Neurohypophyseal Hormone-responsive Renal Adenylate Cyclase

III. RELATIONSHIP BETWEEN AFFINITY AND INTRINSIC ACTIVITY IN NEUROHYPOPHYSEAL HORMONES AND STRUCTURAL ANALOGS*

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Binding and adenylate cyclase activation with neurohypophyseal hormones (NHH) and synthetic analogs were examined in two bovine renal medullary membrane preparations. The relationship between binding affinity of NHH and 10 analogs (as determined by competitive binding studies with tritiated [Lys]

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vasopressin, [3

H]LVP and adenylate cyclase activation was studied in one membrane preparation under identical conditions where hormonal stimulation of cyclase was at steady state and binding was at (or near) equilibrium. A linear relationship was demonstrated between the negative logs of peptide concentration required for half-maximal binding (K

D

) and for half-maximal enzyme activation (K

U

). This finding extends previous evidence indicating that: (a) the bovine membrane binding sites detected by [3

H]LVP have specificity characteristics expected for functional receptors coupled to adenylate cyclase, and (b) these analogs all occupy the same receptor “pocket.” Study of adenylate cyclase activation by NHH analogs, with and without a preliminary preincubation, showed that the magnitude of enzyme stimulation achieved (percentage of LVP, relative to LVP) with certain low affinity analogs was increased when these peptides were assayed without preincubation (in the 0- to 10-min period) without significant influence on K

D

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To maximize the magnitude of adenylate cyclase stimulation achieved with low affinity analogs, the relationship between the apparent affinity and intrinsic activity of 30 NHH analogs was studied in a second membrane, where adenylate cyclase was assayed without preincubation. It was found that the apparent affinity of NHH analogs may be decreased about 5 orders of magnitude with only minimal (20%) reduction in intrinsic activity. With further decrease in affinity to a critical K

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value, there is an abrupt decrease in intrinsic activity so that peptides with K

D

~ 2 x 10

4

M are either weak partial agonists or competitive antagonists.

Studies of [N'-acylated Lys]

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vasopressin analogs have revealed it is possible to introduce carboxyl functions in the Lys

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side chain with unexpectedly high retention of affinity; these analogs may therefore be useful intermediates for affinity chromatography of NHH receptors. Retro-11 analogs of [Gly]

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deaminooxytocin and deaminotocinamide, with a free NH

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group, were shown to be weak partial agonists; N-formylation, resulting in charge removal, led to loss of agonistic activity with development of antagonistic activity.

Structural differences between the bovine and porcine renal receptor have previously been established with respect to position 8 of the vasopressin molecule. The present studies indicate a second species difference, involving the NH

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terminus of the peptide. The NH

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terminus increases binding affinity in the porcine receptor but not in the bovine receptor where deaminopressinamide has higher affinity than pressinamide, and deaminooxytocin has higher affinity than oxytocin.

In a previous paper of this series (1), substantial evidence was presented that specific hormone binding sites in bovine renal medullary membranes appear to be functional neurohypophyseal hormone receptors coupled to adenylate cyclase. In the present studies, we have extended our peptide selectivity...
studies and describe the effects of 30 synthetic analogs of AVP and OT. These studies with NHH analogs have served to define the relationship between: (a) the apparent affinity for peptide binding to receptor sites and for adenylate cyclase activation; (b) affinity and intrinsic activity (percentage of maximum activation achieved by maximally stimulating concentrations of peptide); and (c) modification of peptide structure and activity in the NHH-responsive adenylyl cyclase system of bovine renal medullary membranes.

**EXPERIMENTAL PROCEDURES**

A radiochemically homogeneous sample of [³H]AVP (specific activity 24.2 Ci/mmol), prepared as described previously (21, was used in these experiments. The source of the unlabeled peptides (and their abbreviations) used in this study are listed in Table I. In this set of analogs, 19 represent previously described peptides whose antidiuretic activities have been reported. In addition, we tested 11 new analogs, including 5 N-acyl-Lys derivatives of LVP (synthesized in miniprint supplement) and 6 retro-o-analogs of Gly-LVP.

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**Table 1**

| Compound                        | Abbreviation | Code No. | Source |
|---------------------------------|--------------|----------|--------|
| [Arg]₁vasopressin               | AVP          | 1        | T.L.   |
| [Arg]₁vasotocin                 | AVT          | 2        | R.W.   |
| [Lys]₁vasopressin               | LVP          | 3        | T.L.   |
| Oxytocin                        | OT           | 4        | T.L.   |
| [Aminosuberic]ᵢ₋₄, Arg]₁vasopressin | Asu-AVP     | 5        | S.S.   |
| [Aminomalic]ᵢ₋₄, oxooxytocin    | Asu-OT       | 6        | S.S.   |
| Cyclo-b-Ala₃, Asp]₁oxoxytocin   | DesSS(CONH)OT | 7       | S.S.   |
| 1-Deaminooxytocin               | DeOT         | 8        | V.du.V.|
| Vasopressinamideᵢ₋₄ (pressaminamide) | P            | 9        | V.du.V.|
| 1-Deaminovasopressinamideᵢ₋₄ (deaminopressaminamide) | DeP       | 10       | V.du.V.|
| 1-Deaminooxytocinamideᵢ₋₄ (deaminotocinamide) | DeT       | 11       | T.L.   |
| [Ala]₁, Arg]₁vasopressin        | [Ala]₁AVP    | 12       | R.W.   |
| [Ala]₁oxoxytocin                | [Ala]₁OT     | 13       | R.W.   |
| [Tyr(Mo)]₁oxoxytocin            | [Mc Tyr]₁OT  | 14       | K.J.   |
| [Val]₁oxoxytocin                | [Val]₁OT     | 15       | R.W.   |
| [d-Leu]₁oxoxytocin              | [d-Leu]₁OT   | 16       | R.W.   |
| [Ala]₁oxycin                   | [Ala]₁OT     | 17       | R.W.   |
| [Val]₁oxycin                   | [Val]₁OT     | 18       | R.W.   |
| [Gly]₁oxycin                   | [Gly]₁OT     | 19       | R.W.   |
| [Arg]₁vasopressinoic acid       | AVP acid     | 20       | R.W.   |
| 1-Deaminovasopressinoic acid    | DeOT acid    | 21       | R.W.   |
| [Des-Leu]-glycinamide]₁oxoxytocin | [Des-Leu-Gly-NH₄]₁OT | 22 | R.W. |
| [Des-glycinamide]₁oxoxytocin   | [Des]-Gly₁OT| 23       | R.W.   |
| [N]-succinamyl-Lys]₁vasopressin | [N]-Succinyl]₁LVP | 24 | T.L. |
| [N]-N-carboxy-Lys]₁vasopressin  | [N]-N-Carboxy]₁LVP | 25 | T.L. |
| [N]-glycyl-Lys]₁vasopressin     | [N]-Gly]₁LVP | 26       | T.L.   |
| [N]-biotinyl-Lys]₁vasopressin   | [N]-Biotinyl]₁LVP | 27 | T.L. |
| [N]-fluoresceinyl-thiocarbamyl-Lys]₁vasopressin | [N]-Fluoresceinyl]₁LVP | 28 | T.L. |
| [o-alle]₁-retro-o-tocinamide    | Retro-o-T    | 29       | T.L.   |
| N-Formyl]-o-alle]₁-retro-o-tocinamide | N-Formyl-retro-o-T | 30 | T.L. |
| [o-alle]₁, Gly]₁-retro-o-deaminoxytocin | Gly₁-retro-o-DeOT | 31 | T.L. |
| [o-alle]₁, Gly]₁-1-Leu]₁-retro-o-deaminoxytocin | [Gly]₁-1-Leu]₁-retro-o-DeOT | 32 | T.L. |
| N-Formyl]-o-alle]₁, Gly]₁-retro-o-deaminoxytocin | N-Formyl]-Gly]₁-retro-o-DeOT | 33 | T.L. |
| [o-alle]₁, Gly]₁, mulsaminamide]₁-retro-o-deaminoxytocin | Gly₁, Mal₁-retro-o-DeOT | 34 | T.L. |

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1 Abbreviations for the various hormones and analogs used in this study are shown in Table 1. DeOT and DeT (4) which had shown only minimal biological activity in the rat uterotonic (5).

2 The synthesis of [N-acyl-Lys]₁vasopressins is presented in miniprint at the end of this paper. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 775-75, cite author(s), and include a check or money order for $1.00 per set of photocopies.

3 Two different membranes (Preparations 5 and 6) each derived from 80 g of heifer renal medulla were used in these studies. Kᵢ values for a group of unlabeled peptide analogs were estimated by study of competitive binding in Preparation 5 using a fixed concentration of [³H]LVP (1.5 x 10⁻⁶ M, approximately equivalent to the Kᵢ value) and varying the concentration of unlabeled peptides, as previously described (1). With OT analogs of very low apparent affinity and intrinsic activity in the NHH-responsive adenylyl cyclase system of bovine renal medullary membranes, it was likewise determined under both conditions, and is designed as percentage of V₅₀ (II) or percentage of V₅₀ (I), respectively, (relative to AVP or LVP designated as 100%).
Affinity and Intrinsic Activity/Neurohypophyseal Hormones

Table II

Characteristics of interaction of [3H]LVP with two membrane preparations used in present study

Membrane Preparations 5 and 6 (40 to 60 μg of protein) were incubated in 50 μl of standard medium with [3H]LVP concentrations ranging from 5 × 10⁻⁶ to 10⁻⁷ M. Specific [3H]LVP binding was corrected for nonspecific binding and NHH-responsive adenylate cyclase activity was corrected for basal activity. V₅₀(II) and (II), Kₛ(II) and (II), Bₕₕₜ, and Kₛ were determined as described under "Methods." The values shown represent the mean of three experiments with each membrane. The apparent Hill coefficients for [3H]LVP binding (nₑ(B)) in both membranes were close to unity; the relationship between hormone concentration and enzyme activity in these membranes had apparent Hill coefficients (nₑ(A)) which do not provide significant evidence for negative cooperativity. (The apparent Hill coefficients for enzyme activation (nₑ(A)) statistically are not significantly different than 1.0.) The membrane preparations were similar with respect to [3H]LVP binding characteristics in terms of the number of binding sites and their affinity for [3H]LVP, but differed with respect to the magnitude of adenylate cyclase activation achieved by maximally stimulating concentrations of [3H]LVP.

| Membrane preparation | [3H]LVP binding | Adenylate cyclase activation |
|----------------------|-----------------|----------------------------|
|                      | Bₕₕₜ | Kₛ | nₑ(B) | 12½/min preincubation | No preincubation |
|                      | pmol/mg | nM |       | Kₛ(II) | V₅₀(II) | nₑ(A) | Kₛ(II) | V₅₀(II) |
| 5                    | 3.4  | 8.1 | 1.0   | 2.7    | 80     | 0.87   | 3.6    | 83     |
| 6                    | 3.6  | 7.8 | 0.96  | 2.3    | 112    | 0.88   | 3.4    | 115    |

Table III

Relationship between specific binding and adenylate cyclase activation under "steady state" and "non-steady state" conditions

Membrane Preparation 6 (30 μg of protein/μl of standard medium) was incubated with various concentrations of NHH and analogs, with and without preliminary 12½/min preincubation, as described under "Methods." In each individual experiment, LVP (or AVP, or both) as well as OT were used as standards to measure affinity and intrinsic activity. The values shown for Kₛ(II) and (II), and V₅₀(II) and (II), are the mean of at least three separate determinations for each NHH analog. The Kₛ values of the peptides determined by competitive binding with [3H]LVP (as described under "Methods") are the mean of at least two experiments with each analog.

| Compound               | Specific binding Kₛ | Adenylate cyclase activation | Kₛ/Kₛ(II) |
|------------------------|---------------------|------------------------------|-----------|
|                        | M M                | M M                          | % %       | % %       |
| AVP                    | 4.4 × 10⁻⁶ 1.0 × 10⁻⁶ | 100 5.0 × 10⁻⁶ 100 | 4.4       |
| LVP                    | 8.1 × 10⁻⁶ 2.1 × 10⁻⁶ | 100 8.9 × 10⁻⁶ 100 | 3.9       |
| Asu-AVP                | 1.4 × 10⁻⁶ 4.0 × 10⁻⁶ | 100 1.9 × 10⁻⁶ 100 | 3.5       |
| AVP acid               | 4.0 × 10⁻⁵ 1.2 × 10⁻⁵ | 90 2.5 × 10⁻⁵ 85-100 | 3.3       |
| DeP                    | 6.0 × 10⁻⁶ 2.1 × 10⁻⁶ | 55 3.2 × 10⁻⁶ 85-100 | 2.9       |
| [N¹-Gly]LVP            | 3.6 × 10⁻⁸ 9.6 × 10⁻⁸ | 100 1.1 × 10⁻⁸ 100 | 3.7       |
| [N¹-fluoresceinyl]LVP  | 3.0 × 10⁻⁷ 1.6 × 10⁻⁷ | 100 1.6 × 10⁻⁷ 100 | 1.9       |
| AVT                    | 1.3 × 10⁻⁶ 3.2 × 10⁻⁶ | 100 1.4 × 10⁻⁹ 100 | 4.2       |
| OT                     | 2.0 × 10⁻⁷ 5.0 × 10⁻⁸ | 100 5.3 × 10⁻⁸ 100 | 4.0       |
| DeOT                   | 1.0 × 10⁻⁷ 3.2 × 10⁻⁸ | 100 2.0 × 10⁻⁸ 100 | 3.1       |
| Asu-OT                 | 2.1 × 10⁻⁷ 8.0 × 10⁻⁸ | 90 1.1 × 10⁻⁷ 100 | 2.5       |
| [V¹-H]OT               | 5.0 × 10⁻⁷ 1.2 × 10⁻⁷ | 65 2.7 × 10⁻⁷ 100 | 4.0       |
| [V¹-Gly]OT             | 5.2 × 10⁻⁷ 2.0 × 10⁻⁷ | 50 3.5 × 10⁻⁷ 85-100 | 1.7       |
| DeT                    | 2.0 × 10⁻⁵ 8.7 × 10⁻⁶ | 50 5.2 × 10⁻⁶ 85-100 | 2.3       |
| [Gly¹]retro-n-DeOT     | ~10⁻⁴ 2 × 10⁻⁴ | 23-32 |

affinity (Kₛ ~ 10⁻⁴ M), it was not possible to estimate low Kₛ values by the competitive binding technique, because at very high peptide concentrations (>10⁻⁴ M), "inactive" peptides (e.g., insulin, angiotensin, glucagon) produce a nonspecific inhibition of [3H]LVP binding (1). With some low affinity partial agonists, or competitive inhibitors, or both, used in this study it was, however, possible to obtain an estimate of binding affinity using the method of Schild (6) to obtain values for Kₛ. For both adenylate cyclase and binding determinations, several analogs were routinely tested in the same experiment, together with LVP (or AVP) and OT which were routinely used as standard reference compounds. The characteristics of [3H]LVP interaction with the two membranes used in this study were defined in terms of [3H]LVP binding parameters (Bₕₕₜ, Kₛ, and the Hill coefficient, nₑ(B)) as well as for adenylate cyclase activation (Kₛ, V₅₀, and the Hill coefficient, nₑ(A)) (Table II). In membrane Preparation 5, 4 NHH and synthetic analogs were assayed for competitive binding activity and adenylate cyclase activation with and without preliminary 12½-min preincubation. The intrinsic activity (percentage of V₅₀ relative to LVP) of several low affinity analogs was found to be significantly greater when the peptide was assayed without preincubation relative to the results obtained with preincubation. Accordingly in membrane Preparation 6, all analogs were assayed without preincubation to maximize the magnitude of adenylate cyclase stimulation achieved with low affinity peptide analogs.

RESULTS

Relationship between Peptide Binding and Adenylate Cyclase Activation under Steady State Conditions — The results with Membrane 5 are shown in Table III and Fig. 1. Fig. 1 shows the relationship between the negative logarithms of Kₛ and Kₛ(II) with NHH and a set of analogs, which differs in apparent affinity by more than 5 orders of magnitude. The linear relationship observed provides further evidence that the specific sites in bovine membranes are receptors coupled to adenylate cyclase. There is no straightforward relationship between affinity (1/Kₛ) and intrinsic activity, in the set of
Table IV
Comparison of OT, DeOT, and DeT on adenylate cyclase activation

In six experiments, the activation of NHH-responsive adenylate cyclase by OT, DeOT, and DeT was studied with membrane preparation 6 (40 to 50 μg of membrane protein/50 μl of standard medium) during the 0- to 10-min period of incubation. DeOT consistently had higher apparent affinity for enzyme activation than OT. The intrinsic activity (V_max(II)) of DeT varied from 85 to 100% (relative to OT = 100%), whereas with 12½-min preincubation DeT intrinsic activity (V_max(III)) was only about 50%.

| Experiment | OT | DeOT | DeT |
|------------|----|------|-----|
|            | K_0 (M) | V_max (1) | K_0 (M) | V_max (1) | K_0 (M) | V_max (1) | Affinity of DeT relative to OT |
| 1          | 3.1 × 10^{-8} (100) | 1.0 × 10^{-4} (105) | 2.3 × 10^{-4} (85) | 1.3 | 0.45 |
| 2          | 3.7 × 10^{-8} (100) | 1.2 × 10^{-9} (102) | 3.6 × 10^{-9} (100) | 1.0 | 0.44 |
| 3          | 4.6 × 10^{-8} (100) | 1.5 × 10^{-9} (100) | 6.0 × 10^{-9} (91) | 0.69 | 0.25 |
| 4          | 2.1 × 10^{-8} (100) | 1.5 × 10^{-9} (100) | 6.0 × 10^{-9} (91) | 0.69 | 0.25 |
| 5          | 3.7 × 10^{-8} (100) | 2.0 × 10^{-8} (100) | 6.0 × 10^{-8} (91) | 0.69 | 0.25 |
| 6          | 3.0 × 10^{-8} (100) | 2.0 × 10^{-8} (100) | 6.0 × 10^{-8} (91) | 0.69 | 0.25 |

Fig. 1. The relationship between the negative logarithms of K_0 and K_0 (II) for NHH and 10 peptide analogs. The K_0 values for NHH analogs (cf. numerical code in Table I) were determined by competitive binding studies with a fixed concentration of [H-LVP (1.3 × 10^{-9} M) as described under Methods. The K_0 (II) values for NHH and analogs were determined by measuring adenylate cyclase activation in the time period 12½ to 17½ min of incubation. The results shown are the mean values of two (or more) individual determinations in membrane Preparation 5.

peptides shown in Table III. Thus, DeP, DeT, and AVP acid have low apparent affinities associated with decreased percentage of V_max, relative to AVP, whereas [Gly^7]OT with even lower apparent affinity has higher intrinsic activity than these three analogs. It was not possible to obtain a K_0 value for the retro-n analog of [Gly^7]DeOT, which has the lowest apparent affinity and intrinsic activity in the series shown in Table III. Consistent with previous studies (1) using four other bovine membranes, adenylate cyclase activation with either NHH or analogs in this study did not exhibit significant negative cooperativity.

Adenylate Cyclase Activation with and without Preincubation - As first shown by Dockeart et al. (7), the sensitivity of porcine renal medullary adenylate cyclase to vasopressin is increased when membranes are assayed using a preincubation period to eliminate the lag periods in enzyme activation observed with low concentrations of hormone; however, with low affinity analogs, only minimal differences in K_0 were observed when porcine membranes were assayed with or without preincubation (7-9). A similar relationship was obtained in the bovine membrane system. Table III shows that with several partial agonists (DeP, DeT, and [Gly^7]retro-n-DeOT) intrinsic activity is increased when membranes are assayed without preincubation. Table IV illustrates this point with DeT; when assayed in six experiments without preincubation, the V_max(I) of DeT ranged from 85 to 100% (relative to AVP or OT), whereas with preincubation, the mean V_max(I) value for DeT was about 50% (range 45 to 56% in five experiments). This change in percentage of V_max with low affinity peptide analogs, results from a secondary decrease in enzyme velocity which occurs after about 10 min incubation with the very high concentrations of these peptides required to achieve maximal enzyme stimulation (Fig. 2). Whereas the enzyme activation produced...
TABLE V

Adenylate cyclase activation by NHH and 19 synthetic analogs determined under “non-steady state” conditions

Various concentrations of peptide analogs (up to $10^{-6}$ M) were tested in membrane Preparation 6 (40 to 60 nM of protein/50 pl of medium), without preliminary preincubation. The values shown for $V_{max}$ (I) and $K_i$ (I) are the mean of at least three log peptide concentration-adenylate cyclase activation curves (subtracting basal activity) for each analog.

| Compound          | Percentage of $V_{max}$ (I) relative to AVP | $K_i$ (I) | $K_i$ |
|-------------------|---------------------------------------------|-----------|-------|
| AVP               | 100                                        | $5.1 \times 10^{-7}$ |       |
| AVT               | 100                                        | $3.4 \times 10^{-7}$ |       |
| LVP               | 100                                        | $9.5 \times 10^{-8}$ |       |
| OT                | 100                                        | $2.2 \times 10^{-5}$ |       |
| Ass-OT            | 90-95                                      | $2.1 \times 10^{-8}$ | 90    |
| Adj-CONH/OT       | 80                                         | $2.4 \times 10^{-8}$ |       |
| DeOT              | 80                                         | $2.1 \times 10^{-8}$ |       |
| P                 | 80-100                                     | $4.5 \times 10^{-6}$ | 50    |
| DeP               | 80-100                                     | $3.0 \times 10^{-6}$ |       |
| DeT               | 80-100                                     | $5.2 \times 10^{-6}$ |       |
| [Ala$^1$-AVP]     | 100                                        | $5 \times 10^{-5}$ |       |
| [Ala$^1$-OT]      | 25-30                                      | $5 \times 10^{-4}$ |       |
| [OMe-Tyr$^1$]-OT  | 100                                        | $1 \times 10^{-4}$ |       |
| [Val$^1$]-OT      | 100                                        | $3 \times 10^{-7}$ |       |
| [n-Leu$^1$]-OT    | 56                                         | $9.8 \times 10^{-4}$ |       |
| [Val$^1$]-OT      | 56                                         | $3.9 \times 10^{-4}$ |       |
| [Gly$^1$]-OT      | 47                                         | $2.8 \times 10^{-4}$ |       |
| [Gly$^1$]-OT      | 90-100                                     | $3.0 \times 10^{-3}$ |       |
| AVP acid          | 90                                         | $3 \times 10^{-6}$ |       |
| DeOT acid         | 90                                         | $2.5 \times 10^{-5}$ |       |
| [Des-Leu-GlyNH$_2$]-OT | 100                        | $4.6 \times 10^{-6}$ |       |
| [Des-GlyNH$_3$]   | 75                                         | $8.2 \times 10^{-5}$ |       |

TABLE VI

Attachment of residues to position 8 of LVP (via $\epsilon$-NH$_2$ group of Lys$^8$)

Various N$^\alpha$-acylated Lys$^8$ derivatives of LVP were compared in membrane Preparation 6, without preliminary preincubation. The values shown for $V_{max}$ (I) and $K_i$ (I) are the mean of at least two experiments, where the analog and LVP were compared in the same experiment.

| Residue        | Charge on tail | Adenylate cyclase activation | Affinity relative to LVP |
|----------------|----------------|-----------------------------|--------------------------|
|                | $V_{max}$ (I)  | $K_i$ (I)                   | $V_{max}$ (I) relative to LVP (%) |
| None (LVP)     | +              | 100                         | 3.0 $\times 10^{-5}$     |
| N$^\alpha$-Glycyl | (+)           | 100                         | 1.0 $\times 10^{-5}$     |
| None (LVP)     |                | 100                         | 1.3 $\times 10^{-5}$     |
| N$^\beta$-Succinamyl | (0)         | 100                         | 7.6 $\times 10^{-5}$     |
| N$^\gamma$-Succinyl | (-)         | 100                         | 2.7 $\times 10^{-5}$     |
| None (LVP)     |                | 100                         | 4.0 $\times 10^{-5}$     |
| N$^\delta$-Biotinyl | (0)          | 100                         | 4.5 $\times 10^{-5}$     |
| None (LVP)     |                | 100                         | 2.0 $\times 10^{-5}$     |
| N$^\gamma$-Fluorecencyl | (+ & -)   | 100                         | 1.6 $\times 10^{-7}$     |

by OT ($10^{-5}$ M) is linear over the 20-min time interval studied, the initial rate with DeT ($10^{-4}$ M) over the first 10 min is about 90% of that achieved with OT, while enzyme velocity in the 12.5- to 17.5-min period is about 50% of that achieved with OT. With the retro-D analog of DeOT ($10^{-2}$ M), enzyme velocity is initially about 32% of OT and then declines during the 12.5- to 17.5-min interval to about 16% of the rate observed with OT. These temporal changes in velocity of enzyme activation may be related to the “self-inhibitory” effects described for certain NHH analogs in the pig membrane system (8, 9) which we have also observed in bovine membranes (3).

TABLE VII

Effect of retro-D analogs of DeT and [Gly$^1$]-DeOT on adenylate cyclase activation

Various retro-D analogs of DeT and [Gly$^1$]-DeOT were studied in membrane Preparation 6, without preliminary incubation. The values shown are the mean of at least three experiments with each of the retro-D analogs tested relative to the standards (DeOT, DeT, and [Gly$^1$]-OT) used in these experiments.

| Charge         | Compound      | $V_{max}$ (I) relative to OT = 100% | $K_i$ (I) | $K_i$ |
|----------------|---------------|-------------------------------------|-----------|-------|
| None           | DeOT          | 100                                 | $2.1 \times 10^{-6}$ |
| NH$_2$*        | [Gly$^1$]-OT  | 100                                 | $1.4 \times 10^{-6}$ |
| None           | DeT           | 100                                 | $3 \times 10^{-6}$   |
| NH$_2$*        | Retro-D-T     | 100                                 | $2 \times 10^{-6}$   |
| None           | N-Formyl-retro-D-T | 0           | $2 \times 10^{-6}$   |
| NH$_2$*        | [Gly$^1$]-retro-D-DeOT | 100          | $2 \times 10^{-6}$   |
| None           | N-Formyl [Gly$^1$]-retro-D-DeOT | 100          | $2 \times 10^{-6}$   |
| None           | [Gly$^1$]-Mal$^1$-retro-D-DeOT | 100          | $2 \times 10^{-6}$   |

TABLE VIII

Comparison of P, DeP, and DeT

Cyclic hexapeptide amides were compared in membrane Preparation 6, without preliminary incubation. The values shown are the mean of three experiments where all cyclic analogs were tested simultaneously with nonapeptide standards (AVP and DeOT).

| Compounds    | Adenylate cyclase activation | Affinity of cyclic ring relative to |
|--------------|------------------------------|------------------------------------|
|              | $K_i$ (I)                   | $V_{max}$ (I) | AVP | DeOT | DeT |
| AVP          | $4.3 \times 10^{-6}$       | 100          | 100 |
| P            | $6.8 \times 10^{-6}$       | 99           | 99  | 0.014|
| DeP          | $2.5 \times 10^{-6}$       | 88           | 99  | 0.017|
| DeOT         | $2.6 \times 10^{-6}$       | 100          | 100 |
| DeT          | $3.9 \times 10^{-6}$       | 100          | 0.29|
relationship between apparent affinity (1/Kᵣ(II) or 1/Kᵣ) and intrinsic activity (percentage of V_max relative to AVP) in this series of 30 analogs and 4 NHH.

**DISCUSSION**

Bovine renal medullary membranes were previously shown to have specific hormone binding sites which behave as a homogeneous population of molecules with a single affinity constant for tritiated LVP or AVP (1). The present studies with NHH analogs provide strong additional evidence that the specific [³H]LVP binding sites in bovine membranes have peptide specificity characteristics expected for functional NHH-receptors coupled to adenylate cyclase. Thus, a linear relationship was demonstrated between the negative logarithm of Kᵣ and Kᵣ with four naturally occurring NHH, and a large number of analogs which vary over more than 5 orders of magnitude in apparent affinity (Fig. 1). This finding which extends previous studies, provides strong evidence that all of these peptides occupy the same "pocket" in a single class of receptor molecules, as discussed previously (1).

The present study extends differences previously noted between bovine (1) and porcine (7-10) renal medullary adenylate cyclase systems, with respect to Kᵣ/Kᵣ ratios and Hill coefficients for enzyme activation (nᵢ(A)) observed with NHH. In the present studies with bovine membranes, the Kᵣ/Kᵣ(II) ratios with AVP, AVT, LVP, and OT were in the same range (4.4 to 3.9); the ratios of Kᵣ/Kᵣ(II) decreased below 2 only with certain low affinity analogs (e.g. [Gly]²OT, [N²-fluorescein]²[LVP]. The peptide concentration-adenylate cyclase activation curves with NHH and analogs in bovine membranes had nᵢ(A) values which statistically were not significantly different than 1.0. In porcine membranes the LVP concentration-enzyme activation curves in porcine membranes has nᵢ(A) values about 0.3, indicative of significant negative cooperativity; with other NHH and analogs there was a tendency for apparent negative cooperativity of activation to decrease as the apparent affinity for adenylate cyclase activation decreased, but there was no strict correlation between these parameters. The Kᵣ/Kᵣ ratios obtained with NHH and analogs in pig membranes appeared to be correlated with nᵢ(A), the greater the degree of apparent negative cooperativity the higher the Kᵣ/Kᵣ ratio (10). Thus in porcine membranes, the reported Kᵣ/Kᵣ ratio was about 50:1 with LVP, while the ratios with AVP and OT were about 9:1 and 1:5:1, respectively; analogs of lower affinity than OT had Kᵣ/Kᵣ ratios approaching 1:1. As previously discussed (1) the basis for the differences in apparent negative cooperativity of hormonal activation observed between porcine and bovine membranes remains to be elucidated.

In the present study, we have been able to define the relationship between the apparent affinity of a peptide analog for receptor binding and its intrinsic activity to stimulate adenylate cyclase in bovine membranes. The Kᵣ values of "very low" affinity analogs (wherein Kᵣ could not be measured from competitive binding studies with [³H]LVP) was approximated by taking advantage of the fact that: (i) the negative log of Kᵣ is linearly related to the negative log Kᵣ(II) (Fig. 1) and that (ii) with low affinity analogs the Kᵣ values for adenylate cyclase activation assayed with or without preincubation are very similar (Table III). Using 1/Kᵣ(II) as a measure of relative affinity, it was demonstrated that a progressive decrease in apparent affinity over about 5 orders of magnitude results in only a minor reduction in intrinsic activity, V_max being 80% or greater, of that achieved with AVP or LVP (Fig. 3). However, further decrease in peptide affinity to a critical region (to Kᵣ(II) or Kᵣ values about 2 to 2.5 x 10⁻⁸ M) results in a dramatic decrease in intrinsic activity, so that peptides in this affinity range are either weak partial agonists (with V_max 10 to 50% relative to AVP) or competitive antagonists. Previous studies (1) have shown that the decrease in affinity of LVP relative to AVP is due to two factors: (a) a decrease in the kinetic association constant (kᵣ), which is associated with (b) an equivalent increase in the kinetic dissociation constant (kᵩ). While we cannot be certain that kᵣ and kᵩ are equivalently modified in all peptide analogs, as a first approximation we may assume that for 2 orders of magnitude of change in Kᵣ (equivalent to Kᵣ = kᵩ/kᵣ) kᵩ is decreased and kᵩ increased by about 1 order or magnitude. Since kᵩ for a given peptide is inversely related to the mean time that a peptide molecule occupies a receptor site, Fig. 3 indicates that peptides with very short occupancy times relative to AVP (about 2 orders of magnitude less than AVP) are able to activate most of the cyclase units in the membrane. Further decrease in affinity appears to lead to shortened mean occupancy time, insufficient to permit full activation of enzyme units, thus low affinity analogs of this type (e.g. [Ala]²OT, [Val]²OT, [Ala]²OT, and some retro-n analogs) are weak partial agonists. In the case of other analogs ([d-Leu]²OT and formylated retro-n analogs) kᵩ may be increased to the point where effective occupancy time is insufficient to permit enzyme activation, so that such analogs are pure competitive antagonists. In examining Fig. 3, three peptides, [Ala]²VP, [Gly]²OT, and [Des-Leu-Gly-NH₂]²OT, appear to have higher intrinsic activity than is expected for their relatively low affinity, suggesting that the modification of structure in these peptides may influence kinetic parameters differentially. Thus, the low affinity of [Gly]²OT and [Ala]²VP may be primarily related to low kᵩ values for association, rather than to relatively high kᵩ values. If this view is correct, Tyr² and...
Pro is important to initial entry and "fit" in the receptor pocket; once "in," these groups may be less important for continued occupancy.

With respect to the structural requirements for binding to the bovine receptor and its subsequent activation leading to stimulation of adenylate cyclase activity, the following main conclusions can be drawn: (i) our studies with analogs with amino acid substitutions or deletions have tended to confirm or extend previous structure-activity relationships (11, 12) which have shown that no single amino acid residue in the nonapeptide sequence of NHH is essential for biological activity in the kidney. The elements of the tripeptide tail provide "attachment" sites to enhance peptide affinity to receptors but are not required for activation of adenylate cyclase (Table VIII). A combination of elements of the hexapeptide ring cooperate to establish biological activity; of these the carboxamide function of Asn and the aromatic moiety of Tyr in the ring appear to play "critical" roles (5, 11, 12). (ii) The present study demonstrates structural differences in the bovine and porcine receptor with respect to position 1 as well as position 8 of the vasopressins, where LVP has greater affinity than AVP for the porcine renal receptor, whereas the reverse is true for the renal receptors of almost all other mammalian species (7, 13). Thus, the NH₂ terminus of NHH contributes positively to peptide binding with the pig receptor (8, 9) but has an opposite effect with the bovine receptor, where DeP has higher affinity than P (Table IV) and DeOT higher affinity than OT (Table VIII). (iii) It has been generally assumed that the basicity of position 8 is important for high antidiuretic potency in the NHH series (11, 12) and that the presence of a carboxyl group in the tripeptide tail results in a marked reduction in affinity (14). In the bovine membrane system, AVP acid and OT acid have affinity reduced to a point where the contribution of the tail to high affinity binding is eliminated (Table V); thus these COOH-terminal carboxyl analogs have apparent affinity essentially equivalent to the hexapeptide cyclic ring moieties. With N-acylated Lys derivatives of LVP (Table VI), it is possible to displace the NH₂ group of the Lys side chain by 3 atoms (as in [N-glycyl-Lys]VP) with only minimal reduction in affinity. It is also possible to introduce a COOH group into the lysine side chain (8 atoms (carbon or nitrogen) from the backbone) with only minimal effect on the contribution of the latter to the affinity parameter. Thus [N'-succinyl-Lys]VP which contains a carboxyl group retains 30% of the affinity of [N'-succinamyl-Lys]VP, a group of approximately similar size, without a negative charge. The latter observations suggest that N-acylated Lys derivatives of LVP may be useful intermediates for affinity chromatography studies with NHH receptors. The finding that [N'-fluoresceinyl-Lys]VP has markedly reduced affinity relative to the N'-succinamyl derivative may be the consequence of steric effects of the bulky fluoresceinyl group as well as its charge characteristics. (iv) The present studies (Table VII) clearly show that retro-β analogs occupy NHH receptor sites (albeit with very low affinity) and act as weak competitive inhibitors or partial agonists (with Kᵣ or Kᵅ in the neighborhood of ~10⁻⁴ M). The retro-β analogs of Gly¹DeOT and DeT (with terminal NH₂ charge groups) are weak partial agonists; formylation of the NH₂ group in these retro-β peptides results in compounds which exhibit activity only as competitive antagonists. The presence of a positive charge in these retro-β peptides may provide for an electrostatic interaction with receptors which serves to increase occupancy time (relative to the neutral formylated retro-β analogs), so that partial activation of adenylate cyclase elements is possible. The malonamide retro-β derivative of Gly¹DeOT, which has no charge had only about 10% of the intrinsic activity of Gly¹OT.

The present structure-activity results are consistent with, but do not establish our previous proposal that occupation and activation of NHH receptors is the resultant of multiple hydrogen-bonding interactions between CO, NH, NH₂, and OH groups of NHH, with complementary groups on the receptor which are made possible because of appropriate hydrophobic bonding (4). The idea that CO and NH elements of the peptide backbone, as well as the topochemistry of amino acid side chains play a role in receptor binding and activation remains as an attractive possibility, whose validity must await studies with highly purified receptors.

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Additional references will be found on p. 3237.

Because retro-β analogs had little or no biological activity either as agonists or antagonists in the uterotonic assay, we had suggested that CO and NH elements of the peptide backbone play a fundamental role in NHH action, while recognizing that there are alternative explanations for the findings with retro-β compounds. Thus, we pointed out that in the retro-β configuration, there are possible differences in steric compression of peptide bond elements about the α carbon atoms which may distort topochemical arrangement of amino acid side chains, so that conformational changes of the peptide required for "fit" at the receptor site are more difficult (5). Attempts to study this issue by conformational analysis of retro-β analogs (15) have only shown that the retro-β ring has a β turn similar to the L-peptide; it was not possible to establish similarity of other elements of the molecule because in solution the conformation of the L or D molecule is not fixed unambiguously.
Affinity and Intrinsic Activity of Neurohypophyseal Hormones

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The affinities and intrinsic activities of the neurohypophyseal hormones were determined using the technique of ligand displacement. The affinities of the hormones were determined by measuring the concentration of hormone required to displace a constant amount of a radiolabeled hormone from its receptor. The intrinsic activities of the hormones were determined by measuring the concentration of hormone required to produce a constant effect on a functional assay. The results showed that the hormones differed in their affinities and intrinsic activities, with some hormones being more potent than others. The results also showed that the affinities and intrinsic activities of the hormones were dependent on their chemical structure.
Neurohypophyseal hormone-responsive renal adenylate cyclase. III. Relationship between affinity and intrinsic activity in neurohypophyseal hormones and structural analogs.
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J. Biol. Chem. 1978, 253:3230-3237.

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