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Identification and characterization of a vasopressin isoreceptor in porcine seminal vesicles

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ABSTRACT **Neurohypophysial hormones stimulate the motility of tunica albuginea, epididymis, and vas deferens acting through oxytocin (OT) and V1 vasopressin receptors. To test the hypothesis that these hormones are involved also in the regulation of seminal vesicle physiology, we studied binding of [^{3}H]OT and [^{3}H] arginine vasopressin ([^{3}H]AVP) to porcine seminal vesicle membranes. Neurohypophysial hormones bind to two different classes of sites. The first class shows low capacity (35 fmol per mg of protein) and a very high affinity (K_d < 1 nM) for both the labeled ligands. The second class is characterized by a high capacity (2000 fmol per mg of protein) and a high affinity for AVP ($K_d \approx 2.5$ nM), whereas OT has 160 times lower affinity. Lysine vasopressin and the V1 antagonist [1-deaminopenicillamine,2-(*O*-methyl)tyrosine]Arg^8-vasopressin compete with high affinity with [^{3}H]AVP binding, whereas the V2 agonist [1-deamino,4-valine]D-Arg^8-vasopressin (dVDAVP) is 110 times less potent than AVP. The OT agonist [Thr^4,Gly^7]OT and the OT antagonist [1(β-mercapto-β,β-cyclopentamethylene propionic acid), 2-(*O*-ethyl)tyrosine, 8-ornithine]vasotocin failed to affect [^{3}H]AVP binding. These findings seem to suggest that AVP interacts with the V1 vasopressin isoreceptor in porcine seminal vesicle membranes. However, AVP stimulates adenylate cyclase activity in a dose-dependent fashion with an EC_{50} of 14 nM, whereas OT or dVDAVP has no effect at 100 nM. Moreover, a well-characterized V1 vasopressin antagonist, [1-(β-mercapto-β,β-cyclopentamethylene propionic acid),2-(*O*-methyl)tyrosine]Arg^8-vasopressin [d(CH$_2$)$_5$Tyr(Me)AVP], competes with [^{3}H]AVP binding with an IC_{50} of 0.17 μM. These pharmacological properties are distinct from the previously described V1 and V2 vasopressin receptors and indicate the presence of a new class of AVP receptors. Although this vasopressin isoreceptor shares some pharmacological characteristics with the V1 (pressor) isoreceptor, it has low affinity for the V1 antagonist d(CH$_2$)$_5$-Tyr(Me)AVP and is linked to the adenylate cyclase system. The extremely high density of AVP receptors in porcine seminal vesicles (2 pmol per mg of protein) is comparable to the density of V2 vasopressin receptors in porcine renal medulla, suggesting a physiological role for vasopressin in the seminal vesicle.**

Vasopressin receptors have been identified and characterized in several mammalian tissues: kidney (1), liver (2), vascular smooth muscle cells (3), brain (4), pituitary gland (5), and the interstitial cells of the rat testis (6). Mitchell *et al.* (7) postulated the presence of two vasopressin isoreceptors, distinguished on the basis of functional criteria. The V1 subtype of receptors regulates cellular calcium metabolism, whereas the V2 subtype is functionally coupled to adenylate cyclase (1). The recent development of potent and selective ligands for these two isoreceptors allowed the identification of V1 isoreceptors in blood vessels, liver, brain, and interstitial cells of the testis (2–4, 6), whereas the V2 isoreceptor has been localized in the renal medulla (1).

Oxytocin (OT) receptors were described in the uterus (8), mammary gland (9), oviduct (10), and hippocampus (11). These receptors, in mammals, are not functionally coupled to adenylate cyclase (12); it has been suggested that the occupancy of OT binding sites is associated with an increase of intracellular calcium, apparently via specific inhibition of Ca^{2+},Mg^{2+}-ATPase (13).

We have recently identified and characterized OT and V1 vasopressin receptors in the tunica albuginea of the testis, epididymis, and vas deferens of the prepubertal pig (14). Both *in vitro* and *in vivo* studies suggest that these receptors are involved in the regulation of the motility of the male genital tract (15). Seminal vesicles have a fibromuscular stroma that appears to be a target for androgens and estrogens (16). To test the hypothesis that these glands are also a target tissue for neurohypophysial hormones, we studied the binding of labeled OT and arginine vasopressin (AVP) and the response of adenylate cyclase to these hormones in membranes prepared from porcine seminal vesicles.

MATERIALS AND METHODS

[^{3}H]AVP (50–70 Ci/mmol; 1 Ci = 37 GBq) and [^{3}H]OT (30–60 Ci/mmol) were purchased from New England Nuclear. The tritiated ligands were aliquoted in plastic tubes, sealed under nitrogen, and frozen at −80°C. AVP was obtained from Calbiochem. [1-(β-Mercapto-β,β-cyclopentamethylene propionic acid),2-(*O*-methyl)tyrosine]Arg^8-vasopressin [d(CH$_2$)$_5$Tyr(Me)AVP] was obtained from Peninsula Laboratories (San Carlos, CA). Lysine vasopressin (LVP), [1-deaminopenicillamine,2-(*O*-methyl)tyrosine]Arg^8-vasopressin [dPenTyr(Me)AVP], OT, and isoproterenol were from Sigma. Ovine corticotropin-releasing factor (CRF) was from Bachem Fine Chemicals (Torrance, CA). [Thr^4,Gly^7]OT, [1(β-mercapto-β,β-cyclopentamethylene propionic acid),2(*O*-ethyl)tyrosine,8-ornithine]vasotocin [d(CH$_2$)$_5$Tyr(Et)OVT], and [1-deamino,4-valine]D-Arg^8-vasopressin (dVDAVP) were synthesized and generously provided by M. Manning (Medical College of Ohio, Toledo, OH).

Porcine seminal vesicles were obtained from the National Institutes of Health Animal Center Section. SLA inbred miniature swine (12–16 weeks old) were sacrificed with sodium pentobarbital, and the seminal vesicles were promptly removed and dissected free of fat and adherent tissue. The

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Abbreviations: LVP, lysine vasopressin; OT, oxytocin; AVP, arginine vasopressin; CRF, corticotropin-releasing factor; d(CH$_2$)$_5$Tyr(Me)AVP, [1-(β-mercapto-β,β-cyclopentamethylene propionic acid), 2-(*O*-methyl)tyrosine]Arg^8-vasopressin; dPenTyr(Me)AVP, [1-deaminopenicillamine,2-(*O*-methyl)tyrosine]Arg^8-vasopressin; d(CH$_2$)$_5$Tyr(Et)OVT, [1-(β-mercapto-β,β-cyclopentamethylene propionic acid),2(*O*-ethyl)tyrosine,8-ornithine]vasotocin; dVDAVP, [1-deamino,4-valine]D-Arg^8-vasopressin.

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seminal vesicles were immediately plunged into ice-cold phosphate-buffered saline (PBS; 0.15 M NaCl/0.01 M Na_2HPO_4, pH 7.4), opened longitudinally, and flushed with PBS. No seminal plasma was detectable in the glands. Seminal vesicles were weighed and minced with fine scissors in 4 vol of buffer 1 (10 mM Tris·HCl, pH 7.4/1.5 mM EDTA/0.5 mM dithiothreitol/1 mM benzamidine/0.01% bacitracin/0.002% soybean trypsin inhibitor). Tissues were homogenized with a Polytron homogenizer (Brinkmann), filtered through cheesecloth, and rehomogenized with a glass Teflon homogenizer (Thomas Scientific, Swedesboro, NJ). Crude membrane fractions were prepared by differential centrifugation between 1000 × *g* (10 min) and 160,000 × *g* (30 min), and washed once in buffer 2 (50 mM Tris maleate, pH 7.6/10 mM $MgSO_4$/1 mM benzamidine/0.01% bacitracin/0.002% soybean trypsin inhibitor). The final pellets were dispersed in buffer 2 to a concentration of 2.5–4 mg of protein per ml. The membrane preparation was divided into small aliquots, frozen in dry ice, and stored at −80°C until assayed.

Binding experiments were performed in buffer 2 containing 0.1% bovine serum albumin, with a final vol of 0.5 ml at 22°C for 60 min. Aliquots of the membranes (final concentration, 0.3 mg/ml) were added to a fixed concentration of [^{3}H]OT and [^{3}H]AVP with or without increasing concentrations of unlabeled ligands. The tracers were present at 0.5–0.6 nM for the incubations with unlabeled ligands, and at 0.04–0.1 nM for the incubation without unlabeled ligands. Measurements were obtained in triplicate. The unlabeled ligands were diluted with a Hamilton Micro Lab M programmable microprocessor-controlled diluter/dispensor. Bound radioactivity was separated by filtration through Whatman GF/B filters presoaked in ice-cold 50 mM Tris·HCl, pH 7.4/0.1% bovine serum albumin using the Brandel M-48R cell harvester (Gaithersburg, MD). Filters were washed twice with 2 ml of ice-cold 50 mM Tris·HCl (pH 7.4) and counted after incubation overnight in 5 ml of Ready-Solv HP (Beckman).

Adenylate cyclase was assayed in the same membrane preparation (20–30 μg of protein) as described (17). [^{32}P]cAMP was isolated from the mixture by the method of Salomon *et al.* (18).

Protein content was measured using the Pierce protein assay reagent (Pierce) based on the method of Bradford (19), with bovine serum albumin as a standard.

The computer program ALLFIT (20) was used for the analysis of sigmoidal displacement curves using the constrained four-parameter logistic model to obtain estimates of EC_{50} or IC_{50} values and the logit–log slope ("pseudo-Hill coefficient"). Families of self- and cross-displacement curves were analyzed simultaneously using the program LIGAND (21). Models involving one, two, or three independent classes of sites were evaluated. Selection among models

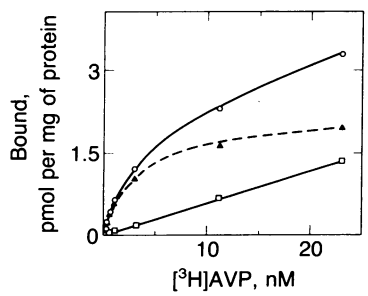

FIG. 1. Equilibrium binding of [^{3}H]AVP to membranes prepared from seminal vesicles of 12- to 16-week-old pigs. Membranes were incubated at 22°C for 60 min with increasing concentrations (0.04–22 nM) of [^{3}H]AVP. Ordinate: Total binding (○); nonspecific binding in the presence of 1 μM AVP (□). Specific binding (▲) was calculated as the difference between total and nonspecific binding. Each point is the mean of triplicate determinations.

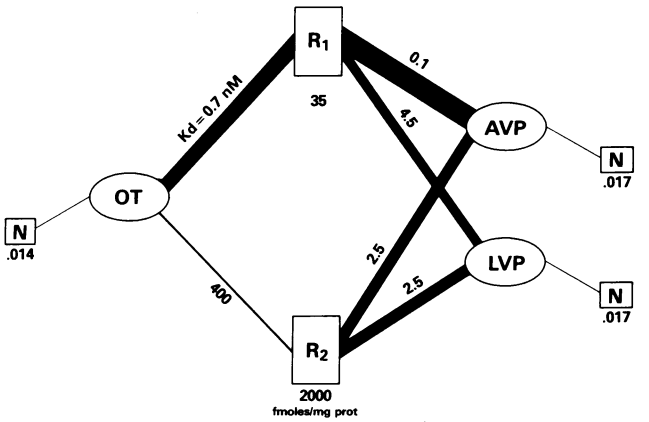

FIG. 2. Best fitting model: The relative affinities of the three ligands OT, AVP, and LVP are schematically represented by the thickness of the lines connecting the ligands with the receptors. The K_d values (nM) are indicated by the numbers on each line. R_1 and R_2 denote the two classes of sites. The numbers below each rectangle indicate the concentration of binding sites (fmol per mg of protein). N, nonspecific binding, expressed as bound/free ratio. Membranes were from seminal vesicles of 12- to 16-week-old pigs. The model was fit using 21 curves from three membrane preparations in four experiments. A model involving two classes of sites was significantly better than a model involving only one site ($P < 0.001$).

was based on the root-mean-square error of each fit using an F-test based on the "extra-sum-of-squares principle," and on tests of randomness of the residuals around the fitted curves (21).

RESULTS

Saturation analysis demonstrates that [^{3}H]AVP binds with high affinity and in a specific and saturable manner to seminal vesicle membranes (Fig. 1). A Scatchard plot appeared to be curvilinear (see below).

Analysis of five competition curves for AVP obtained from four different membrane preparations using the program ALLFIT indicated that the logit–log slope (pseudo-Hill coefficient) of the curves was significantly less than unity ($b = 0.86 \pm 0.03$; $P < 0.01$), which is consistent with heterogeneity of receptors.

Computer modeling of 21 curves of self- and cross-displacement using labeled AVP and OT, and unlabeled AVP, LVP, and OT, confirmed the presence of at least two classes of binding sites for the neurohypophysial hormones. The introduction of a second independent class of sites dramatically improved the goodness-of-fit ($P << 0.001$). Fig.

Table 1. Concentration of two classes of binding sites (R_1, R_2) (fmol per mg of protein) and pK = −log(K_d) for AVP, LVP, and OT for the two binding sites in membranes from porcine seminal vesicle

	Binding site	
	R_1	R_2
	Binding capacity, fmol per mg of protein	
	35 ± 12	2000 ± 120
Ligand	Affinity, pK	
AVP	9.9 ± 0.24	8.6 ± 0.04
LVP	8.3 ± 0.26	8.6 ± 0.06
OT	9.1 ± 0.26	6.4 ± 0.06

Values ± SEM are derived from the simultaneous computer modeling of 21 curves obtained using three different membrane preparations.

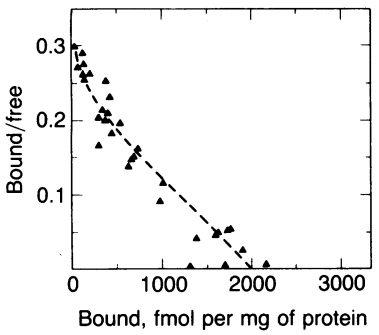

FIG. 3. Scatchard plot of [^{3}H]AVP specific binding to porcine seminal vesicle membranes. This plot was derived from the fit of 21 curves of self- and cross-displacement of AVP, LVP, and OT. Each point is the mean of triplicate determinations.

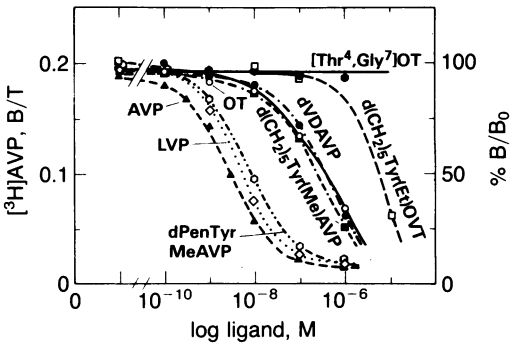

FIG. 4. Displacement of specific [^{3}H]AVP binding to seminal vesicle membranes by neurohypophysial hormones and several synthetic analogs. Membranes (0.3 mg/ml) were incubated for 60 min at 22°C with 0.6 nM [^{3}H]AVP in the presence or absence of increasing concentrations of the following peptides: AVP (▲), LVP (◇), OT (○), dVDAVP (●), [Thr^4,Gly^7]OT (●), d(CH$_2$)$_5$Tyr(Et)OVT (□), d(CH$_2$)$_5$Tyr(Me)AVP (■), dPenTyr(Me)AVP (○). B/T, bound/total; % B/B$_0$, normalized response.

2 summarizes the results of modeling. AVP binds to a very high-affinity low-capacity class of site (K_d = 0.1 nM; binding capacity = R_1 = 35 fmol per mg of protein) and to a high-affinity high-capacity class of site (K_d = 2.5 nM; R_2 = 2000 fmol per mg of protein). OT binds with high affinity to the first class of sites (K_d = 0.7 nM) but shows 1/160th the affinity (K_d = 400 nM) of AVP and LVP for the second site. LVP is nonselective for the two classes of sites. Table 1 summarizes the affinities (expressed as pK values) and binding capacities. Fig. 3 shows the curvilinear Scatchard plot for AVP derived from the analysis of four self- and cross-displacement studies (a total of 21 curves) in membranes from seminal vesicles using a model involving two independent classes of binding sites.

To better characterize the [^{3}H]AVP binding to membranes from seminal vesicles, we generated several families of competition curves using analogs with different specificities for the V1 and V2 vasopressin receptors and for the OT receptor. Fig. 4 shows a typical example of multiple competition curves. Table 2 shows the IC_{50} and the logit–log slope of the curves for each peptide tested. LVP (the natural form of vasopressin in pig), AVP, and the V1 vasopressin antagonist dPenTyr(Me)AVP compete with high affinity with [^{3}H]AVP binding, while OT and the selective antidiuretic (V2) vasopressin analog dVDAVP are 110 times less potent than AVP. The OT antagonist d(CH$_2$)$_5$Tyr(Et)OVT does not compete with [^{3}H]AVP at concentrations up to 1 μM. The selective OT agonist [Thr^4,Gly^7]OT failed to affect [^{3}H]AVP binding at any concentrations tested. Surprisingly, the V1 vasopressin antagonist d(CH$_2$)$_5$Tyr(Me)AVP competes with AVP with an IC_{50} of 167 ± 19 nM, a value 25-fold higher than the IC_{50} of the other V1 antagonist tested—namely, dPenTyr(Me)AVP (IC_{50} = 6.6 ± 0.4 nM). To confirm this important property, the studies with d(CH$_2$)$_5$Tyr(Me)AVP were repeated four times in four different membrane preparations using two different batches of the peptide. The same antagonist displaced [^{3}H]AVP from membranes prepared from tunica albuginea and epididymis of the same pigs with K_d values of 0.99 and 0.43 nM (14).

Table 2 also shows the IC_{50} and the logit–log slope for [^{3}H]OT competition curves for the neurohypophysial hormones and several analogs. Several OT agonists and antagonists compete with high affinity for the [^{3}H]OT binding. Remarkably, both AVP and LVP also showed high affinity for this site.

AVP was able to increase adenylate cyclase activity in seminal vesicle membranes in a dose-dependent fashion, with an EC_{50} of 14 nM, while no significant increase was observed for OT and dVDAVP at concentrations of 100 nM (Fig. 5). Using 100 nM AVP, a 63% ± 1.7% increase above the basal values ($P < 0.01$) was recorded in two different experiments. Porcine seminal vesicle membranes were assayed for β-adrenergic binding sites, using [^{125}I]iodocyanopindolol. The binding capacity was 43 ± 9 fmol per mg of protein in two experiments (S.K., unpublished data). The β-adrenergic agonist isoproterenol (10 μM) caused a 47.2% ± 1.2% increase of adenylate cyclase activity relative to control ($P < 0.01$) in two different experiments. Table 3 shows the effect of 100 nM AVP, 10 μM isoproterenol, and 50 nM CRF on adenylate cyclase activity in porcine seminal vesicles with and without 2 μM GTP. The combined effect of the various hormones with AVP is also shown. AVP and isoproterenol alone or in combination significantly increase adenylate cyclase activity, compared with control values in the presence of 2 μM GTP ($P < 0.05$ by Duncan's multiple range test). No additivity of effects was observed when membranes were

Table 2. Analysis of competition curves using the four-parameter logistic model

	[^{3}H]AVP			[^{3}H]OT		
	Exp.	IC_{50}, nM	Slope	Exp.	IC_{50}, nM	Slope
AVP	5	2.9 ± 0.2	0.86 ± 0.03	3	1.1 ± 0.2	0.82 ± 0.08
LVP	3	4.2 ± 0.4	0.88 ± 0.03	3	2.5 ± 1.2	0.83 ± 0.03
OT	3	335 ± 29	0.77 ± 0.03	3	3.3 ± 0.7	0.63 ± 0.1
dVDAVP	2	313 ± 26	0.85 ± 0.03	2	>1000	
[Thr^4,Gly^7]OT	2	>1000		2	8.0	0.50 ± 0.2
d(CH$_2$)$_5$Tyr(Me)AVP	4	167 ± 19	0.60 ± 0.07	—		—
dPenTyr(Me)AVP	2	6.6 ± 0.4	0.73 ± 0.03	—		—
d(CH$_2$)$_5$Tyr(Et)OVT	1	>1000		1	13	0.44 ± 0.1

IC_{50} and logit–log slope of neurohypophysial hormones and several analogs binding to porcine seminal vesicle membranes. Values ± SEM derived from the analysis of the competition curves with [^{3}H]AVP and [^{3}H]OT, using the program ALLFIT (20). Number of experiments is indicated. All slopes are significantly less than unity ($P < 0.05$).

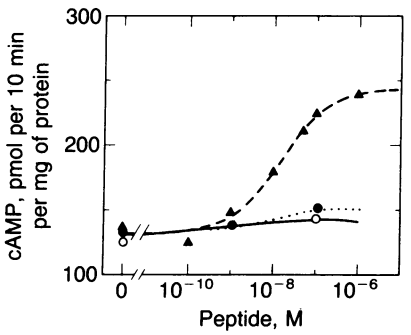

FIG. 5. Effect of increasing concentrations of AVP (▲) and dVDAVP (●) on adenylate cyclase activity (pmol per 10 min per mg of protein) of porcine seminal vesicle membranes. The effect of 100 nM oxytocin (○) is also shown. Each point is the mean of triplicate determinations in the presence of 2 μM GTP. For comparison, NaF-stimulated activity was 1041 ± 26.

stimulated with both 100 nM AVP and 10 μM isoproterenol. Ovine CRF neither increased adenylate cyclase activity nor potentiated the AVP-induced response. The 10-fold response of adenylate cyclase activity to NaF (Table 3) indicates that the membrane preparation has an active adenylate cyclase system.

DISCUSSION

We have identified and characterized two classes of binding sites for OT and vasopressin in membranes prepared from seminal vesicles from prepubertal pigs. AVP and LVP bind to a high-affinity high-capacity binding site (2000 fmol per mg of protein), while OT and AVP bind relatively nonselectively to a very high affinity binding site of considerably lower capacity (35 fmol per mg of protein).

To our knowledge, this is the first observation of an extremely high concentration (2 pmol per mg of protein) of AVP receptors in seminal vesicles of prepubertal pigs. These values are actually 2-fold higher than the concentration of vasopressin receptors detected in plasma membrane preparations from porcine renal medulla (1). The large amount of vasopressin receptors and the affinity constants in excess of $10^8\ M^{-1}$ detected in the seminal vesicles strongly suggest that these AVP receptors may have a physiological role.

The neurohypophysial hormones OT and vasopressin do not affect the contractility of seminal vesicles either *in vivo* (22–24) or *in vitro* (25), while the same hormones stimulate the motility of seminiferous tubules, tunica albuginea, epididymis, and vas deferens (15). We have recently characterized the OT and AVP receptors in the pig tunica albuginea, epididymis, and vas deferens (14). The AVP receptors present in these tissues appear to belong to the V1 (pressor) subtype of vasopressin receptors, since (*i*) they do not activate adenylate cyclase; (*ii*) they have high affinity for the potent vasopressor antagonist $d(CH_2)_5Tyr(Me)AVP$; and (*iii*) the selective antidiuretic (V2) peptide dVDAVP binds with only low affinity to this site.

Table 3. Effect of AVP, CRF, and isoproterenol (ISO) on adenylate cyclase activity (pmol of cAMP per 10 min per mg of protein) in seminal vesicle membranes with and without 2 μM GTP

Addition to medium	Adenylate cyclase activity, pmol of cAMP per 10 min per mg of protein	
	−GTP	+GTP (2 μM)
None (control)	123 ± 18	254 ± 25
AVP (0.1 μM)	197 ± 6*	411 ± 28*
ISO (10 μM)	149 ± 5	372 ± 25*
CRF (0.05 μM)	134 ± 7	290 ± 1
AVP (0.1 μM) + ISO (10 μM)	—	442 ± 16*
AVP (0.1 μM) + CRF (0.05 μM)	—	384 ± 33*
NaF (10 μM)	1166 ± 21*	—

Each value is the mean of triplicate determinations ± SEM.
*Statistically significantly different from control ($P < 0.05$) using Duncan's multiple range test.

In contrast, the AVP receptor in porcine seminal vesicles show distinct properties: (*i*) AVP increases adenylate cyclase activity in a dose-dependent fashion with a 63% increase at 1 μM. The EC_{50} for AVP was 14 nM, a value that is close to the K_d (2.5 nM) for the predominant class of sites. (*ii*) The V1 vasopressor antagonist $d(CH_2)_5Tyr(Me)AVP$ competes with [^{3}H]AVP with relatively low affinity (IC_{50} = 167 nM).

The present results suggest several similarities between the AVP receptors present in seminal vesicles and the V2 antidiuretic receptors (26). However, two main differences should be pointed out: (*i*) The selective antidiuretic (V2) agonist dVDAVP displaces [^{3}H]AVP with an IC_{50} of 313 ± 26 nM, and at a concentration as high as 100 nM it does not significantly increase adenylate cyclase activity. (*ii*) The V1 antagonist dPenTyr(Me)AVP has a high affinity for the [^{3}H]AVP binding site (6.6 ± 0.4 nM) of seminal vesicles.

Antoni (5) has recently characterized AVP receptors present in membranes prepared from rat pituitary. These receptors showed high affinity for AVP and for the V1 vasopressor antagonist dPenTyr(Me)AVP (IC_{50} = 3 nM), while the other V1 antagonist tested, $d(CH_2)_5Tyr(Me)AVP$, showed a much lower potency (IC_{50} = 310 nM). Antoni *et al.* (27) reported the same rank order of potency in terms of biological potency of corticotropin/β-endorphin release from rat pituitary *in vitro*. Giguere and Labrie (28) demonstrated that in rat pituitary cells in culture the AVP agonist [$Asu^{1,6}$,Arg^8]vasopressin does not stimulate cAMP accumulation but potentiates the effects of CRF on cAMP. Furthermore, Knepel *et al.* (29) have reported that AVP stimulates cAMP accumulation in rat anterior pituitary quarters.

CRF binding sites have been demonstrated to be present in rat testis and prostate (30). To test the hypothesis that AVP might potentiate the CRF-induced increase in adenylate cyclase activity, we stimulated pig seminal vesicle membranes with 50 nM CRF with or without 100 nM AVP. CRF neither stimulated adenylate cyclase nor potentiated the AVP-induced increase (Table 3). Isoproterenol (10 μM), acting through the β-adrenergic receptors, stimulated adenylate cyclase activity but was not additive with the response to 100 nM AVP (Table 3). Thus, the AVP receptors present in porcine seminal vesicles seem to be distinct from both the previously described V1 and V2 subtypes. These results, together with the properties of AVP receptors in anterior pituitary (5, 27–29, 31, 32), indicate the presence of another class of vasopressin receptors in the seminal vesicles.

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