Specific Binding of $[^3H]$Lysine-Vasopressin to Pig Kidney Plasma Membranes

RELATIONSHIP OF RECEPTOR OCCUPANCY TO ADENYLATE CYCLASE ACTIVATION*

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SUMMARY

A plasma membrane fraction was prepared from pig kidney medulla. It contained lysine-vasopressin-sensitive adenylate cyclase activity (maximum activation: 4- to 6-fold, apparent $K_m$ value for the hormone: about $5 \times 10^{-8} \text{M}$). Lysine-vasopressin was not inactivated by the membrane preparation even at low hormonal concentrations.

The specific $[^3H]$lysine-vasopressin (vasopressin) binding sites found are probably involved in adenylate cyclase activation; binding occurred in a concentration range giving dose-dependent activation of the adenylate cyclase. Relative affinities of vasopressin, oxytocin, (O-methyl)-tyrosine$^2$-oxytocin and angiotensin (vasopressin > oxytocin > (O-Me)-Tyr$^2$-oxy > angiotensin) for binding and adenylate cyclase activation were similar. Nonspecific binding, i.e. binding which could not be inhibited by $10^{-8} \text{M}$ unlabeled vasopressin, represented 12.5% of the specific binding at $10^{-8} \text{M} [^3H]$vasopressin.

For all hormonal concentrations, $[^3H]$vasopressin binding increased with time up to an equilibrium value. The binding was reversible. The time course of hormone-receptor complex formation was highly temperature- and hormone concentration-dependent. Dissociation and association rate constants at $30^\circ$ varied from 0.02 to 0.088 min$^{-1}$ for $k_1$, and from $1.3 \times 10^{-7}$ to $3.0 \times 10^{-7} \text{M}^{-1} \text{min}^{-1}$ for $k_2$. For the same enzyme preparation, the $k_2/k_1$ ratio was very close to the value deduced from the hormonal concentration giving half the maximum binding at equilibrium.

When adenylate cyclase activation was measured as a function of time after the addition of hormone, activation was found to be progressive. The time required to reach maximum activation is similar to that needed to reach an equilibrium value for $[^3H]$vasopressin binding at the same concentration. These data suggest that adenylate cyclase activation is a function of receptor occupancy. Reversal of adenylate cyclase activation was also progressive.

When adenylate cyclase activation at equilibrium was measured for different values of receptor occupancy, it was found that for the same increment in receptor occupancy, the increment in adenylate cyclase activation drops as total receptor occupancy increases.

It has been clearly shown that cyclic adenosine 3':5'-monophosphate is the second intracellular messenger of the antidiuretic effect of antidiuretic hormone on the mammalian kidney. Two series of experiments (2, 3) showed that exogenous cyclic AMP or theophylline mimic the increase by antidiuretic hormone of the water permeability of isolated rabbit collecting tubules. Furthermore, antidiuretic hormone increases the intracellular cyclic AMP content of kidney slices (4, 5) or of renal cell suspension (6). This cyclic AMP accumulation probably results from enhancement of nucleotide production, since antidiuretic hormone activates the adenylate cyclase system of particulate fractions or partially purified kidney plasma membranes (4, 7-17).

Despite the fact that the antidiuretic hormone concentrations required to stimulate the adenylate cyclase system are much higher than the circulating hormone levels reported (18), the hormonal receptor present in the plasma membrane fractions retains many of the characteristics of the receptor present in the intact structure, especially as regards its specificity toward neurohypophysial hormones and their structural analogues (12).

In the present investigation, binding sites for $[^3H]$vasopressin were found in pig kidney plasma membranes containing adenylate cyclase highly sensitive to lysine-vasopressin, the natural antidiuretic principle in this species. Several correlations between the binding of vasopressin or analogues and the adenylate cyclase activation were shown which suggest that the binding sites detected are the receptors coupled to the adenylate cyclase system. The kinetic parameters of the interaction between the hormonal molecule and its receptor were defined. A precise examination of the correlations between binding and adenylate cyclase activation led to the conclusion that activation is a function of receptor occupation, thus excluding the validity of the rate theory in this case. Furthermore, the relationship between occupation and activation is a complex and nonlinear one.

The abbreviations used are: cyclic AMP, cyclic adenosine 3':5'-monophosphate; vasopressin, lysine-vasopressin.
Experimental Procedure

Materials

Lysine-vasopressin (vasopressin) (lysine-vasopressin chloride, 285.5 u. per mg) and oxytocin chloride hydrate (374.1 u. per mg) were kindly donated by Dr. Boissonnas of Sandoz. ATP (disodium salt) was purchased from P-L Biochemicals Inc. and cyclic adenosine 3':5'-monophosphate, Tris, and EDTA from Sigma; creatine kinase, phosphocreatine (disodium salt) were purchased from Boehringer. Cytochrome c, NADPH, and succinate (sodium salt) were purchased from Calbiochem. Neutral aluminium oxide (activity grade I, Batch 4420) was purchased from M. Woelm Eshwege.

Radiochemicals

Cyclic [3H]AMP (ammonium salt, 19 Ci per mmole) and [α-32P]ATP (sodium salt 880 to 1027 mCi per mmole) stored at −25 ° in 50% ethanol, were purchased from the Commissariat à l'Energie Atomique (Saclay, France).

[3H]Vasopressin was prepared by Pradelles et al. (19). The peptide was labeled on the tyrosine residue in position 2. Its specific activity was 10 Ci per mmole and its biological activities, respectively, determined by the rat vasopressor assay (20) and pig kidney adenylate cyclase activation, were 228 and 285 i.u. per mg. The [3H]vasopressin was stable upon storage for at least 6 months when kept in liquid nitrogen in diluted form.

Methods

Enzyme Preparation—Pig kidneys collected under sterile conditions were obtained from the local slaughterhouse and kept at 4 °. Internal medulla was rapidly dissected at 0 ° and immediately homogenized.

Using the technique of Emmcot et al. (21), the tissue (40 g) was homogenized in a tight glass Potter-Elvehjem in 1 ml sodium bicarbonate at 4 °. The crude homogenate was filtered on glass wool and the filtrate made up to 500 ml with sodium bicarbonate and centrifuged. The 1200 × g pellet was dispersed with a loose Teflon Potter-Elvehjem in 1 ml sodium bicarbonate at 4 °, made up to 500 ml, and again centrifuged at 1200 × g at 4 °. This washing procedure was repeated five or six times. The membranes contained in the washed 1200 × g pellet were separated by flotation in a nonlinear sucrose gradient composed of layers at the following densities: 1.16, 1.18, 1.20, and 1.30, and subjected to 60-min centrifugation at 60,000 × g. The membranes were collected at the 1.16, 1.18 interface and washed in 1 ml sodium bicarbonate. This fraction is defined under “Results” as “purified plasma membranes.”

Most of the experiments described in this paper were performed on a membrane fraction prepared using a modified version of the technique just described. The tissue was homogenized in a medium containing 250 mM sucrose, 5 mM Tris-HCl, pH 8.0, 3 mM MgCl2, and 1 mM EDTA, pH 8.0. After filtration on glass wool, the 300 × g pellet was collected and suspended in the homogenization medium made hypotonic by dilution of sucrose and stirred for 10 min at room temperature. The 300 × g pellet was homogenized after each centrifugation with a loose Teflon Potter-Elvehjem in the same hypotonic medium at 4 °, made up to 400 ml, and again centrifuged at 300 × g. This washing procedure of the 300 × g pellet was repeated five or six times. The final pellet, suspended in the same medium, was stored in liquid nitrogen. This fraction is referred to as “enzyme” in the results.

In some experiments, the enzyme was subjected to 60-min, 60,000 × g centrifugation in a nonlinear sucrose gradient, as described above, and the material present at the interfaces collected.

Enzymatic Determinations—Adenylate cyclase activity was measured as previously described (22). To sum up the method, the incubation medium (final volume: 100 μl) contained 100 mM Tris-HCl, pH 8.0, 10 mM MgCl2, 0.25 mM ATP, 0.65 μCi of [α-32P]ATP, 1 mM cyclic AMP, 20 mM phosphocreatine, and 1 mg per ml of creatine kinase. Except where otherwise indicated, enzyme (160 to 320 μg), kept at 0 °, was added at zero time and incubation carried out for 5 min at 30 °. Cyclic [32P]AMP was separated by filtration on dry aluminium oxide columns as proposed by Ramachandran et al. (23). Controls of this method have already been described (22).

Na1- and K1-activated ATPase was measured by the method of Post and Sen (24) and the Pi released was measured by the method of Fiske and Subbarow (25). NADPH-cytochrome c reductase and succinate-cytochrome c reductase were determined according to the method of Fleischer and Fleischer (26) except for a few minor modifications. Protein determinations were carried out according to the procedure of Lowry et al. (27) with bovine serum albumin as a standard.

Measurements of [3H]Vasopressin Binding—The enzyme was incubated in the same medium as that used for adenylate cyclase assays, without [α-32P]ATP, creatine kinase, and phosphocreatine.

The absence of this ATP-regenerating system did not modify the binding process of [3H]vasopressin. Incubations were carried out at 30 ° except where otherwise indicated. At the end of the incubation period, 2 ml of Solution A containing 100 mM Tris-HCl, pH 8.0, 10 mM MgCl2, 0.25 mM ATP, and 1 mM cyclic AMP kept at 0 ° were added. The samples were then filtered through Millipore filters (EAWP 0.45 μ) prewashed with 5 ml of cold B solution containing 0.1% bovine serum albumin, 10 mM Tris-HCl, pH 8.0, and 1 mM MgCl2. The filters were washed at 0 ° three times with 10 ml of the same solution. The time between the end of incubation and the end of filtration did not exceed 30 s. The Millipore filters were dried and their radioactivity content measured by liquid scintillation in 8 ml of the following medium: 50 mg of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene and 4 g of 2,5-diphenyloxazole in 1000 ml of toluene. For each experimental condition, two determinations were made for specific binding and one for nonspecific. Nonspecific binding corresponds to the binding measured when 10−6 M unlabeled vasopressin is added to the incubation medium. Specific binding is total binding measured in a given experimental condition, corrected for a nonspecific value determined in the same condition. When the filters were washed once, twice, or three times with 10 ml of rinsing solution, the amounts of [3H]vasopressin bound at 2.5 × 10−9 M were, respectively, 0.647, 0.597, and 0.585 pmole of [3H]vasopressin bound per mg of protein. Blank values for the filters alone were negligible (0.005 pmole/mg of protein). Nonspecific binding was 0.029 in the first case and 0.026 pmole [3H]vasopressin bound per mg of protein in the second and third cases. Nonspecific binding was found to increase linearly with the [3H]vasopressin concentration and represented 12.5 to 16% of specific binding at 10−8 M. Nonspecific binding reached a submaximum value almost instantaneously, in less than 0.5 min. After 0.5 min the nonspecific binding represented 80% of the nonspecific value measured 4 hours later. Moreover, at a given incubation time, this nonspecific binding was the same at 0 and 30 °.
Table I
Evolution of enzymatic profile, adenylate cyclase activity, and [3H]vasopressin binding capacity during enzyme preparation

Activities are expressed in micromoles of substrate transformed per mg of protein per hour except for adenylate cyclase activities which are expressed in picomoles of cyclic AMP formed per mg of protein per 5 min. [3H]Vasopressin binding is expressed in picomoles of [3H]vasopressin bound per mg of protein after a 10-min incubation period.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Adenylate cyclase</th>
<th>(3H)Vasopressin binding</th>
<th>Na⁺,K⁺-activated ATPase</th>
<th>Ratio of Na⁺,K⁺- to Mg²⁺-activated ATPase</th>
<th>Succinate-cytochrome c reductase</th>
<th>NADPH-cytochrome c reductase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Vasopressin (10⁻⁸ M)</td>
<td>NaF (10⁻² M)</td>
<td>Specific</td>
<td>Nonspecific</td>
<td>Specific</td>
</tr>
<tr>
<td>Homogenate (A)</td>
<td>36</td>
<td>65</td>
<td>173</td>
<td>0.040</td>
<td>0.065</td>
<td>0.57</td>
</tr>
<tr>
<td>Lysate pellet (B)</td>
<td>75</td>
<td>298</td>
<td>433</td>
<td>0.152</td>
<td>0.049</td>
<td>1.03</td>
</tr>
<tr>
<td>Enzyme (C)</td>
<td>88</td>
<td>420</td>
<td>444</td>
<td>0.138</td>
<td>0.020</td>
<td>0.52</td>
</tr>
<tr>
<td>C:B</td>
<td>1.27</td>
<td>1.51</td>
<td>1.02</td>
<td>0.91</td>
<td>0.41</td>
<td>0.27</td>
</tr>
<tr>
<td>C:A</td>
<td>2.45</td>
<td>4.95</td>
<td>2.56</td>
<td>3.45</td>
<td>0.57</td>
<td>0.91</td>
</tr>
</tbody>
</table>

Results

Enzymatic Profile of Kidney Plasma Membrane Fractions—The technique of Emmelot et al. (21) made it possible to obtain a fraction possessing enriched plasma membrane-bound enzymatic activities and diminished mitochondrial and microsomal activities. Thus the respective specific activities of the basal or fluoride-sensitive adenylate cyclase, and of the Na⁺,K⁺-activated ATPase increased about 5-fold compared to the starting homogenate. However the adenylate cyclase was poorly sensitive to vasopressin stimulation. The activation ratio tended to drop during the purification procedure. The osmotic pressure of the homogenization medium appears to be a crucial factor. When 250 mM sucrose was added to the homogenization medium the response to vasopressin was much higher and the apparent affinity for the hormone increased. This homogenization technique was retained for the preparation of membrane fractions. As indicated in Table I, extensive washing of the low speed crude homogenate sediment in hypotonic media led to a significant increase in adenylate cyclase and Na⁺,K⁺-activated ATPase specific activities. Washing gradually eliminated vasopressin-insensitive adenylate cyclase activity, as indicated by the progressive increase in the activation ratio. In addition, the adenylate cyclase activity measured in the presence of a maximum vasopressin concentration was very similar to that measured in the presence of fluoride. Similarly, the nonspecific [3H]vasopressin binding was reduced whereas the specific binding activity increased. The final fraction was not enriched in Na⁺,K⁺-activated ATPase specific activity but the ratio of Na⁺,K⁺-dependent over Mg²⁺-dependent activities increased from 0.13 to 0.53, suggesting gradual elimination of the mitochondrial Mg²⁺-dependent activity. High speed centrifugation of the final washed pellet by flotation in a nonlinear sucrose gradient resolved the pellet into three bands containing adenylate cyclase activity; however, in none of these bands was the specific activity of the vasopressin-sensitive adenylate cyclase enhanced.

The experiments described below were performed with the washed pellet, referred to as "enzyme."

Control experiments showed that the amount of [3H]vasopressin bound at equilibrium as well as the amount of cyclic AMP formed increased linearly with the enzyme concentration in the incubation medium (Fig. 1).

The concentration of enzyme used for the different experiments varied from 1.6 to 3.3 mg per ml. At these concentrations, cyclic AMP accumulation under basal or maximum NaF or vasopressin-stimulated conditions was linear with time up to 15 min at least. As shown in Fig. 2, there is no indication of any deterioration of vasopressin even when inframaximal doses (2.0 × 10⁻⁸ or 10⁻⁹ M) were incubated in the presence of enzyme for 5, 10, or 20 min. Finally, the enzyme was found to be stable when stored in liquid nitrogen for at least 2 months. However, significant variability was observed from one enzyme preparation to the other with respect to their apparent affinity for vasopressin, determined either from [3H]vasopressin binding curves or activation curves of the adenylate cyclase. The maximal activation ratio varied from 4- to 6-fold for the different enzyme preparations used in this study. Correlations between binding and adenylate cyclase activation were always established with the same enzyme preparation.

Qualitative Correlations between Vasopressin Binding and Adenylate Cyclase Activation—Comparison of adenylate cyclase activations by vasopressin, oxytocin, (O-Me)Tyr²-oxytocin, and angiotensin (Fig. 3) showed that the adenylate cyclase was highly specific for lysine-vasopressin. (O-Me)Tyr²-oxytocin was able to stimulate the adenylate cyclase and maximal activation was similar to that induced by vasopressin, but the apparent affinity for this peptide was about 1500 times lower than the
FIG. 2. Control of lysine-vasopressin stability in the presence of membranes. The four dose-response curves for adenylate cyclase activation by lysine-vasopressin were obtained as follows. For each determination, 100-μl aliquots of an assay medium without [γ-32P]ATP containing 1.55 mg per ml of enzyme were incubated for 0, 5, 10, and 20 min at 30°. At the end of preincubation time, the samples were centrifuged for 10 min at 3000 × g at 0°. Twenty-five microliters of the supernatant were collected in test tubes containing [γ-32P]ATP, lysine-vasopressin, and all the other components required for standard assays. Reaction was initiated by adding 1.55 mg per ml of fresh enzyme. A second group of samples containing 2 × 10⁻⁴ M or 10⁻⁵ M of lysine-vasopressin were preincubated for 0, 5, 10, and 20 min in the presence of enzyme. As in the case of the first group, 25 μl of the preincubation medium, separated from the enzyme by centrifugation, were collected for final assay. The final hormonal concentrations expected, in the presence of fresh enzyme, were 5.0 × 10⁻⁹ M (1) and 2.5 × 10⁻⁹ M (2). The values obtained are plotted on the different graphs using the abscissae corresponding to the concentrations expected if no deterioration of vasopressin had occurred. Experimental values are the mean of three independent determinations.

apparent affinity for vasopressin (1.5 × 10⁻⁹ M). Oxytocin has an apparent affinity for the receptor 500 times lower than lysine-vasopressin. On the other hand, oxytocin was unable to provide maximal adenylate cyclase stimulation and the activation ratio decreased at high concentrations, as several authors previously observed (12, 22, 28). Angiotensin induced slight stimulation but for very high concentrations (10⁻⁴ M).

The same enzyme preparation was able to bind [3H]vasopressin. For a given hormonal concentration, binding increased with time up to an equilibrium value. Binding at equilibrium was saturable with the hormonal concentration in the medium. As in the case of the receptors coupled to the adenylate cyclase, the hormonal binding sites were highly specific toward vasopressin. Unlabeled vasopressin inhibited (Fig. 4) the binding of 5 × 10⁻⁹ M [3H]vasopressin, a concentration producing about 60% of maximal binding. Inhibition occurred in the range of concentrations which were active when tested on the adenylate cyclase. Almost complete inhibition was obtained at 10⁻⁷ M vasopressin, the concentration that gave maximal adenylate cyclase activation. [3H]Vasopressin binding was also inhibited by unlabeled oxytocin and (O-Me)Ty2-oxytocin. In this case too, inhibition only occurred in the range of concentrations active when tested on the adenylate cyclase. Angiotensin was unable to inhibit [3H]vasopressin binding even at 10⁻⁴ M. These good qualitative correlations between adenylate cyclase activation and [3H]vasopressin binding suggest that the binding sites detected are those involved in the adenylate cyclase activation.

Characterization of Vasopressin Binding Sites—[3H]Vasopressin binding is a time-dependent process (see Fig. 5). At the hormone concentration tested (10⁻⁸ M), about 10 min were needed to reach equilibrium, the half-time for binding being 2 min. [3H]Vasopressin binding was reversible by 20-fold dilution of the
FIG. 5. Time course of the association and dissociation of [3H]vasopressin with renal plasma membrane receptors. Enzyme (3.10 mg of protein per ml) was incubated in a final volume of 100 µl with 10⁻⁸ mol of [3H]vasopressin and the binding was measured as a function of time at 30° (association curve: left part). To test the reversibility of binding, the enzyme was first incubated at 30° for 15 min in the presence of [3H]vasopressin (10⁻⁸ M). The hormonal concentration was then lowered to 5 X 10⁻¹⁰ M by dilution with Solution A without hormone at 30° (see "Methods"). Residual binding was measured as a function of time after dilution. The semilogarithmic plots of the association and dissociation curves are shown in the lower part of the figure.

The hormone is washed out from the incubation medium; this decreases the hormonal concentration to 5.0 X 10⁻¹⁰ M which at equilibrium gives less than 5% of the maximum binding at 10⁻⁸ M. Under these experimental conditions, reversibility is almost complete.

Both the association and dissociation curves can be adequately described in terms of reversible binding of the hormone on a homogeneous population of independent binding sites

\[ R + H \xrightleftharpoons[k_l]{k_{-l}} RH \]  

(1)

in which \( R \) and \( RH \) are free and bound receptors, respectively, \((R + RH = R_T; \text{total amount of receptor})\) and \( k_l \) and \( k_{-l} \) are the association and dissociation constants, respectively. As indicated above, the fraction of hormonal molecules bound to the receptor at equilibrium is less than 2.5%, so that the hormonal concentration in the medium \((H)\) can be taken as a constant. In this situation, the formation of \( RH \) is rendered by the following equation.

\[ \log_{10} \frac{[RH]_{eq}}{[RH]_{eq} - [RH]} = (k_l[H] + k_{-l})t \]  

(2)

the corresponding equation for the dissociation being

\[ \log_{10} \frac{[RH]_{eq}}{[RH]_{eq} - [RH]} = -k_{-l}t \]  

(3)

in which \( [RH]_{eq} \) is the concentration of the hormone-receptor complex at equilibrium. Equation 3 only applies to the case where the hormone is washed out from the incubation medium after the equilibrium state for association \([RH]_{eq}\) is achieved.

The logarithmic transformations of the experimental association and dissociation curves led to good linear relationships (Fig. 6, lower part). Calculation of \( k_l \) and \( k_{-l} \) from the slopes of these two linear regression lines gives the following values: \( k_l = 2.3 \times 10^{+7} \text{ M}^{-1} \text{ min}^{-1} \), and \( k_{-l} = 0.035 \text{ min}^{-1} \). This would correspond to the equilibrium constant \( k_{eq} = 1.5 \times 10^{+5} \text{ M} \), which agrees very well with the apparent \( K_a \) for adenylate cyclase activation determined on the same enzyme preparation. However in order to use the experimental association and dissociation curves to estimate \( k_l \) and \( k_{-l} \), we had to prove that hormone-receptor interaction is the rate-limiting process and not the hormone's accessibility to the receptor sites by diffusion. This latter possibility seems unlikely, since the time course of \( RH \) formation was highly temperature-dependent (Fig. 6). At 0 and 15° the half-times for binding of [3H]vasopressin (10⁻⁸ M) were 30 and 9 min, respectively, compared to 2 min at 30°. The equilibrium values tended to increase slightly when the temperature was lowered. In addition, the time course of the binding was also modified by lowering the pH to 7.4 (half-time = 1.3 min); however, the equilibrium value of binding \([RH]_{eq}\) was significantly lower under this experimental condition. (see also Fig. 11, for instance). Furthermore, as may be expected from Equation 2, the time course of [3H]vasopressin binding was hormone concentration-dependent (Fig. 7). Determination of \( k_l[H] + k_{-l} \) for two different values of \( H \) permits the calculation of \( k_l \) and \( k_{-l} \).

Table III gives the \( k_l \) and \( k_{-l} \) values obtained on different enzyme preparations using either simultaneous determination of association and dissociation curves or the determination of two association curves for two different hormone concentrations. The differences observed in the experimental values reflect the differences noted between various enzyme preparations. For a given enzyme preparation (see for instance Enzyme 3, table III) there is good agreement between equilibrium constants determined from \( k_{eq} = k_l k_{-l} \times 7.0 \times 10^{-9} \text{ M} \) and from the hormonal concentration giving half-saturation of the receptor when binding of [3H]vasopressin was measured after 15-min incubation periods (1.4 \times 10^{-9} M). The small difference observed can probably be accounted for by the fact that true equilibrium was not reached in 15 min.
It may be noted (see Table II, Enzyme 3) that both $k_1$ and $k_2$ were reduced by lowering the temperature from 30–15°, the equilibrium constant remaining unchanged. This is consonant with the observation (see Fig. 8) that the apparent $K_m$ values for vasopressin determined from adenylate cyclase activation curves at 30 and 15° are identical. It should be noted that the activation ratio (stimulated over control) is higher at 30° than at 15°. pH dependency of [3H]vasopressin binding at $10^{-8}$ M is illustrated by Fig. 9. [3H]Vasopressin binding at $10^{-8}$ M was almost completely eliminated at pH 6.5 and an optimum was observed at a pH nearing 8.5. Marked pH dependency is also observed for adenylate cyclase activation. However, the optimal pH is lower than the optimal pH for the binding. This probably reflects the combination of several pH effects on the hormone binding, on the catalytic activity of the enzyme and on the receptor adenylate cyclase coupling.

**Relations between Hormone Binding to Receptors and Adenylate Cyclase Activation**—The experimental data already described strongly suggest that the [3H]vasopressin binding sites present in pig kidney medulla membrane fractions are involved in activating the adenylate cyclase system. Parallel measurements on the same enzyme preparation of [3H]vasopressin binding and adenylate cyclase activation offer a possible experimental approach to the receptor-cyclase coupling problem. In order to study this coupling process it is essential to find out whether adenylate cyclase activation is a function of receptor occupation or of the turnover of the hormonal molecules on the receptor sites, in accordance with the rate theory proposed by Paton (29). As several authors have pointed out (e.g. Waud (30)), the best

**TABLE II**

*Kinetic parameters of [3H]vasopressin binding to enzyme under different experimental conditions*

For enzyme Preparations 1 and 2, association and dissociation rate constants were calculated from independent association and dissociation curves (see “Results”). For enzyme Preparation 3, the rate constants were calculated from two association curves obtained with two hormonal concentrations (see “Results”).

<table>
<thead>
<tr>
<th>Enzyme preparation No.</th>
<th>Experimental conditions</th>
<th>Association $k_{1/2}$</th>
<th>$k_1H + k_2$</th>
<th>$k_1$</th>
<th>$k_2$</th>
<th>$k_{-1}/k_1$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$\text{min}$</td>
<td>$\text{min}^{-1}$</td>
<td>$\text{M}^{-1}\text{min}^{-1}$</td>
<td>$\text{min}^{-1}$</td>
<td>$\text{M}^{-1}$</td>
</tr>
<tr>
<td>1</td>
<td>30°; pH 8 $H = 10^{-8}$</td>
<td>1.9</td>
<td>0.364</td>
<td>2.3 $\times 10^{-7}$</td>
<td>0.035</td>
<td>1.5 $\times 10^{-9}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.3</td>
<td>0.258</td>
<td>2.0 $\times 10^{-7}$</td>
<td>0.020</td>
<td>8 $\times 10^{-10}$</td>
</tr>
<tr>
<td>2</td>
<td>30°; pH 8 $H = 5 \times 10^{-9}$</td>
<td>2.0</td>
<td>0.349</td>
<td>3.0 $\times 10^{-7}$</td>
<td>0.006</td>
<td>1.2 $\times 10^{-9}$</td>
</tr>
<tr>
<td>3</td>
<td>30°; pH 8 $H = 2.0 \times 10^{-9}$</td>
<td>4.5</td>
<td>0.152</td>
<td>1.3 $\times 10^{-7}$</td>
<td>0.088</td>
<td>7.0 $\times 10^{-9}$</td>
</tr>
<tr>
<td>3</td>
<td>15°; pH 8 $H = 2.0 \times 10^{-9}$</td>
<td>14.5</td>
<td>0.048</td>
<td>3.5 $\times 10^{-4}$</td>
<td>0.03</td>
<td>8.7 $\times 10^{-2}$</td>
</tr>
</tbody>
</table>

Fig. 7. Effect of hormonal concentration on the association time course at 15°. Enzyme (3.30 mg per ml) was incubated at 15° in a final volume of 100 μl in the presence of [3H]vasopressin, $9 \times 10^{-8}$ μ (●●●●) and $5 \times 10^{-8}$ μ (○○○○). It may be noted that the initial velocity is proportional to the hormonal concentration. The semilogarithmic plots of the two association curves are shown in the lower part of the figure.

Fig. 8. Adenylate cyclase activation by vasopressin. Dose-response curves at 15 and 30°. Enzyme concentration was 3.3 mg per ml. Incubation periods were 5 min at 30° and 10 min at 15° to obtain sufficient cyclic AMP production. Apparent $K_m$ values are indicated by the broken lines.
criteria for discriminating between the two possibilities is to determine the time course of the response. If the occupation theory is valid, one may expect the time course of the response to follow that of the hormonal binding; the response should increase with time after the addition of hormone up to an equilibrium value. If, on the contrary, the rate theory is valid, one may expect the response to reach its maximum immediately after the addition of hormone and gradually drop to an equilibrium value, since the response which is a function of the rate of binding decreases as the total receptor occupation grows.

The gradual increase in adenylate cyclase activation was also apparent when cyclic AMP accumulation was studied as a function of time after adding the hormone. The linear part of the cyclic AMP accumulation curve did not extrapolate to zero time (see Fig. 11), as does the control curve. The lag time for adenylate cyclase activation is longer for the lowest vasopressin concentration used. This is consonant with the fact (see above) that the time required to reach equilibrium for binding increases as the hormonal concentration drops. Similarly, the adenylate cyclase activation was not immediately reversible when the hormonal concentration in the incubation medium was instantaneously reduced to one-tenth of its initial nonmaximum value (10^{-8} M); 15 min afterwards, the response was at an intermediate point between the equilibrium values at 10^{-3} and 10^{-4} M (Fig. 12).

Considering that adenylate cyclase activation is a very early consequence of hormone-receptor interaction, the above data strongly suggest that the generation of the stimulus is a function of receptor occupation; they virtually exclude the validity of the rate theory in this case. In the occupation theory we include the possibility that the response is a function of the number of hormonal molecules leaving the receptor during the unit of time (= k_{-a}[RH]).

Comparison of the binding curve and the adenylate cyclase activation curve at equilibrium makes it possible to determine the function relating the cyclase activation to receptor occupation. [3H]Vasopressin binding after a 15-min incubation period was measured as a function of vasopressin concentration in the medium. On the same enzyme preparation, adenylate cyclase activity was measured for 3 min (from the 14th to 17th min) after the addition of hormone. As shown by Fig. 13, the hormonal binding and cyclase activation curves are not superimposable but it may be noted that maximum concentrations were identical for both. When relative cyclase stimulation is plotted as a function of relative receptor occupation, a significant deviation from line of identity is observed, suggesting a nonlinear relationship between receptor occupation and response. The same increment in receptor occupation generates a stimulus whose magnitude decreases as the total receptor occupation grows.
The present experiments showed that the hormone-receptor interaction was rate-limiting for the binding population of receptor sites. The association and dissociation curves presented in this paper are similar to those reported very recently by Campbell et al. (17) for the preparation of kidney plasma membranes, the procedure used in this study enables vasopressin-deteriorating systems to be eliminated. Such elimination is of great importance for accurate examination of vasopressin binding or adenylate cyclase activation, especially when very low hormonal concentrations are used. Several correlations between vasopressin binding to pig kidney medulla plasma membranes and adenylate cyclase activation strongly suggest that the vasopressin binding sites are receptors coupled to the adenylate cyclase system. Thus, there is a striking similarity between the apparent relative affinities for vasopressin, oxytocin, and (O-Me)Tyr2-oxytocin, estimated either from their ability to activate the adenylate cyclase or to compete with [3H]vasopressin for binding. In addition, gradual receptor saturation by vasopressin occurs in a dose range identical with that producing dose-dependent activation of the adenylate cyclase. Complete saturation of the receptors and maximum activation of the enzyme are obtained for the same vasopressin concentration in the medium.

The binding of [3H]vasopressin is completely reversible. The association and dissociation curves can be accounted for by reversible binding of the hormonal molecules on a homogeneous population of receptor sites. The association and dissociation curves presented in this paper are similar to those reported very recently by Campbell et al. (17). The present experiments showed that the hormone-receptor interaction was rate-limiting for the binding of the hormone to the receptor and for its release. The time course of the binding is highly temperature- and hormone concentration-dependent. For a given enzyme preparation there is good agreement between the equilibrium constant deduced from binding curves at equilibrium and the \( k_{-1}k_1 \) ratio calculated from independent determinations of \( k_{-1} \) and \( k_1 \). This ratio was always found to be slightly lower than the apparent \( K_m \) deduced from the hormonal concentration giving half the maximum binding after 15-min incubation periods. This is in line with a fraction of a minute for the \( 10^{-7} \) m vasopressin concentration and several min for the \( 2.10^{-9} \) m concentration. Comparison of the curves obtained for these two concentrations shows almost immediate activation by vasopressin at \( 10^{-7} \) m, whereas in the case of \( 2.10^{-9} \) m, activation is gradual. The fact that the lines joining the experimental data obtained for basal and NaF conditions pass through 0.5 min rather than zero time is due to the equilibration period necessary to raise the assay sample's temperature from 0 to 30°. Enzyme concentration was 3.3 mg per ml.

**DISCUSSION**

As compared with the method proposed by Campbell et al. (17) for the preparation of kidney plasma membranes, the procedure used in this study enables vasopressin-deteriorating systems to be eliminated. Such elimination is of great importance for accurate examination of vasopressin binding or adenylate cyclase activation, especially when very low hormonal concentrations are used. Several correlations between vasopressin binding to pig kidney medulla plasma membranes and adenylate cyclase activation strongly suggest that the vasopressin binding sites are receptors coupled to the adenylate cyclase system. Thus, there is a striking similarity between the apparent relative affinities for vasopressin, oxytocin, and (O-Me)Tyr2-oxytocin, estimated either from their ability to activate the adenylate cyclase or to compete with [3H]vasopressin for binding. In addition, gradual receptor saturation by vasopressin occurs in a dose range identical with that producing dose-dependent activation of the adenylate cyclase. Complete saturation of the receptors and maximum activation of the enzyme are obtained for the same vasopressin concentration in the medium.

The binding of [3H]vasopressin is completely reversible. The association and dissociation curves can be accounted for by reversible binding of the hormonal molecules on a homogeneous population of receptor sites. The association and dissociation curves presented in this paper are similar to those reported very recently by Campbell et al. (17). The present experiments showed that the hormone-receptor interaction was rate-limiting for the binding of the hormone to the receptor and for its release. The time course of the binding is highly temperature- and hormone concentration-dependent. For a given enzyme preparation there is good agreement between the equilibrium constant deduced from binding curves at equilibrium and the \( k_{-1}k_1 \) ratio calculated from independent determinations of \( k_{-1} \) and \( k_1 \). This ratio was always found to be slightly lower than the apparent \( K_m \) deduced from the hormonal concentration giving half the maximum binding after 15-min incubation periods. This is in line with a fraction of a minute for the \( 10^{-7} \) m vasopressin concentration and several min for the \( 2.10^{-9} \) m concentration. Comparison of the curves obtained for these two concentrations shows almost immediate activation by vasopressin at \( 10^{-7} \) m, whereas in the case of \( 2.10^{-9} \) m, activation is gradual. The fact that the lines joining the experimental data obtained for basal and NaF conditions pass through 0.5 min rather than zero time is due to the equilibration period necessary to raise the assay sample's temperature from 0 to 30°. Enzyme concentration was 3.3 mg per ml.

**Fig. 11.** Linearity with time of cyclic AMP accumulation under basal, NaF, and vasopressin-stimulated conditions. Binding \( t/2 \) is a fraction of a minute for the \( 10^{-7} \) m vasopressin concentration and several min for the \( 2.10^{-9} \) m concentration. Comparison of the curves obtained for these two concentrations shows almost immediate activation by vasopressin at \( 10^{-7} \) m, whereas in the case of \( 2.10^{-9} \) m, activation is gradual. The fact that the lines joining the experimental data obtained for basal and NaF conditions pass through 0.5 min rather than zero time is due to the equilibration period necessary to raise the assay sample's temperature from 0 to 30°. Enzyme concentration was 3.3 mg per ml.

**Fig. 12.** Reversal of vasopressin-induced adenylate cyclase activation. Four aliquots of the same enzyme preparation (0.51 mg each) were preincubated at 30° in standard assay media (0.25 ml total volume) without [\( \alpha-^32\)P]ATP. Two of them contained \( 10^{-8} \) m vasopressin, another \( 10^{-9} \) m vasopressin, and the last no vasopressin. After a 10-min incubation period with stirring at 30°, the four samples were brought to a final volume of 2.5 ml by means of a solution ensuring that each component of the adenylate assay medium remained unchanged. In each of the three control samples, the lysine-vasopressin concentration was respectively kept to its initial value: \( 10^{-8} \) m (---), \( 10^{-9} \) m (----), and no vasopressin (-----), whereas in the experimental group used to test the reversal of adenylate cyclase activation lysine-vasopressin concentration dropped from \( 10^{-8} \) to \( 10^{-9} \) m (----). Duplicate aliquot samples of 0.15 ml were taken at different times after the volume of all the samples had been brought to 2.5 ml, and immediately assayed for 2 min at 30° in test tubes containing 6 &i of [\( \alpha-^32\)P]ATP in a volume of 0.02 ml. The reaction was stopped as indicated in the legend of Fig. 10.

**Fig. 13.** Effect of hormonal concentration on [3H]vasopressin binding and adenylate cyclase activation measured near equilibrium conditions. [3H]Vasopressin binding (■, ■) was measured after 15-min incubation at 30°. The apparent \( K_m \) value for binding (1.5 \( \times 10^{-8} \) m). For measurement of adenylate cyclase activation (O) near equilibrium conditions, the enzyme and vasopressin were incubated at 30° for 14 min in the standard assay medium without [\( \alpha-^32\)P]ATP. [\( \alpha-^32\)P]ATP (0.01 ml was then added and the adenylate cyclase reaction stopped 3 min later. Basal adenylate cyclase activity was 13.5 pmoles of cyclic AMP per min per mg of protein. ---, the apparent \( K_m \) value for adenylate cyclase activation by vasopressin (6.0 \( \times 10^{-8} \) m). For binding and adenylate cyclase measurements, enzyme concentration was 3.3 mg per ml.
small difference can be accounted for by the fact that the true equilibrium value is not reached in 15 min especially for the lowest hormone concentrations. In several systems where cyclic AMP accumulation was followed as a function of time after the addition of hormone, a linear relationship was obtained which crosses the time axis at zero. Such data, obtained for instance in the case of the glucagon-sensitive adenylate cyclase from liver plasma membranes (31, 32) or oxytocin-sensitive adenylate cyclase from frog bladder epithelial cells (22), was taken as evidence that cyclase activation is an almost instantaneous process. However, under these experimental conditions, lag time in the activation might not be detected, especially when high hormonal concentrations are used (see Fig. 11). When the adenylate cyclase activity was measured for 2-min periods as a function of time after the addition of hormone, it became clear that the activation is time-dependent. Since adenylate cyclase activation may reasonably be considered as a very early consequence of hormone-receptor interaction, the above observation may be taken as strong evidence in favour of the validity of the occupation theory in the system under study.

Usually dose-response curves of hormone-sensitive adenylate cyclase systems were determined from measurements of cyclic AMP accumulation during short periods of time following addition of the hormone to the incubation medium. Keeping in mind that for an hormonal concentration equal to the $K_m$, the half-time for hormonal binding (and adenylate cyclase activation) is given by $t_{1/2} = 0.69/t_{1/2}$, it is clear that in all systems in which $t_{1/2}$ is not negligible with respect to the duration of the incubation period, all experimental determinations of cyclase cyclase activation are in fact underestimated especially those corresponding to the lowest hormone concentrations. In the system under study as shown in Fig. 14, it is not possible to superimpose the adenylate cyclase dose-response curves determined during three periods of cyclic AMP production: from 0 to 3 min after the addition of the hormone, from 0 to 15 min after such addition, and from the 14th to the 17th min following the initial hormonal contact. With respect to the above discussion, the difference in shape between the dose-response curves for (O-Me)Tyr$^2$-oxytocin, vasopressin and oxytocin shown in Fig. 3 might reflect different time dependencies of binding for these three analogues, the dose-response curves being constructed from the measurement of cyclic AMP accumulation under nonequilibrium conditions (between 0 and 5 min after the addition of peptide to the incubation medium). Underestimation of cyclase activation by low hormone concentrations when the reaction's mean velocity is measured for a short period after adding the hormone helps to account for the difference in apparent sensitivity between the intact structure and broken cell preparations. Due to the great temperature dependency of the association and dissociation constants, it appears very likely that the half-time for binding at $37^\circ$ is greatly reduced and is thus compatible with the observation that the antidiuretic response of the intact animal appears almost instantaneously. In any case, data obtained on membrane fractions should be applied with caution to the functioning of the intact structure, since the properties of the adenylate cyclase system and possibly the number of accessible receptor sites may be altered by the preparation of membranes.

Comparison of the time course of $[RH]$ of vasopressin binding with the time course of adenylate cyclase activation (Fig. 10) suggests the existence of a linear relationship between receptor occupancy and response. However, this conclusion is valid only if one assumes that adenylate cyclase activation follows instantaneously the hormone-receptor interaction. In addition, in the experiment described only a small range of variation in receptor occupancy was explored. The first determination of adenylate cyclase activation between 0 and 2 min corresponded to a mean receptor occupancy of about 10% of maximal receptor capacity. The equilibrium value corresponded to about 30% of maximal receptor capacity. The equilibrium value corresponded to about 30% of maximal receptor capacity for the enzyme preparation tested.

In fact when adenylate cyclase activation was measured after 15 min incubation in the presence of hormone (an experimental situation which approached equilibrium state for hormonal binding), it is clear (Fig. 13) that the function relating cyclase activation to receptor occupancy is not a simple linear relationship.

Several explanations can be given for such nonlinear coupling, a concept originally introduced by Stephenson (33). They are:

1. The population of receptors is composed of several kinds of receptors which differ as regards their affinity for the hormone and their ability to generate a stimulus, the receptors with the highest affinity being more effectively coupled to the adenylate cyclase. If this is the case, the nonlinear coupling may result from partial denaturation during the enzyme preparation and have no physiological significance. However there is no experimental evidence suggesting heterogeneity of the receptor population (good semilogarithmic transformation of association and dissociation curves; Hill coefficient of binding curve at equilibrium = 1). They are:

   ![Fig. 14. Effect of measuring adenylate cyclase activation in nonequilibrium conditions on the apparent $K_m$ for vasopressin. The first and second dose adenylate cyclase activation curves were obtained by measuring cyclic AMP production between zero and 3 min (○), or zero and 15 min (△). The third curve (□) was obtained after 14 min preincubation of membranes with different vasopressin concentrations. $[\text{ATP}]$ was added (0.01 ml) at the end of preincubation time and adenylate cyclase reaction continued for 3 min. Enzyme concentration was 3.3 mg protein per ml. In the three experimental conditions, results are expressed in picomoles of cyclic AMP per min per mg of protein. Hill coefficients calculated for these three curves were respectively 0.75 (○); 0.52 (△); and 0.3 (□).](http://www.jbc.org/)

2. The nonlinear coupling is an intrinsic property of the system reflecting the molecular mechanisms involved in the activation. Among several possibilities, one can assume that the hormone-receptor interaction induces the generation of an intermediary active compound, whose liberation exhibits saturation kinetics toward $RH$. Alternatively, the Hill coefficient is $0.43 \pm 0.13$ ($n = 4$) for the dose-cyclase activation relationship at equilibrium suggests a negative cooperativity phenomenon as defined by
Koshland et al. (34, 35). If one assumes that the cyclase is a polymer of subunits containing one catalytic site and one vasopressin receptor, and that the transition between inactive and active conformations for the catalytic moiety can be induced either by receptor occupation or by transition of neighboring subunits, it is also possible to account for a nonlinear coupling as well.

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