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Neurohypophyseal Hormone-Responsive Adenylate Cyclase from Mammalian Kidney

(arginine-vasopressin/lysine-vasopressin/oxytocin/analogs/hormone receptor)

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ABSTRACT **The investigation was undertaken to evaluate the direct stimulatory effects of neurohypophyseal hormones upon adenylate cyclase activity in a cell-free, particulate fraction derived from the kidney medulla of various mammalian species. The relative affinity of neurohypophyseal hormones for the receptor component of the adenylate cyclase system (as defined by the concentration of hormone required for half-maximal stimulation) had the order [8-arginine]-vasopressin > [8-lysine]-vasopressin ≫ oxytocin (AVP > LVP ≫ OT) for rat, mouse, rabbit, and ox; in the pig, the order was LVP > AVP ≫ OT. The relative affinities of the three hormones in rat and pig cyclase systems were found to correspond with the relative antidiuretic potencies of these hormones in the intact rat and pig. These findings show that the renal receptor for neurohypophyseal hormones in a particular species exhibits the highest affinity for the specific antidiuretic hormone that occurs naturally in that species.**

Some of the molecular requirements for the stimulation of rabbit adenylate cyclase were defined by studies of several neurohypophyseal analogs possessing structural changes in positions 1, 2, 3, 4, 5, 8, and 9.

This investigation introduces the particulate preparation of renal medullary adenylate cyclase as a tool for the analysis of neurohypophyseal hormone-receptor interactions and indicates that this preparation can be adapted to serve as an *in vitro* bioassay system for antidiuretic hormonal activity.

The mammalian antidiuretic hormones (vasopressins) promote water reabsorption from the nephron by increasing the permeability to water of the distal convoluted tubules and collecting ducts (1, 2). There is substantial evidence that vasopressins and related analogs act via the adenylate cyclase–cyclic AMP system in the mammalian kidney as well as in amphibian membranes, namely, (*a*) 3′:5′-cyclic AMP mimics the effects of vasopressin upon isolated kidney tubules (2) and amphibian urinary bladders (3); (*b*) neurohypophyseal hormones stimulate adenylate cyclase in membrane preparations derived from renal medulla of dog (4), rat (5–7), mouse (6), rabbit (6, 8–10), and hamster (11), and from the epithelium of toad bladder (12, 13); and (*c*) the stimulation of toad bladder adenylate cyclase effected by a set of neurohypophyseal peptides can be correlated with the hydroosmotic response evoked by these peptides in the intact bladder (12).

Abbreviations: AVP, [8-arginine]-vasopressin; LVP, [8-lysine]-vasopressin. Neurohypophyseal hormones are denoted in accordance with the IUPAC–IUB [*Tentative Rules* (*Biochemistry*, 6, 362 (1967)].

The availability of adenylate cyclase preparations from renal medulla makes it possible to study vasopressin action in a cell-free system, with the elimination of many of the factors (14) that complicate the bioassay of antidiuretic activity in the whole animal. This paper presents: (*a*) properties of particulate vasopressin-sensitive preparations of adenylate cyclase from the renal medulla of rat, mouse, rabbit, ox, and pig; (*b*) a comparison of the relative stimulatory effects on these cyclase systems of the natural mammalian neurohypophyseal hormones, [8-arginine]-vasopressin (AVP), [8-lysine]-vasopressin (LVP), and oxytocin; (*c*) a comparison of the relative stimulatory effects of AVP, LVP, and oxytocin on the renal medullary cyclase systems of rat and pig with the relative antidiuretic activities measured *in vivo* in these species; and (*d*) a detailed study of the chemical structural requirements for hormonal stimulation of a rabbit renal medullary cyclase preparation.

METHODS

Preparation of adenylate cyclase

Renal medullary adenylate cyclases were prepared as previously described (8) from rats, rabbits, and mice. Kidneys from oxen and domesticated pig, obtained at the slaughter house and transported in ice to the laboratory, were prepared in a similar manner. Medullary tissue dissected free of renal cortex was minced and homogenized in a medium of the following composition: 0.25 M sucrose, 5 mM Tris·HCl (pH 7.5), 3 mM $MgCl_2$, and 1 mM EDTA (for rat, rabbit, and mouse preparations the medullary tissue of three animals was pooled). After filtration through gauze, the homogenate was centrifuged at 600 × *g* for 10 min. The 600 × *g* pellet was washed twice (in a sucrose-free medium otherwise identical to that used for homogenization) and resuspended in an amount of sucrose-free medium required to yield concentrations of 4–7 mg protein per ml (as determined by the procedure of Lowry *et al.*, ref. 15). As a routine, the freshly prepared adenylate cyclase fraction was divided into small portions, rapidly frozen at −70°C (dry ice–acetone), and stored at −50°C. Vasopressin-sensitive cyclase activity was found to be stable under these conditions for at least 6 months.

Assay of adenylate cyclase activity

Adenylate cyclase activity was assayed according to the method of Bär and Hechter (16), with modifications (8). Adenylate cyclase preparations (30–80 μg of protein) were

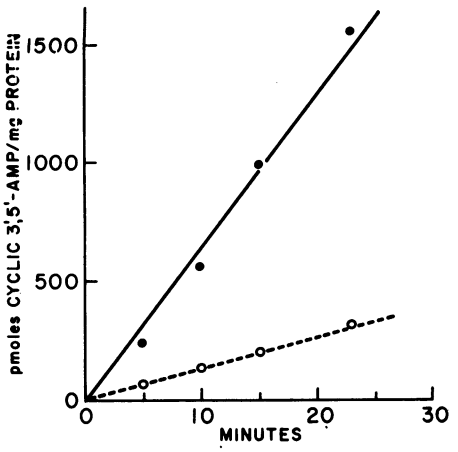

FIG. 1. Cyclic AMP production as a function of time. The rabbit kidney adenylate cyclase assay was conducted under standard conditions in a total volume of 0.2 ml; 0.05-ml aliquots were removed at 5-, 10-, 15-, and 25-min intervals. Basal rate of cyclic AMP formation (O- - -O) and cyclic AMP formation in the presence of 10^{-6} M [8-arginine]-vasopressin (●——●) are depicted.

incubated in a total volume of 0.05 ml with the following components: 0.1 mM [α-^{32}P]-ATP (about 10^6 cpm), 4 mM $MgCl_2$, 0.1% bovine serum albumin, 0.1 mM EDTA, 40 mM Tris·HCl buffer (pH 7.5), 0.5 mM unlabeled cyclic AMP, 25 mM creatine phosphate, and 1.0 mg/ml creatine kinase. Unless otherwise stated, incubations were carried out at 37°C for 15–20 min at pH 7.5. ATP levels were maintained at about 70% of the initial value, in the absence or presence of added hormone. Reactions were terminated by heat treatment.

RESULTS

The basic properties of renal medullary adenylate cyclase have been assessed in rabbit preparations; the formation of cyclic AMP is linear for at least 20 min, both in the absence and presence of 10^{-6} M AVP (Fig. 1). Both basal and AVP-stimulated adenylate cyclase activities are directly proportional to the protein concentration of the enzyme preparation in the range 0.6–2.5 mg/ml. Accordingly, adenylate cyclase activity is expressed in terms of picomoles per minute per mg protein. The pH optima for both basal and AVP-stimulated adenylate cyclase activity is between 7.5 and 8.0 in Tris·HCl buffer tested over the pH range 7–9; the pH optimum is approximately 8.0 in Tris maleate–glycine buffer tested over the pH range 5–10. Renal medullary cyclase was found to be selectively activated by neurohypophyseal hormones; parathyroid hormone, epinephrine, ACTH, β-MSH, and glucagon were found to be virtually inactive when tested in concentrations from 1 to 100 μM. Growth hormone (4.0 μg/ml), insulin (0.5 μg/ml), and TSH likewise did not affect renal medullary adenylate cyclase.

The stimulating activity of AVP, LVP, and oxytocin on renal medullary adenylate cyclase preparations from the different species was determined by graphic analysis of dose–response curves. The data were analyzed in terms of (*a*) the hormone concentration required for half-maximal adenylate cyclase stimulation (regarded as a measure of the affinity of hormone for the receptor component of the system) and (*b*) the maximal stimulation of adenylate cyclase achieved with saturating concentrations of hormone (regarded as an index of the intrinsic activity of the hormone) (17, 18). Fig. 2 depicts typical log dose–response curves obtained for AVP, LVP, and oxytocin with adenylate cyclase preparations from rat, rabbit, and pig renal medulla. Table 1 summarizes doses of AVP, LVP, and oxytocin required for half-maximal stimulation of cyclase preparations obtained from five mammalian species. In all these species the half-maximal response to AVP required a dose 100 times smaller than that of oxytocin. The order of the relative affinities was AVP > LVP ≫ oxytocin in mice, rat, rabbit, and ox; however, LVP had a higher affinity than AVP for the adenylate cyclase obtained from pig. Results summarized in Table 2 show that the relative stimulatory activities of AVP, LVP, and oxytocin upon rat and pig adenylate cyclases correlate directly with the known antidiuretic potencies of these hormones in the intact animals when assessed in terms of the affinity parameter.

The effects of neurohypophyseal hormone analogs on the rabbit renal medullary cyclase preparation are summarized in Table 3. The values for affinity and intrinsic activity were obtained from a series of dose–response studies; in each experiment a dose–response curve with AVP serves as control.

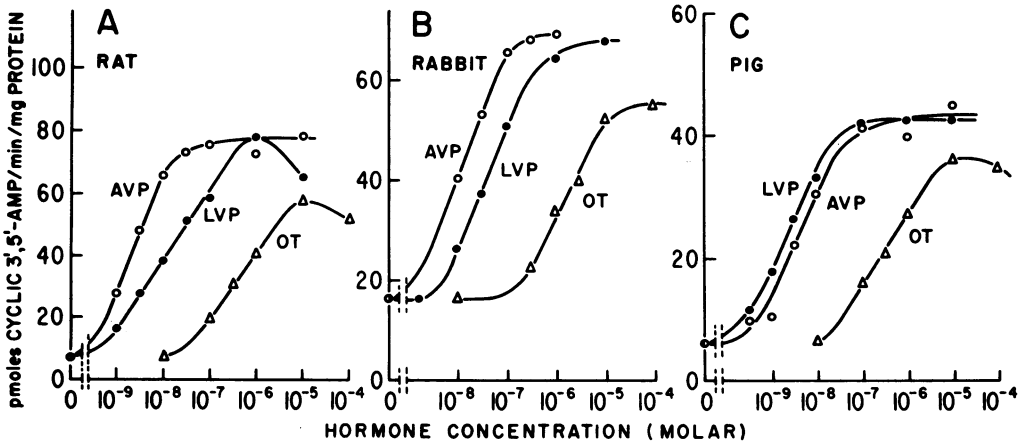

FIG. 2. Cyclic AMP production by renal medullary adenylate cyclase preparations of rat (*A*), rabbit (*B*), and pig (*C*) as a function of neurohypophyseal hormone concentration: [8-arginine]-vasopressin (-O-), [8-lysine]-vasopressin (-●-), and oxytocin, OT (—Δ—). All assays were conducted with the same enzyme preparation on the same day under standard conditions.

TABLE 1. *Neurohypophyseal hormonal stimulation of adenylate cyclase preparations from renal medulla of several mammals*

	Rat	Mouse	Rabbit	Ox	Pig
AVP	2.0	0.98	34	20	4.0
	4.5	1.6	15	22	5.6
	2.5				
LVP	12	2.1	45	50	2.1
	16	4.0	63	50	2.6
	25				
Oxytocin	430	130	1800	500	630
	320	96	1100	2000	320
	320				

Values are hormone concentrations (nanomolar) that evoke half-maximal adenylate cyclase stimulation.

DISCUSSION

These studies show that cell-free preparations of renal medullary adenylate cyclase possess distinctive advantages for the investigation of the mode of action of antidiuretic agents. These preparations are stimulated by neurohypophyseal hormones with high specificity, which indicates that the medullary receptor is highly selective. As the concentration of hormone is increased, the adenylate cyclase activity of the preparations increases until a maximum rate is reached, so that it is possible to determine the relative stimulatory effects of different neurohypophyseal peptides in terms of affinity and intrinsic activity.

Intrinsic antidiuretic activities of neurohypophyseal hormones cannot be determined *in vivo* because of the many complexities involved in experiments utilizing the response of the whole animal. However, it is possible to compare relative antidiuretic activities of neurohypophyseal hormones (in terms of potency values) with relative activities of the same compounds in the cell-free adenylate cyclase system (in terms of the affinity parameter). Such a comparison in the present study has resulted in the demonstration of a close parallelism between the relative activities of these hormones in the isolated cyclase system and in the whole animal (Table 2).

The observed difference of rat and pig receptors linked to vasopressin-sensitive adenylate cyclase may account for the observation by Sawyer (24) that in the rat the duration of LVP-induced antidiuresis is shorter than the duration of AVP-induced antidiuresis of equal maximum intensity. Conversely, in the pig the effect of LVP is of equal or even greater duration than the effect of AVP (25). In both cases the phenomena may be related to the corresponding differences in the affinity of the hormonal peptides for the receptor sites (26).

It is noteworthy that, with renal medullary adenylate cyclase preparations from rabbit (21), mouse (22), and beef (23) in which species AVP (as in rat) is the natural antidiuretic principle, the relative order of potency of neurohypophyseal hormones is AVP > LVP $\gg$ oxytocin, while in the domesticated pig, where the natural antidiuretic hormone is LVP (20), the order of potency was LVP > AVP $\gg$ oxytocin. These results suggest that the renal effector system for antidiuretic hormone is most sensitive to the naturally occurring vasopressin and that change in the structure of vasopressin (probably resulting from genetic mutation in phylogeny) is associated with a complementary change in the receptor moiety of the distal nephron.

TABLE 2. *Comparison of the action of neurohypophyseal peptides on renal medullary adenylate cyclase from rat and pig with their respective antidiuretic activities in vivo*

	Rat		Pig	
	Cyclase assay[a]	Antidiuresis[b]	Cyclase assay[a]	Antidiuresis
AVP	3.0	500[c]	4.8	LVP > AVP[d]
LVP	16	~250[e]	2.3	
Oxytocin	360	5[e]	470	

[a] Hormone concentrations (nanomolar) which evoke half-maximal adenylate cyclase stimulation. Values are the average of three independent experiments with different enzyme preparations. [b] Mean potencies (units/mg).

[c] Value reported by Meienhofer, J., A. Trzeciak, R. T. Havran, and R. Walter, *J. Amer. Chem. Soc.*, 92, 7199 (1970). [d] See ref. 24. [e] Boissonnas, R. A., S. Guttmann, B. Berde, and H. Konzett, *Experientia*, 17, 377 (1961).

The availability of an *in vitro* vasopressin-sensitive renal cyclase system has also made it possible to study the effects of structural modification of neurohypophyseal hormones in terms of affinity and intrinsic activity. We have examined various structural analogs of neurohypophyseal hormones in an attempt to assess the structural requirements for activation of the vasopressin-sensitive renal medullary cyclase in the rabbit. The significance of these results (see Table 3) may be summarized as follows: [deamino-1,6-dicarba]-vasopressin, an analog of AVP in which the N-terminal amino group has been replaced by hydrogen and the disulfide bridge by an ethylene bridge, has approximately the same affinity and the same intrinsic activity in the rabbit adenylate cyclase preparation as AVP. Thus the disulfide bridge of the AVP molecule is not essential for the activation of renal medullary adenylate cyclase, a finding which corroborates our experience with the toad bladder cyclase system (12). This finding is also in accord with the reported antidiuretic activity of [deamino-1,6-dicarba]-vasopressin (27) and related neurohypophyseal peptides (28–31) in the intact animal and in model *in vitro* preparations of amphibian bladder (29) and skin (30). All these observations show that the disulfide bond is not a prerequisite for neurohypophyseal hormone-induced permeability changes.

Modification of the amino-acid residue in position 8 of AVP was studied with several analogs. We have previously noted (6) that the replacement of arginine by lysine in AVP leads to a decrease in affinity with retention of intrinsic activity. In the present studies the significance of position 8 has been studied in detail. Replacement of L-arginine by its D-isomer leads to moderate reduction in affinity and intrinsic activity. The substitution of the basic chain in position 8 by a neutral side chain, such as in [8-alanine]-oxypressin, leads to drastic reduction in affinity and some reduction in intrinsic activity. An additional reduction in affinity and intrinsic activity is observed upon substitution of the phenylalanine residue in position 3 by isoleucine ([8-alanine]-oxytocin). The corresponding deamino analogs, [deamino-8-alanine]-oxypressin and [deamino-8-alanine]-oxytocin, which differ solely in position 3 (Phe vs. Ile residue), exhibit about the same intrinsic activity but differ with respect to the concentration required for half-maximal response, the oxypressin

TABLE 3. *Stimulatory effects of several neurohypophyseal hormone peptides on adenylate cyclase of rabbit kidney medulla*

Compound[a]	Peptide concn for half-max. response[b]	Max. response (% of AVP)[c]
1. [8-Arginine]-vasopressin (AVP)[d, e]	20	100
2. [8-Lysine]-vasopressin (LVP)[d, f]	70	100
	40	100
3. Oxytocin[g, h]	2500	62
	3900	63
4. [8-Arginine]-vasotocin[d, i]	57	89
	125	90
5. [Deamino-1,6-dicarba]-vasopressin[g, j]	23	94
	30	94
6. [Deamino-8-D-arginine]-vasopressin[g, k]	30	68
	71	91
7. Vasopressinoic acid[c, l]	850[m]	22
		22
8. [8-Alanine]-oxypressin[g, n]	230	75
	530	80
9. [Deamino-8-alanine]-oxypressin[g, n]	390	60
	270	69
10. [8-Alanine]-oxytocin[g, n]	4000	45
	11,800	42
11. [Deamino-8-alanine]-oxytocin[g, n]	3400	76
	6300	76
12. Deamino-oxytocin[d, o]	4300	90
	3100	88
13. [Deamino-2-alanine]-oxytocin[g, p]	—	~4 (weak inhibitor of AVP at 10^{-4} M)
14. [4-N^4-Methylasparagine]-oxytocin[g, q]	10,000[m]	26
		22
15. [Deamino-4-N^4-methyl-asparagine]-oxytocin[g, q]	10,000[m]	26
		22
16. [5-Valine]-oxytocin[g, r]	—	~5 (weak inhibitor of AVP at 10^{-4} M)
17. Oxytocinoic acid[g, s]	3900[m]	23
		11

[a] *Deamino* stands for the β-mercaptopropionyl residue. [b] AVP was used as reference standard in each experiment; concentration for half-maximal response to AVP has been determined in 11 experiments and an average value of 21 ± 1.6 nM; this value was used to normalize the half-maximal concentrations found for the peptide used in each trial. [c] Response produced by saturating concentrations of peptides. Standard errors were below 15%. [d] Prepared by the solid-phase method of peptide synthesis introduced by Merrifield, R. B., *J. Amer. Chem. Soc.*, **85**, 2149 (1963). [e] Meienhofer, J., A. Trzeciak, R. T. Havran, and R. Walter, *J. Amer. Chem. Soc.*, **92**, 7199 (1970). [f] Meienhofer, J., and Y. Sano, *J. Amer. Chem. Soc.*, **90**, 2996 (1968). [g] Prepared by conventional method of peptide synthesis. [h] du Vigneaud, V., C. Ressler, J. M. Swan, C. W. Roberts, P. G. Katsoyannis, and S. Gordon, *J. Amer. Chem. Soc.*, **75**, 4879 (1953). [i] Havran, R. T., C. Meyers, I. L. Schwartz, and R. Walter, in *Peptides, Proc. Tenth Europ. Peptide Symp.*, 1969, ed. E. Scoffone (North-Holland Publ. Co., Amsterdam, 1971), in press. [j] Hase, S., T. Morikawa, and S. Sakakibara, *Experientia*, **25**, 1239 (1968). [k] Zaoral, M., J. Kolc, and F. Šorm, *Collect. Czech. Chem. Commun.*, **31**, 382 (1966). [l] See ref. 32. [m] Because of the low intrinsic activity of this peptide, the concentration for half-maximal response should be considered an approximation. [n] Walter, R., and V. du Vigneaud, *Biochemistry*, **5**, 3720 (1966). [o] Takashima, H., V. du Vigneaud, and R. B. Merrifield, *J. Amer. Chem. Soc.*, **90**, 1323 (1968). [p] Walter, R., and I. L. Schwartz, *Life Sci.*, **7**, 545 (1968). [q] Havran, R. T., I. L. Schwartz, and R. Walter, *Mol. Pharmacol.*, **5**, 83 (1969). [r] Walter, R., and I. L. Schwartz, *J. Biol. Chem.*, **241**, 5500 (1966). [s] Ferrier, B. M., and V. du Vigneaud, *J. Med. Chem.*, **9**, 55 (1965).

analog being the more potent in the *in vitro* system.

Substitution of the glutamine residue in position 4 of oxytocin (or deamino-oxytocin) by its isomer, 4-N^4-methyl-asparagine, results in analogs of low intrinsic activity and low affinity, while [deamino-2-alanine]-oxytocin and [5-valine]-oxytocin were unable under the conditions employed in this study to stimulate renal medullary adenylate cyclase.

Vasopressinoic acid and oxytocinoic acid, hormone analogs in which the C-terminal carboxamide is replaced by a free carboxyl group, possess a low intrinsic activity in the adenylate cyclase preparation; these compounds have also been found to be weak agonists *in vivo* (32). We have reported previously that vasopressinoic acid, in the cell-free rabbit renal medullary cyclase system, is a potent inhibitor of AVP as well as of LVP and oxytocin (8). In contrast, oxytocinoic acid did not inhibit the AVP-induced activation of adenylate cyclase probably as a result of the low affinity of oxytocinoic acid for the kidney receptor; this contention is supported by the finding that oxytocinoic acid is also a weak antagonist of the oxytocin-induced stimulation of adenylate cyclase (Table 3). The inhibition of AVP-stimulated adenylate cyclase by vasopressinoic acid is pH-dependent and increases with an increase in pH from 7 to 9 (unpublished data).

The high specificity and reproducible response of the cell-free renal medullary adenylate cyclase preparation suggest that this system may be promising for *in vitro* assay of neurohypophyseal hormones—analogous to the adenylate cyclase bioassay for parathyroid hormone (33).

The present study indicates that the AVP-sensitive adenylate cyclase preparation is a valuable system for the investigation of the early events in the mechanism of action of neurohypophyseal hormones on the kidney. The striking parallelism of the *in vitro* action (adenylate cyclase activation) and the *in vivo* action (antidiuresis) of AVP, LVP, and oxytocin in several species indicates that the potential for selective quantitative modulation of the ultimate response to the hormone can only be realized at the initial hormone–receptor interaction. Furthermore, the use of isolated adenylate cyclase systems for structure–activity studies has yielded information not only about the relative affinity of neurohypophyseal hormonal peptides for their receptor, but also about their maximal stimulatory capacity, a parameter which cannot be evaluated by antidiuretic assays *in vivo*.

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