Studies on Receptor-mediated Activation of Adenylyl Cyclases

I. PREPARATION AND DESCRIPTION OF GENERAL PROPERTIES OF AN ADENYLYL CYCLASE SYSTEM IN BEEF RENAL MEDULLARY MEMBRANES SENSITIVE TO NEUROHYPOPHYSEAL HORMONES*

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SUMMARY
With use of a discontinuous sucrose density gradient centrifugation in the presence of 1 mM EDTA and 10 mM Tris-HCl, pH 7.5, membrane particles were prepared from beef renal medulla that are 4- to 6-fold enriched in fluoride-sensitive and 8- to 12-fold enriched in neurohypophyseal hormone (NHH)-sensitive adenylyl cyclase activity. Average yield of the NHH-sensitive adenylyl cyclase activity was 30 to 35% ; average yield of membrane protein, starting with 80 g of beef renal medulla, was 200 to 250 mg. These membranes also contain a prostaglandin-sensitive adenylyl cyclase system, the activity of which is additive to that sensitive to NHH.

Arginine-vasopressin, lysine-vasopressin, and oxytocin stimulate enzyme activity half-maximally at $10^{-9}$, $10^{-8}$, and $10^{-7}$ M, respectively. The response of the renal medullary adenylyl cyclase activity to NHH is extremely dependent on the concentrations of magnesium and ATP used in the assay. Response is maximal at low concentrations of magnesium and at concentrations of ATP varying from 0.07 to 0.10 mM. Increasing the concentration of magnesium results in selective stimulation of basal activity with concomitant loss of the relative stimulation by NHH. Increasing or decreasing the concentration of ATP results in selective loss of activity due to NHH stimulation, also leading to reduced relative stimulation by NHH. GTP at $10^{-5}$ M inhibits stimulation of the beef renal medullary adenylyl cyclase by NHH.

5'-Adenylyl-imidodiphosphate (AMP-PNP), an analogue of ATP that is not hydrolyzed by membrane ATPases, was evaluated as substrate for renal adenylyl cyclase. Depending on the concentration of the analogue and magnesium used, AMP-PNP yields activities that are between 10 and 25% of those obtained with ATP. Maximal hormonal stimulation requires higher concentrations of AMP-PNP than ATP; excess AMP-PNP does not result in inhibition of hormonal stimulation. Activities with the analogue exhibit higher pH optima than with ATP. The possibility that these differences in activity are due to a different exit rate of inorganic imidodiphosphate as compared to Pi, or to the different divalent cation binding properties of AMP-PNP and ATP, or both, is discussed.

The extreme sensitivity of hormonal stimulation to variations of nucleotide concentration, be it ATP or GTP, coupled to the relatively high yield of activity from beef renal medulla, make this preparation useful for the study of modes of regulation of hormonal stimulation of adenylyl cyclase systems.

In addition, several minor modifications of the procedure of Krishna et al. (KRISHNA, G., WEISS, B., AND BRODIE, B. B. (1968) J. Exp. Ther. Pharmacol. 163, 379-385) for isolation and determination of cyclic adenosine 3':5'-monophosphate (cAMP) formed during incubations for adenylyl cyclase are described. They result in higher yields of cAMP (60 to 65%) and in lower reaction blanks (0.0005 to 0.0010% of initially added ATP).

Adenylyl cyclases (ATP:pyrophosphate-lyase (cyclizing), EC 4.6.1.1) are important models for the elucidation of molecular events involved in initiation of hormone action and have therefore received much attention during recent years. Studies with fat cells have revealed that these enzyme systems are multimolecular complexes asymmetrically arranged within the plasma membrane and have led to the clear recognition of at least two functional components: (a) a hormone receptor, proteic in nature and located on the outer surface of the membrane; and (b) a catalytic unit exposed at the inner side of the membrane and accessible to its substrates (1-3). In addition, studies with rat liver adenylyl cyclase revealed that (a) both the catalysis of ATP to cAMP by enzyme and the translation of receptor-hormone interaction into activation of the catalytic unit are dependent on membrane lipids (4, 5); (b) the interaction of hormone with its receptor is rapidly reversible (6); (c) the process or processes coupling hormone receptor to catalytic unit require a guanyl nucleotide for proper operation (7); and (d) both the hormone receptor and the enzyme exist in rapidly reversible

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states of activity (6). Finally, studies with adenylyl cyclases from cat heart and adrenal cortex have revealed that certain phospholipids and divalent cations may also play specific roles in regulating hormonal stimulation (8, 9). For a recent general review of the subject see Perkins (10).

Despite all of these studies, the information gained is indirect and it has not yet been possible to isolate and characterize any of the molecular components comprising these complex membrane-bound systems. Precise information as to the nature of the coupling process(es) connecting hormone receptor to catalytic unit is missing.

Neurohypophyseal hormone (NHH)-sensitive adenylyl cyclase systems may be appropriate to gain insight into the processes and components. Chase and Aurbach (11) demonstrated the existence, of a NHH-sensitive adenylyl cyclase system, and to present its general characteristics with respect to the molecular components comprising these complex membrane-bound systems. Precise information as to the nature of the coupling process(es) connecting hormone receptor to catalytic unit is missing.

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30 units per mg), and 25 mm Buffer B (2,2-bis(hydroxymethyl)-2,2',2''-nitriloethanol-propane-HCl) pH 8.0. Incubations were for 10 min at 37° and were terminated by the addition of 0.1 ml of “stopping solution,” composed of 10 ml of HEPES (approximately 10,000 cpm), 40 mM ATP, and 1.0% sodium dodecyl sulfate, followed by immediate boiling for 3 min.

Isolation of cAMP

The [32P]cAMP formed was isolated by a slight modification of the method of Krishna et al. (20) that yields better reproducibility between replicates and lower reaction blanks. H2O (1.0 ml) was added to the incubated samples which were then transferred with Pasteur pipettes to Dowex 50 columns (0.6 cm inside diameter, 1.5 ml of packed Bio-Rad AG 50-X4, 200 to 400 mesh, H+ form) that have been subjected to at least three “regeneration cycles” (see below). The columns were then sequentially washed with 1.0, 3.0, and 3.0 ml of H2O. The last 3.0 ml, containing approximately 80% of the applied cAMP, were collected and 0.2 ml each of 0.15 M Ba(OH)2 and about 4% ZnSO4 was added in that sequence. The resulting precipitates were removed by filtering through Bar filters (see below). The BaSO4 filters were then washed once with 1.0 ml of H2O. The filtrates (pH 9.0 to 9.5) and the washing fluids were directly collected in counting vials containing 15 ml of Aquasol or 11 ml of PCS or 3a-70B.

Regeneration of Dowex 50 Columns—This process was carried out three times before the columns were used for the first time and once between each use in the adenyl cyclase assay. One regeneration cycle is formed by successive washing of the column packed with 1.5 ml of Bio-Rad AG 50W-X4 (900 to 400 mesh) with 20 ml of H2O-20 ml of 2 x NaOH, 20 ml of H2O, 20 ml of 2 N HCI, and 3 x 20 ml of H2O. Using this regime a single Dowex column has been used in up to 100 separate adenyl cyclase assays yielding good recovery of cAMP without noticeable increase of the reaction blank of the assay.

BaSO4 Filters—These are prepared by simultaneous addition of 0.4 ml each of 0.15 M Ba(OH)2 and 4% ZnSO4 to tight glass wool plugs placed in the tip of glass columns (0.6 x 15 cm), followed by a single washing step with 20 ml of H2O.

The above methodology differs from that previously described (21, 22) in that (a) sodium dodecyl sulfate was included in the stopping solution, (b) Dowex 50W-X8 (200 to 400 mesh) was substituted for Dowex 50W-X8 (100 to 200 mesh), (c) the volume of resin bed was increased from 1.0 to 1.5 ml, (d) the resin was extensively washed with alternating solutions of NaOH and HCl before use, and (e) the radioactive mixture of BaSO4 and Zn(OH)2 formed during the negative adsorption step was removed by more efficient filtration method rather than by centrifugation. Over-all recovery of AMP-PNP was 80% to 95%. Significantly lower reaction blanks were obtained under these conditions, ranging, in the presence of up to 150 μg of membranes protein, from 0.0005 to 0.0010% of the initially added ATP. Replication of duplicate samples was within 5% of the mean under all conditions of assay, except when basal activity was determined at times shorter than 2 min. Under these conditions the counts per min of ATP converted to cAMP were equal to or less than the reaction blank and reproducibility of the assay was reduced to within 10% of the mean.

To decrease reaction blanks in experiments with AMP-PNP, the final boiling step was omitted. The sodium dodecyl sulfate-containing stopping solution alone was sufficient to stop the reactions effectively. Under these conditions, the reaction blanks with the synthetic nucleotide were between 0.0008 and 0.0025% of the initially added [β-32P]AMP-PNP and reproducibility was within 10% of the mean.

Expression of Results

Adenylyl cyclase activity in figures and tables refers to the conversion of both substrates of adenyl cyclase, 5'-adenylyl-imidodiphosphate (AMP-PNP) to cAMP and PPi (or PNP) expressed, unless otherwise indicated, in nanomoles of cAMP formed per 10 min per mg of membrane protein.

Marker Enzymes

5'-Nucleotidase (EC 3.1.3.5) was measured by the method of Bodansky and Schwartz (23) and glucose 6-phosphatase (EC 3.1.3.9) by the method of Swanson (24), with the addition of 1 mM EDTA. These enzymes were assayed in 0.1 ml of appropriate medium and incubations were at 30°. Incubation time varied between 10 and 90 min depending on enzyme fraction and activity measured. Activities were proportional to enzyme concentration. Reactions were stopped by addition of 0.025 ml of 50% trichloroacetic acid. The precipitate formed was removed by centrifugation and the liberated P2 was determined in 0.05-μl aliquots by the method of Fiske and SubbaRow (25). Succinate-cytochrome c reductase was measured by the method described by Fleischer and Fleischer (26).

Protein was measured by the Lowry procedure (27) using bovine serum albumin as standard.

RESULTS

Preliminary experiments indicated that stimulation of adenylyl cyclase by [Arg8]vasopressin in crude homogenates of beef renal medulla, as well as in low speed particulate fractions, varied from as little as 20% up to 200%, depending on the kidney used. Mean stimulation was approximately 50%. Fractionation on a continuous sucrose gradient showed that low speed particles contain in addition to [Arg8]vasopressin-stimulated adenylyl cyclase system other adenylyl cyclase activities not responsive to the neurohypophyseal hormone (Fig. 1). Based on the data obtained from the continuous sucrose gradient we designed the purification scheme described under “Experimental Procedures” and used a discontinuous gradient formed by (from bottom to top of tube) 41.5, 37.0, and 32.5% sucrose in 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA. As can be seen in Table I membranes collected at the interface between 37.0 and 41.5% sucrose showed maximal [Arg8]vasopressin responsiveness. Purification of activity due to [Arg8]vasopressin addition (last two columns of Table I) was in the order of 8- to 11-fold, with a yield ranging between 80 and 55%. Freezing resulted in approximately 20 to 30% loss of all expressions of adenylyl cyclase activity (basal,
Adenylyl cyclase activities in the crude homogenate and the various fractions described under "Experimental Procedures" were measured under standard assay conditions. When present, [Arg\(^*\)]vasopressin was \(10^{-7}\) M and NaF was 10 mM.

Table I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Protein</th>
<th>Adenylyl cyclase activity in the presence of</th>
<th>Increase in activity due to [Arg(^*)]vasopressin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml</td>
<td>mg/ml</td>
<td>Nil</td>
<td>[Arg(^*)]vasopressin</td>
</tr>
<tr>
<td>Crude homogenate</td>
<td>1560</td>
<td>2.25</td>
<td>0.034</td>
<td>0.065</td>
</tr>
<tr>
<td>S100</td>
<td>1530</td>
<td>1.65</td>
<td>0.026</td>
<td>0.051</td>
</tr>
<tr>
<td>P160</td>
<td>80</td>
<td>6.40</td>
<td>0.070</td>
<td>0.154</td>
</tr>
<tr>
<td>S160-1400</td>
<td>1520</td>
<td>1.50</td>
<td>0.007</td>
<td>0.010</td>
</tr>
<tr>
<td>P160-1000</td>
<td>200</td>
<td>4.00</td>
<td>0.146</td>
<td>0.258</td>
</tr>
<tr>
<td>32.5/37.0 interface</td>
<td>58.5*</td>
<td>1.25</td>
<td>0.148</td>
<td>0.232</td>
</tr>
<tr>
<td>37.0/41.5 interface</td>
<td>88.4*</td>
<td>2.85</td>
<td>0.077</td>
<td>0.376</td>
</tr>
<tr>
<td>41.5 pellet</td>
<td>13.4*</td>
<td>5.00</td>
<td>0.096</td>
<td>0.182</td>
</tr>
</tbody>
</table>

* Two consecutive discontinuous sucrose gradient centrifugations were carried out and the various interfaces and pellets from the 12 tubes pooled to give the listed volumes.

As shown in Fig. 2, under standard assay conditions (1.0 mM ATP and 5.0 mM total added MgCl\(_2\)) the adenylyl cyclase reaction proceeded linearly with respect to time and to protein concentration, both in the absence and the presence of a maximally stimulating concentration of [Arg\(^*\)]vasopressin.

**Dependence of NHH-stimulated Adenylyl Cyclase on Substrate and Magnesium Concentration**

Initial experiments that tested the dependence of the enzyme system on magnesium ion showed that adenylyl cyclase activity was essentially undetectable at any concentration of added MgCl\(_2\) below that of added EDTA. Similar basal and NHH-stimulated activities were obtained at 2.0 mM MgCl\(_2\) in the presence of 1.4 mM EDTA as with 3.0 mM MgCl\(_2\) in the presence of 2.4 mM EDTA. We have therefore expressed magnesium ion concentrations in millimoles per liter (mM) in excess of the EDTA concentration present in the assay.

**Studies with ATP**

The effect of varying the concentration of ATP in the incubation medium at different magnesium concentrations on the renal medullary membrane adenylyl cyclase, determined in the absence and presence of [Arg\(^*\)]vasopressin, is shown in Fig. 3, A and B. Two main findings emerged from this study: (a) the basal, hormonally unstimulated activity of the system is stimulated by increasing concentration of Mg\(^{2+}\) more than the hormonally stimulated activity, a fact that leads to decreasing hormone stimulation relative to basal with increasing concentration of magnesium; and (b) at any given concentration of Mg\(^{2+}\), basal and hormonally stimulated activities respond differently to varying concentrations of ATP. This finding is further illustrated in Fig. 3C where the [Arg\(^*\)]vasopressin-dependent stimulation is expressed relative to the respective basal activity. It can be seen that the enzyme system is maximally stimulated by [Arg\(^*\)]vasopressin at concentrations of ATP ranging from 0.07 to 0.10 mM, and that at both higher and lower concentrations of ATP, the relative stimulation by [Arg\(^*\)]vasopressin is less. Thus, while "standard" assay conditions of 1.0 mM ATP and 3.6 Mg\(^{2+}\) (in excess of 1.4 mM EDTA) give a relative stimulation of about 3- to 4-fold over basal, reduction of the concentrations of ATP and Mg\(^{2+}\) to 0.05 and 0.6 mM, respectively, results in an increase of the relative stimulation by [Arg\(^*\)]vasopressin to 7- to 9-fold over basal.
FIG. 3. A and B, effect of ATP concentration on basal and [Arg\textsuperscript{8}]vasopressin (AVP)-stimulated adenylyl cyclase activities determined at various Mg\textsuperscript{2+} concentrations (expressed as millimoles per liter (mM) in excess of 1.4 mM EDTA). Activities were determined at pH 8.0. Membrane protein was 58 \mu g per assay. The specific activity of the [\alpha-\textsuperscript{32}P]ATP used as substrate was varied to give 10,032, 5,896, 2,414, 859, 320, 90, and 33 cpm per pmole at 0.0058, 0.0107, 0.077, 0.216, 0.72 and 2.33 mM, respectively. ——, basal activities; ——, activities obtained in the presence of 10\textsuperscript{-7} M [Arg\textsuperscript{8}]vasopressin; numbers represent Mg\textsuperscript{2+} concentrations. For rest of conditions, see “Experimental Procedures.” C, [Arg\textsuperscript{8}]vasopressin-stimulated activities shown in A and B are expressed relative to their respective basal activities.

FIG. 4. pH dependence of basal and [Arg\textsuperscript{8}]vasopressin (AVP)-stimulated activities (expressed in millimoles per liter (mM) in excess over 1.4 mM EDTA) using ATP and AMP-PNP as substrate. Incubation conditions were those described under “Experimental Procedures” except for the following changes: (a) the concentrations of [\alpha-\textsuperscript{32}P]ATP (1000 cpm per pmole) and AMP-PNP (1800 cpm per pmole) were those shown on the figure; (b) the concentration of Buffer B was increased to 50 mM; and (c) the concentrations of Mg\textsuperscript{2+} were those shown on the figure next to the corresponding curves. The indicated pH values are actual values after 5 min of incubation. The nucleoside triphosphate regenerating system was omitted from incubations with AMP-PNP. When present, [Arg\textsuperscript{8}]vasopressin was 10\textsuperscript{-7} M; membrane protein was 55 \mu g per assay in the experiment with ATP and 47 \mu g per assay in the experiment with AMP-PNP.

Effect of pH at Different Concentrations of Mg\textsuperscript{2+}—At low concentrations of Mg\textsuperscript{2+} (0.6 mM), the pH optimum of both basal and [Arg\textsuperscript{8}]vasopressin-stimulated adenylyl cyclase activities is broad, extending from pH 8.0 to 9.5. Increasing concentrations of Mg\textsuperscript{2+} result in a narrower pH curve with a maximum between pH 7.8 and 8.0 and the appearance of an inhibitory effect of Mg\textsuperscript{2+} at pH values exceeding 9.6 (see Fig. 4, right).

Studies with AMP-PNP

This nucleotide was first shown to be a substrate for adenylyl cyclase activity in liver plasma membranes by Rodbell et al. in 1971 (7) and subsequently also in human platelet membranes (29) and more recently in fat cell membranes (30). It was therefore of interest to see whether also the renal medullary membrane adenylyl cyclase system reacted with this synthetic analogue of ATP and if so, how it compared to ATP, a comparison which has not yet been made in other systems. It was found that the synthetic nucleotide is also a substrate for this adenylyl cyclase. A study of the dependence of the renal adenylyl cyclase system on AMP-PNP concentration, carried out both in the absence and presence of a nucleoside triphosphate regenerating system and comparison to the dependence of the system on ATP concentration is illustrated in Fig. 5. In this and other studies it was established that although AMP-PNP is capable of supporting the adenylyl cyclase reaction and allows for hormonal stimulation, the absolute adenylyl cyclase activities...
that can be obtained with the synthetic substrate are significantly less than those that are obtained under similar conditions using ATP, regardless of whether the AMP-PNP reaction is carried out in the absence or the presence of a nucleoside triphosphate regenerating system. In the particular experiment shown in Fig. 5 basal and [Arg⁸]vasopressin-stimulated activities driven by AMP-PNP were about 50 and 20%, respectively, of those driven by ATP, varying somewhat with the concentration of substrate used.

*Effect of pH*—At 0.6 mM Mg²⁺ the AMP-PNP-driven basal adenylyl cyclase activity has a pH optimum of 9.6. This pH optimum is shifted toward lower values with increasing concentrations of Mg²⁺ so that at 18.6 mM the pH optimum is 8.3. Similarly, in the presence of [Arg⁸]vasopressin, the pH optimum shifts from 9.0 at 0.6 mM Mg²⁺ to 8.3 at 18.6 mM Mg²⁺. The activities obtained with AMP-PNP were less than those obtained with ATP under all conditions tested. These findings are illustrated in Fig. 4.

*Effect of AMP-PNP and Mg²⁺ Concentration on Stimulation of Renal Adenylyl Cyclase by AVP—*Using AMP-PNP (pH 9.0, absence of regenerating system) the dependence of the system on Mg²⁺ followed a pattern similar to that seen with ATP, i.e., the basal activity is stimulated to a greater extent by increasing concentrations of the divalent action than the [Arg⁸]vasopressin-stimulated activity. The dependence of the system on AMP-PNP concentration, however, differed from that seen with ATP in that (a) hormonal stimulation required, to be detectable, the presence of at least 0.05 mM AMP-PNP, and (b) no inhibition of hormonal stimulation (relative to basal) was observed with concentrations of AMP-PNP up to 1.0 mM, regardless of the Mg²⁺ concentration used. This is illustrated in Fig. 6. In other experiments no inhibition by excess AMP-PNP was seen even at concentrations as high as 2.5 mM, which is about 20-fold higher than the concentration of ATP at which a clear diminution of hormone action can be detected.

**Stimulation of Renal Medullary Membranes by Other Neurohypophyseal Hormones and Prostaglandin B₂**

Under conditions that were found optimal for studying the response to [Arg⁸]vasopressin, renal medullary adenylyl cyclase activity is also stimulated by two other natural neurohypophy-
adenylyl cyclases not sensitive to these hormones and which separates the NHH-sensitive adenylyl cyclase system from other membrane protein) and is therefore adequate for future preparation of renal medullary membranes has several advantages. (4) It gives a high yield of membranes (approximately 250 mg of membrane protein) and is therefore adequate for future preparation. (5) It separates the NHH-sensitive adenylyl cyclase system from other adenylyl cyclase not sensitive to these hormones and which account for up to two-thirds of the total renal medullary adenylyl cyclase activity (see Table I). (c) It yields a NHH-sensitive system that is both highly responsive (up to 10-fold stimulation under adequate assay conditions) and highly sensitive (apparent $K_m$ of activation for [Arg]$^8$vasopressin, $10^{-9}$ M). (d) It yields a NHH-sensitive system that has preserved some of its regulatory properties as seen by its complex modulation by ATP and Mg$^{2+}$, and by its response to GTP. It should be mentioned that to successfully obtain the membrane fraction enriched in NHH-sensitive adenylyl cyclase it was necessary to (a) include 1 mM EDTA in sucrose gradient used for separation, and (b) have gone through a hypotonic dilution step before preparing the low speed particulate fraction (P100,100) used as source of adenylyl cyclase in the sucrose gradient separation. Selective enrichment with NHH-sensitive adenylyl cyclase could not be obtained when the homogenate was prepared in either isotonic (0.25 M) or hypertonic (0.8 M) sucrose, be it in the absence or presence of 10 mM MgCl$_2$, 1.0 mM EDTA, 10 mM Tris-HCl, pH 7.5, or combinations thereof. While this and subsequent studies on the beef renal adenylyl cyclase system were well under progress, Finn et al. (34) working with beef adrenals and using a zonal centrifugation technique published a method for separating mitochondrial membranes and fragments from adrenocorticotropin hormone-sensitive adenylyl cyclase-containing membranes. We have not yet tested whether the method of Finn et al. is also applicable to our renal medullary membrane preparations; however, in view of the presence of large amounts of mitochondrial fragments in our renal medullary membrane preparation, ultimate inclusion of a rate zonal centrifugation step into the purification scheme may well be advantageous. We are currently investigating this possibility of improving the relative purity of NHH-sensitive adenylyl cyclase-containing membranes.

One striking property of our preparation of renal medullary membranes is that its NHH-sensitive adenylyl cyclase system is profoundly influenced by ATP and Mg$^{2+}$, to the extent that, to establish optimal condition for the assay of NHH-dependent stimulation, it became essential to carry out an extensive study in which both substances were varied simultaneously. From this study it became apparent that hormonal stimulation is best seen at low Mg$^{2+}$ concentrations and depends on an optimal concentration of ATP. Recently three other reports have appeared dealing with the partial purification of renal medullary membranes. Campbell et al. (35) prepared membranes from porcine renal medulla using a differential centrifugation method described by Fitzpatrick (28) for the preparation of ATPase-containing membrane fractions, and obtained a NHH-sensitive adenylyl cyclase-containing membrane that is 5-fold purified with respect to the starting homogenate and which responds to [Lys]$^8$vasopressin with a 3- to 3.5-fold increase in activity when determined at 1.2 mM ATP and 6.0 mM MgCl$_2$; half-maximal response to [Lys]$^8$vasopressin was found at 2.2 x $10^{-8}$ M. Neer (36) prepared membranes from rat renal medulla and obtained a NHH-sensitive adenylyl cyclase system that is 5-fold purified with respect to the starting homogenate and responds with a 6 fold increase in activity when determined at about 1.0 mM ATP and 9.0 mM MgCl$_2$; half-maximal stimulation by [Lys]$^8$vasopressin varied for different membrane preparation between 4 x $10^{-8}$ M and 15 x $10^{-8}$ M. Finally, Bockaert et al. (37) have also partially purified membranes from porcine renal medulla using the technique described by Emmelot et al. (38) for liver plasma membranes and obtained a NHH-sensitive adenylyl cyclase system that is about 7-fold purified with respect to the starting homogenate and responds with a 5-fold stimulation when determined at 0.05 mM ATP and 10 mM MgCl$_2$; half-maximal stimulation by [Lys]$^8$vasopressin was found at 5 x $10^{-8}$ M. In none of these studies was a regulatory effect of either Mg$^{2+}$ or ATP reported that resembled our findings. On the contrary, the rat renal medullary system

### Table II

<table>
<thead>
<tr>
<th>Additions</th>
<th>Adenylyl cyclase activity$^a$</th>
<th>Change in adenylyl cyclase activity due to addition of [Arg]$^8$vasopressin</th>
<th>PGE$_1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>92 ± 3</td>
<td>294</td>
<td>146</td>
</tr>
<tr>
<td>[Arg]$^8$vasopressin</td>
<td>386 ± 2</td>
<td>292</td>
<td>144</td>
</tr>
<tr>
<td>PGE$_1$</td>
<td>238 ± 4</td>
<td></td>
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<tr>
<td>[Arg]$^8$vasopressin + PGE$_1$</td>
<td>530 ± 1</td>
<td></td>
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</tbody>
</table>

$^a$ Values are mean ± standard deviation of quadruplicate determinations and are expressed in picomoles of cAMP formed per 10 min per mg of membrane protein.

### Table III

<table>
<thead>
<tr>
<th>Additions</th>
<th>Adenylyl cyclase activity$^a$</th>
<th>[Arg]$^8$vasopressin</th>
<th>PGE$_1$</th>
<th>AVP-stimulated activity relative to basal$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>67 ± 3</td>
<td>445 ± 6</td>
<td>6.65</td>
<td></td>
</tr>
<tr>
<td>GTP</td>
<td>80 ± 2</td>
<td>275 ± 4</td>
<td>3.44</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Values are mean ± standard deviation of quadruplicate determinations and are expressed in picomoles of cAMP formed per 10 min per mg of membrane protein.

$^b$ Ratio of activation is obtained by dividing adenylyl cyclase activity obtained in the presence of $10^{-7}$ M [Arg]$^8$vasopressin by that obtained in the absence of [Arg]$^8$vasopressin.

by maximally stimulating concentrations of [Arg]$^8$vasopressin ($10^{-7}$ M) and of PGE$_1$ ($3 \times 10^{-4}$ M) were additive.

**Effect of GTP**—This nucleotide has been shown in several adenylyl cyclase systems to enhance, promote or stimulate the action of hormones (7, 29–33). As shown in Table III, addition of $10^{-4}$ M GTP to the incubation medium resulted in inhibition, rather than stimulation of [Arg]$^8$vasopressin response.

### DISCUSSION

The methodology described in this report for the preparation of renal medullary membranes has several advantages. (a) It gives a high yield of membranes (approximately 250 mg of membrane protein) and is therefore adequate for future preparation. (b) It separates the NHH-sensitive adenylyl cyclase system from other adenylyl cyclases not sensitive to these hormones and which account for up to two-thirds of the total renal medullary adenylyl cyclase activity (see Table I). (c) It yields a NHH-sensitive system that is both highly responsive (up to 10-fold stimulation under adequate assay conditions) and highly sensitive (apparent $K_m$ of activation for [Arg]$^8$vasopressin, $10^{-9}$ M). (d) It yields a NHH-sensitive system that has preserved some of its regulatory properties as seen by its complex modulation by ATP and Mg$^{2+}$, and by its response to GTP. It should be mentioned that to successfully obtain the membrane fraction enriched in NHH-sensitive adenylyl cyclase it was necessary to (a) include 1 mM EDTA in sucrose gradients used for separation, and (b) have gone through a hypotonic dilution step before preparing the low speed particulate fraction (P100,100) used as source of adenylyl cyclase in the sucrose gradient separation. Selective enrichment with NHH-sensitive adenylyl cyclase could not be obtained when the homogenate was prepared in either isotonic (0.25 M) or hypertonic (0.8 M) sucrose, be it in the absence or presence of 10 mM MgCl$_2$, 1.0 mM EDTA, 10 mM Tris-HCl, pH 7.5, or combinations thereof. While this and subsequent studies on the beef renal adenylyl cyclase system were well under progress, Finn et al. (34) working with beef adrenals and using a zonal centrifugation technique published a method for separating mitochondrial membranes and fragments from adrenocorticotropin hormone-sensitive adenylyl cyclase-containing membranes. We have not yet tested whether the method of Finn et al. is also applicable to our renal medullary membrane preparations; however, in view of the presence of large amounts of mitochondrial fragments in our renal medullary membrane preparation, ultimate inclusion of a rate zonal centrifugation step into the purification scheme may well be advantageous. We are currently investigating this possibility of improving the relative purity of NHH-sensitive adenylyl cyclase-containing membranes.

One striking property of our preparation of renal medullary membranes is that its NHH-sensitive adenylyl cyclase system is profoundly influenced by ATP and Mg$^{2+}$, to the extent that, to establish optimal condition for the assay of NHH-dependent stimulation, it became essential to carry out an extensive study in which both substances were varied simultaneously. From this study it became apparent that hormonal stimulation is best seen at low Mg$^{2+}$ concentrations and depends on an optimal concentration of ATP. Recently three other reports have appeared dealing with the partial purification of renal medullary membranes. Campbell et al. (35) prepared membranes from porcine renal medulla using a differential centrifugation method described by Fitzpatrick (28) for the preparation of ATPase-containing membrane fractions, and obtained a NHH-sensitive adenylyl cyclase-containing membrane that is 5-fold purified with respect to the starting homogenate and which responds to [Lys]$^8$vasopressin with a 3- to 3.5-fold increase in activity when determined at 1.2 mM ATP and 6.0 mM MgCl$_2$; half-maximal response to [Lys]$^8$vasopressin was found at 2.2 x $10^{-8}$ M. Neer (36) prepared membranes from rat renal medulla and obtained a NHH-sensitive adenylyl cyclase system that is 5-fold purified with respect to the starting homogenate and responds with a 6 fold increase in activity when determined at about 1.0 mM ATP and 9.0 mM MgCl$_2$; half-maximal stimulation by [Lys]$^8$vasopressin varied for different membrane preparation between 4 x $10^{-8}$ M and 15 x $10^{-8}$ M. Finally, Bockaert et al. (37) have also partially purified membranes from porcine renal medulla using the technique described by Emmelot et al. (38) for liver plasma membranes and obtained a NHH-sensitive adenylyl cyclase system that is about 7-fold purified with respect to the starting homogenate and responds with a 5-fold stimulation when determined at 0.05 mM ATP and 10 mM MgCl$_2$; half-maximal stimulation by [Lys]$^8$vasopressin was found at 5 x $10^{-8}$ M. In none of these studies was a regulatory effect of either Mg$^{2+}$ or ATP reported that resembled our findings. On the contrary, the rat renal medullary system
appeared to follow Michaelis-Menten kinetics for all three activities tested (basal, [Lys]vasopressin- and fluorode-stimulated), which ours did not. The reason for this difference is not clear, but may probably be found in the method of preparation of membrane fractions which may alter the regulatory properties of the adenylyl cyclase system(s) embedded in them. It is possible that in these studies some of the ATP-dependent effects seen in our preparation were masked by the presence of NHH-insensitive adenylyl cyclases that significantly contribute to the experimentally determined basal activity. Neither in the study by Campbell et al. (35), nor in those of Neer (36) or Bockaert et al. (37), has the presence of other hormone-sensitive adenylyl cyclase systems in the final membrane preparation been tested. In this sense it should be pointed out that even though our adenylyl cyclase preparation is selectively enriched with respect to the NHH-sensitive system, it is not free from that complication either; there is at least one other adenylyl cyclase system present that responds to PGP, and whose basal activity must be contributing to some extent to the total basal activity determined in the adenylyl cyclase assay. The recent report by Storm and Dolginow (39), demonstrating a glucagon-stimulated inactivation of the glucagon-sensitive adenylyl cyclase activity in liver plasma membranes by iodoacetamide may have provided a tool to explore quantitatively the question of relative contribution of several adenylyl cyclase systems to "basal activity."

Relatively low adenylyl cyclase activities were obtained with AMP-PNP as substrate. Two possible explanations come to mind. One relates to the fact that the products of the reaction catalyzed by adenylyl cyclase are cAMP and PNP; when AMP-PNP is used as substrate as opposed to cAMP plus PP, that are the natural products obtained with ATP. It is not known which is the rate-limiting step of the reaction catalyzed by adenylyl cyclase, but if the exit of PNP is sufficiently slow to either become the rate-limiting step or is slower than an already rate-limiting exit of PP, then a slower product removal from the catalytic site would be one explanation for the lower activities observed with AMP-PNP. Another explanation is based on the chemical properties of AMP-PNP itself. Yount et al. (18) have demonstrated at least two important characteristics of AMP-PNP to be different from those of ATP. (a) Under conditions of maximal ionization, i.e., above pH 8.5, where both ATP and AMP-PNP exist mainly in their -4 net charge form, AMP-PNP binds Mg++ only one-half as strongly as ATP. (b) The pK of the terminal phosphate of AMP-PNP is 7.7 as opposed to 7.1 in ATP. Both differences tend to decrease the fraction of AMP-PNP in the bound form, substrate for adenylyl cyclase, and to increase proportionately the free form of the nucleotide, inhibitory to the reaction (1, 40), and therefore conspire against optimal catalytic activities. The finding of a higher pK optimum for basal adenylyl cyclase activity using AMP-PNP as substrate is consistent with this hypothesis. These explanations are, of course, speculative and a definitive answer will have to come from studies with a purified system exploring the mechanism of the catalytic process converting ATP (or AMP-PNP) to cAMP plus PP; (or PNP).

Adenylyl cyclases are stimulated by an active hormone receptor. Studies from this and other laboratories have established that this stimulation is not only dependent on the formation of a hormone-receptor complex, but also on the presence or absence of certain nucleotides. In this study (Fig. 3) we found that ATP exerts two effects on the NHH receptor-dependent activation of beef renal medullary adenylyl cyclase. One effect, seen when concentrations of ATP are increased from $10^{-6}$ M to $10^{-3}$ M, results in enhancement of hormonal stimulation; the other effect, seen when concentrations of ATP are increased from $10^{-3}$ M to $10^{-2}$ M, results in inhibition of hormonal stimulation. In this second effect is not seen with AMP-PNP as substrate (see Fig. 6). These findings suggested that activation of adenylyl cyclases by hormone receptors may very well be under control by nucleotides in a positive manner, as seen in earlier studies with glucagon-sensitive adenylyl cyclases from liver and pancreatic β cells, as well as in a negative manner as suggested by the inhibitory effect of high concentrations of ATP on NHH stimulation of the renal medullary adenylyl cyclase. The finding that GTP at $10^{-4}$ mimicked the effect of millimolar concentrations of ATP supports this hypothesis. A detailed study on the characteristics of the GTP effect as well as that of adenosine, found to mimic the stimulatory effect of low concentrations of ATP, is reported in the accompanying paper.

In conclusion, this report describes the method of preparation of a beef renal medullary membrane fraction in relatively large quantities and the basic properties of a NHH-sensitive adenylyl cyclase system present in these membranes. These membranes have subsequently been used to study the regulation of the receptor-dependent activation of adenylyl cyclase by nucleosides and nucleotides, to determine rate constants of the hormone-receptor interaction as seen through activation of adenylyl cyclase, and to define several conformational states of the catalytic unit of the system as induced by the free receptor, the NHH-occupied receptor and GTP. These studies are the subject of some of the accompanying reports (41-43), all dealing with the mechanism(s) by which hormone receptors affect adenylyl cyclase systems.

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Lutz Birnbaumer and Po-Chang Yang


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