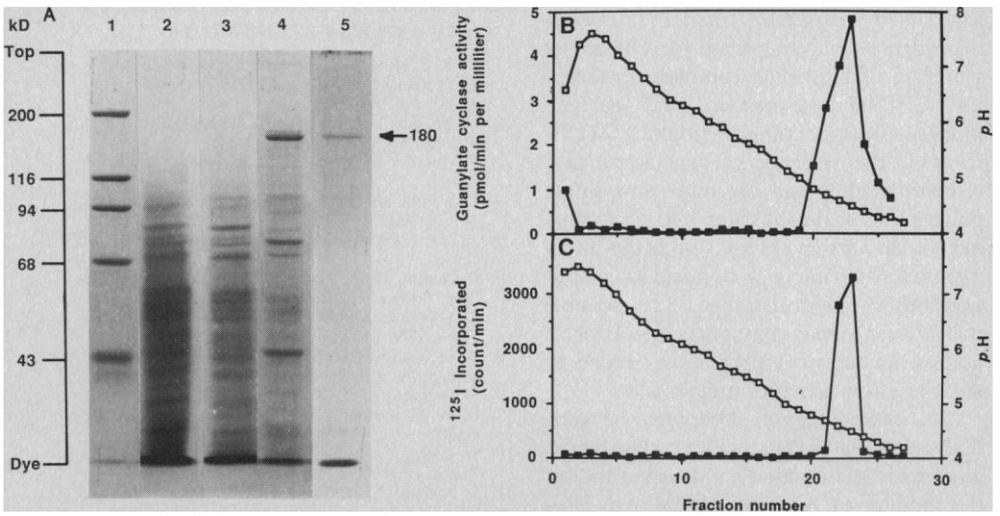

Fig. 1. Purity of the particulate guanylate cyclase–coupled ANF receptor. (**A**) SDS-PAGE of membrane guanylate cyclase at successive purification steps. (Lane 1) Molecular weight markers, (lane 2) membranes (starting material), (lane 3) solubilized membranes, (lane 4) GTP-affinity step, and (lane 5) crossed affinity purification step (0.12 μg of protein). The gel was stained with Coomassie blue. The molecular weight markers were myosin (120,000). β-galactosidase (116,000), phosphorylase b (94,000), bovine serum albumin (68,000), and ovalbumin (43,000). (**B**) Purified guanylate cyclase (3 ng) was subjected to nondenaturing isoelectric focusing electrophoresis. Nondenaturing isoelectric focusing was performed with pure noniodinated enzyme and the ^{125}I-labeled enzyme as previously described (*24*). The gel was sliced into 2-mm slices; the enzyme was extracted with 50 m*M* tris (*p*H 7.5) containing 10 m*M* $MgCl_2$ and 1 m*M* CHAPS, and assayed for guanylate cyclase activity (*4*). (**C**) Nondenaturing isoelectric focusing of radioiodinated guanylate cyclase was performed as described above and counted for radioactivity. Data are means (± SD) from two individual experiments each on the native and ^{125}I-labeled enzyme. Each assay was done in duplicate (*25*).

cyclic GMP formed per minute per milligram of protein (Fig. 2). That the enzyme is an authentic guanylate cyclase is demonstrated by immunological studies, which show that antibody to the 180-kD protein blocks up to 90% of the guanylate cyclase activity of the purified enzyme. It is noteworthy that the antibody blocks neither soluble guanylate cyclase nor adenylate cyclase activities, indicating specificity for the membrane enzyme.

The pure guanylate cyclase binds ^{125}I-labeled ANF. Bound ANF is displaceable by unlabeled ANF. Increases in the specific binding activity of ANF parallel the purification steps of the guanylate cyclase, reaching a mean ± SEM of 4.01 ± 0.45 nmol per milligram of protein in the pure enzyme (Fig. 2). This value approaches the theoretical binding activity of 5.55 nmol per milligram of protein, if it is assumed that 1 mol of the ligand binds 1 mol of the receptor protein. Because of the limited quantity of purified enzyme, we could not determine the detailed ANF binding kinetics, but the Scatchard analysis of the particulate fraction showed a high ANF affinity (K_D, 1.5 p*M*) and one binding site. Although the purified guanylate cyclase binds ANF, the enzyme is not stimulated by ANF. These results are similar to those for the lung enzyme (*16*). Since the loss in response to ANF stimulation occurred during the detergent solubilization of the receptor, it is possible that a lipid component or an accessory protein necessary for original hormonal stimulation is lost in this purification step.

During the course of our investigations Kuno *et al.* (*16*) showed that in a highly purified rat lung preparation, ANF receptor and guanylate cyclase are copurified. Although the dual presence of the receptor and

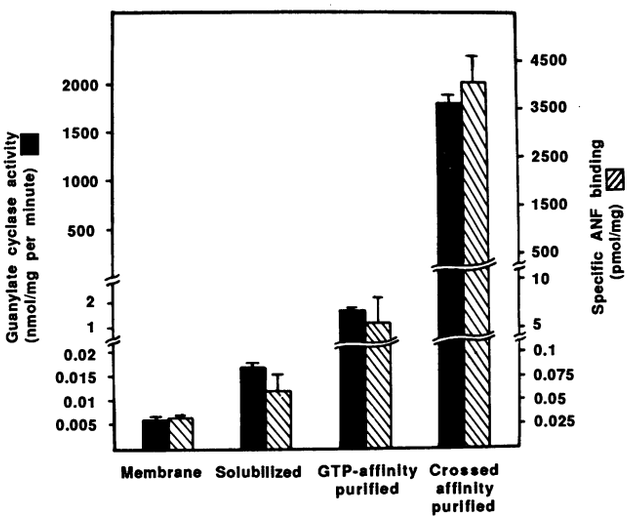

Fig. 2. Coexistence of the particulate guanylate cyclase and ANF receptor at the successive purification steps. Guanylate cyclase activity was measured as in (*4*). The ANF receptor binding assays were performed by incubating ^{125}I-labeled ANF (0.2 pmol per tube; specific activity 93 Ci/mmol) with samples at different purification steps at 25°C for 1 hour in a total volume of 500 μl of incubation buffer [5 m*M* $MgCl_2$, 50 m*M* tris-HCl (*p*H 7.5), 0.2% heat-inactivated bovine serum albumin, 0.1 m*M* EDTA, and aprotinin (700 IU/ml)]. For determination of nonspecific binding, the sample buffers contained, in addition, 1 μ*M* nonradioactive ANF. The reaction was terminated by the addition of 3 ml of ice-cold 0.9% NaCl (w/v) followed by immediate filtration through Whatman GF/C glass fiber filters for the particulate fraction, and through Whatman GF/B filters treated with 0.32% polyethyleneimine for solubilized and other purified fractions. The filter paper was washed three times with 5 ml of ice-cold buffer, dried, and counted for radioactivity. Results are shown as the means ± SEM (n = 6).

enzyme has to be established in a homogeneous lung protein, there are striking biochemical and kinetic differences between the lung and the tumor receptor–coupled enzymes: the subunit molecular mass of the lung protein is 120 kD; the lung enzyme is stimulated by hemin (*17*) and is absolutely dependent on Mg^{2+}-GTP (*16*), whereas the tumor enzyme is able to substitute Mn^{2+}-GTP for Mg^{2+}-GTP and is not stimulated by hemin. In addition, the *p*I of the lung protein is 6 (*14*), whereas that of the tumor enzyme is 4.7. In contrast to the near 1:1 stoichiometry of the binding of ANF to the tumor enzyme, the lung enzyme bound only 14.5% of ANF at the noted theoretical value. The structural and kinetic differences between the two enzymes suggest that these two receptor–guanylate cyclases may be isozymes.

Studies done with affinity-labeling techniques (*18–20*) have shown a 120-kD ANF binding protein and those done with ANF cross-linking techniques (*19*) have shown 60- to 70-kD proteins in various tissues. Preliminary evidence suggests that only certain ANF receptor sites may be coupled to guanylate cyclase (*21*). It will be of interest to scrutinize the structural and functional features of these receptors.

Coexistence of the ANF receptor and guanylate cyclase activities on a single polypeptide chain indicates that the mechanism of transmembrane signal transduction involving mediation by second messenger, cyclic GMP, is different from the well-estab-

lished adenylate cyclase system. In hormone-dependent adenylate cyclase there is an assemblage of individual components–receptor, GTP-binding protein, and catalytic moiety—for signal transduction (*22, 23*). In contrast, the presence of dual activities—receptor binding and enzymic—on a single polypeptide chain indicates that this transmembrane protein contains both the information for signal recognition and its translation into a second messenger. It is possible that a third signal component (probably a lipid or an accessory protein) is needed to link these two activities functionally.

Note added in proof: Although the antibody to the 180-kD guanylate cyclase blocks guanylate cyclase activity, it does not inhibit the binding of ANF to the protein. This indicates that either the antibody is solely against the guanylate cyclase epitope of the protein or that there are two tightly coupled 180-kD proteins which are inseparable by the present techniques.

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26. These studies were carried out between 1982 and 1986 and constitute a portion of A.K.P.'s dissertation. We thank R. Nutt, Merck Sharp and Dohme Research Laboratories, for the sample of synthetic ANF; W. Y. Cheung, St. Jude Research Hospital, and M. Rodbell, National Institute of Environmental Health Sciences, for the critical review of the manuscript, and J. M. Sharma for editing the manuscript. Supported by NSF grant 8609867 and NIH grant NS23744.

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Technical Comments

Computing with Neural Networks

Hopfield and Tank (*1*) refer to "A new concept for understanding the dynamics of neural circuitry" using the equation (in a slightly different notation)

$$C_i\frac{du_i}{dt} = -\frac{1}{R_i}u_i + \sum_{j=1}^{n} T_{ij}f_j(u_j) + I_i \qquad (i = 1,\ldots, n) \qquad (1)$$

for the neuron state variables u_i. The concept is that the variables $u_i(t)$ approach equilibrium as $t \to \infty$ if the connections T_{ij} are symmetric ($T_{ij} = T_{ji}$). Hopfield and Tank also state that "a nonsymmetric circuit . . . has trajectories corresponding to complicated oscillatory behaviors . . . but as yet we lack the mathematical tools to manipulate and understand them at a computational level" (*1*, p. 629), and that "the symmetry of the networks is natural because, in simple associations, if A is associated with B, B is symmetrically associated with A" (*1*, p. 629).

Associations are often asymmetric, as in the asymmetric error distributions arising during list learning (*2*). Neural network models (*3*) explain these distributions when one uses Eq. 1 supplemented by an associative learning equation for the connections T_{ij}

$$\frac{dT_{ij}}{dt} = -AT_{ij} + Bu_if_j(u_j) \qquad (2)$$

Because of the nonlinear term $u_if_j(u_j)$ in Eq. 2, $T_{ij} \neq T_{ji}$.

Stability theorems (*4*) have been proved about neural networks which include and generalize Eqs. 1 and 2. Thus symmetry is not necessary to prove associative learning and memory storage by neural networks. Nor is symmetry needed to design stable neural networks for adaptive pattern recognition (*5*). Methods have also been developed (*6*) for analyzing the oscillatory behavior of neural circuits. We believe that the relation between symmetry and stability in neural networks is much more subtle and better understood than Hopfield and Tank (*1*) suggest.

Nonetheless, symmetry does help to analyze the system represented by Eq. 1. In fact, we (M.A.C. and S.G.) (*7*) independently discovered an energy function for neural networks "designed to transform and store a large variety of patterns. Our analysis includes systems which possess infinitely many equilibrium points" (*7*, p. 818), examples of which have been constructed (*8*). These networks are

$$\frac{du_i}{dt} = a_i(u_i)\Big[b_i(u_i) - \sum_{j=1}^{n} c_{ij}d_j(u_j)\Big] \qquad (i = 1, \ldots, n) \qquad (3)$$

Given symmetric connections ($c_{ij} = c_{ji}$), the energy function is

$$V = -\sum_{i=1}^{n}\int_0^{u_i} b_i(\xi_i)d_i'(\xi_i)d\xi_i + \frac{1}{2}\sum_{j,k=1}^{n} c_{jk}d_j(u_j)d_k(u_k) \qquad (4)$$

Along system trajectories

$$\frac{d}{dt}V = -\sum_{i=1}^{n} a_i(u_i)d_i'(u_i)\Big[b_i(u_i) - \sum_{k=1}^{n} c_{ik}d_k(u_k)\Big]^2 \qquad (5)$$

If $a_i(u_i) \geq 0$ and $d_i'(u_i) \geq 0$, then $\frac{d}{dt}V \leq 0$, which is the key property of an energy function. We (M.A.C. and S.G.) have noted that "the simpler additive neural networks . . . are also included in our analysis" (*7*, p. 819). The system represented by Eq. 3 reduces to the additive network (Eq. 1) when $a_i(u_i) = C_i^{-1}, b_i(u_i) = -1/R_i\ u_i + I_i$, $c_{ij} = -T_{ij}$ and $d_j(u_j) = f_j(u_j)$. Then

$$V = \sum_{i=1}^{n}\frac{1}{R_i}\int_0^{u_i}\xi_if_i'(\xi_i)d\xi_i - \sum_{i=1}^{n} I_if_i(u_i) - \frac{1}{2}\sum_{j,k=1}^{n} T_{jk}f_j(u_j)f_k(u_k) \qquad (6)$$

which includes the energy functions used in (*1*). We (M.A.C. and S.G.) (*7*) also analyzed the more difficult and physiologically important cases where the cells obey membrane, or shunting, equations and the signal functions $d_j(u_j)$ may have output thresholds.