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CHARACTERIZATION OF HUMAN PLATELET VASOPRESSIN RECEPTORS

by

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THESIS

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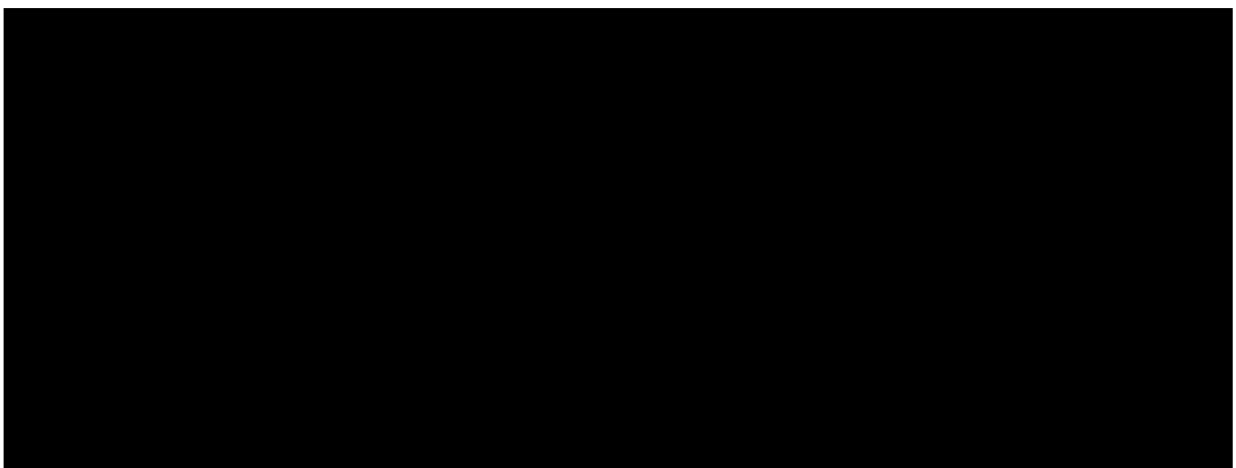

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INTRODUCTION

By the turn of the 19th century, Oliver and Schaffer demonstrated that the posterior pituitary gland contained a vasopressor peptide which subsequently was named vasopressin (AVP). Shortly thereafter, the powerful water reabsorption action of this peptide at the renal level was recognized and it therefore was referred to as the antidiuretic hormone. This peptide was isolated and synthetized by du Vigneaud in 1951, but it was only in 1974 that a sensitive radioimmunoassay for this hormone was developed. Eventually, potent and specific antagonists of this hormone at the vascular level were designed in 1980.

AVP plays a major role in the regulation of the volume and osmolality of body fluids via its ability to promote water reabsorption in the kidney but also provokes vasoconstriction and blood pressure increase (1). The physiological relevance of the hemodynamic actions of AVP often has been denied and its vasoconstrictor action long has been regarded as a pharmacological property used for:

- the induction of an experimental coronary spasm,
- the treatment of digestive hemorrhages and sickle cell disease attacks of hematuria.

However, there is now growing experimental evidence that

endogenous AVP plays a role in blood pressure control. This assertion is supported by the following experimental evidences:

- in isolated vessel preparation in vitro, AVP is the most powerful vasoconstrictor agent known, more potent even than angiotensin II and catecholamines (2). In addition, AVP potentiates the vasoactive action of the preceding vasoconstrictor agents.
- in conscious animal, physiological circulating levels of AVP have obvious cardiovascular effects (3).
- the development of specific and sensitive radioimmunoassays for AVP has revealed abnormal levels of this hormone in various physiological and pathophysiological states challenging the cardiovascular homeostasis (dehydration, hemorrhage, cardiac failure, adrenal insufficiency, hypertension, (4)).
- Finally, the development of potent and specific peptidic antagonists of the vasoconstrictor effect of AVP has made it possible specifically and reversibly to block the cardiovascular action of endogenous AVP (5-7).

For instance, in hypertensive models characterized by high AVP circulating levels, AVP antagonists induce the hypotensive response expected from the blocking of the effects of the endogenous hormone on vascular tone.

To better delineate this vasoactive action of AVP in human physiology and pathophysiology, it is mandatory to develop additional tools such as:

- easily accessible models of AVP receptors,
- orally active specific and potent AVP antagonists acting at either the renal or vascular level.

The characterization of AVP receptors will certainly help design this new class of pharmacologically active agents.

A large array of biological effects for AVP has been described, including water reabsorption, contraction of smooth muscle cells and glomerular mesangial cells, liver glycogenolysis and neoglucogenesis, cardiac positive chronotropic and negative inotropic effects, platelet aggregation, mitogenic action on several cell lines and memory processes alterations. The use of AVP structural analogues suggest that all the observed effects of this hormone are mediated through specific receptors at the cellular level.

Two classes of AVP receptors can be distinguished on the basis of their mode of action at the cellular level (9-12):

1) the vascular type (or V1 type) which acts through an increase of the intracytosolic calcium concentration and a stimulation of phosphatidyl inositol turnover. This class of receptors is known to mediate the effect of AVP on blood vessel constriction, liver glycogenolysis and blood platelet aggregation.

2) the renal type (or V2 type) which interacts with the membrane adenylate cyclase system and which is responsible for the water reabsorption action of this hormone at the renal

level.

In animal preparations, smooth muscle cells or liver extracts are used for investigating AVP specific receptors of the vascular type (9,10), whereas kidney preparations are used to study AVP renal type receptors (11). These tissues are rarely available from man. For this reason, Block et al (12) used human mononuclear phagocytes to characterize specific AVP receptors which presumably are of the renal type.
Because Haslam (13) showed that AVP aggregates human platelets in the presence of calcium and thus suggested "a parallel between platelet aggregation and the contraction of smooth muscle", we attempted to demonstrate that AVP would bind specifically to these human cells and to identify the type of receptor involved. In fact, human platelet membranes possess specific binding sites for 3H-Arginine-8-AVP ([3H]AVP). The pharmacological characteristics of these binding sites are consistent with those of the V1-vascular type of AVP receptor. Accordingly, platelets may become a useful tool for exploring further the cardiovascular effects of AVP in man.

MATERIAL AND METHODS

- Pharmacological agents

The radioligand [3H]AVP was purchased from New England Nuclear (Boston, Mass). Its radiochemical purity was checked by high pressure liquid chromatography (HPLC) with a reverse phase C18

column from Waters using a gradient from 23% to 29% acetonitrile and 0.01M ammonium acetate, pH 6.7. The specific activity of [3H]AVP was close to 55 Ci/mmol. Bovine serum albumin, AVP, creatine phosphate, creatine phosphokinase, EGTA, beta-mercaptoethanol, ATP, cyclic AMP, GTP, epinephrine, 5'-guanylylimidodiphosphate (GppNHp), forskolin and prostaglandin E1 were obtained from Sigma Chemical Co (St Louis, Mo). The AVP analogues listed in table 1 were generous gifts of Dr. Hofbauer (Ciba-Geigy, Switzerland), Dr. Mulder (Ferring AB, Malmo, Sweden) and Dr. Manning (Toledo, Ohio) or were purchased from Peninsula laboratories (San Carlos, Ca).

- Platelet particulate preparation

Individual 50 ml units of 5-day old platelet concentrates were purchased from the local blood bank. These units were prepared from 450 ml of human blood collected into polypropylene bags containing 63 ml of a citrate-phosphate-dextrose-adenine solution (CPDA each 63 ml contains 2 g glucose, 1.66 g sodium citrate anhydrous, 206 mg citric acid, 140 mg sodium biphosphate and 17.3 mg adenine). The platelet concentrates were spun at room temperature for 15 min at 180 g to sediment the residual erythrocytes. The platelet rich plasma was removed and spun at room temperature for 15 min at 1165 g to sediment platelets. The platelet pellet was suspended in 1.5 ml buffer (Tris Hcl 50 mM + EDTA 5 mM pH 7.4) then frozen in liquid nitrogen. The preparation was allowed to thaw at room temperature and transferred to high speed centrifuge tubes.

High speed centrifugation (30,000*g for 15 min at 4 degrees C) was repeated 3 times after rinsing the pellet with 5 ml of cold buffer. The washed pellet was resuspended in 2.5 ml of buffer and stored frozen at -70 degrees. All the previous steps were carried out with plastic material, and repeated saturation analysis showed that the receptor was stable under these conditions for a period of up to 3 months.

- Hormone binding assays

For the binding assays, platelet particulate aliquots were thawed and diluted (.8 to 1 mg/ml final concentration determined with Bradford's method (14)) in the assay buffer containing 50 mM Tris-HCl and 4 mM MgCl2, pH 7.4. Duplicate samples were incubated at 30 degrees in 5 ml polypropylene plastic tubes (Sarstedt, West Germany) in a final volume of 250 ul containing 1mg/ml bovine serum albumin and different concentrations of [3H]AVP ranging from .3 to 12 nM.
For saturation and competition analysis, the incubation time was 30 min. For competition experiments with the structural analogues of AVP, 13 concentrations of these compounds were added to the reaction mixture containing 1 to 2 nM [3H]AVP. Incubation was terminated by adding 5 ml of ice-cold assay buffer and free separated from bound [3H]AVP by rapid (1 ml/sec) filtration over Whatman GF/C glass fiber filters previously soaked in assay buffer plus albumin 1 mg/ml for 1 hour. The filters were rinsed 4 times with 5 ml buffer and transferred to vials containing 4 ml of Hydrofluor (National

diagnostics, N.J.) as scintillation fluid and the radioactivity was determined in a Packard Tricarb scintillation counter at an efficiency of 52%. The stability of the tracer in the incubation medium was checked before and at the end of the incubation period by HPLC and thin layer chromatography. Unspecific binding of [3H]AVP was defined as radioactivity bound to platelet particulates which was not competed by 100 nM of unlabelled AVP. Specific binding was therefore defined as total binding minus unspecific binding.

- Adenylate cyclase activity assay

Adenylate cyclase activity was determined by measuring the formation of [32P]cyclic AMP from [alpha-32P]ATP (Amersham, Des Plaines, Ill., 23 Ci/mmol)in an incubation mixture (total volume 100 ul) containing 50mM HEPES, pH 8.0, 4mM MgCl2, 0.2 mM EGTA, 100 mM NaCl, 2 mM beta-mercaptoethanol, 0.1% bovine serum albumin, 10 mM creatine phosphate, 10 U/ml creatine phosphokinase, 0.4 mM ATP, 1 mM cyclic AMP, 1 uCi of [alpha-32P]ATP, 100 uM GTP and human platelet preparation (40 ug of protein per tube). [3H]cyclic AMP (New England Nuclear, Boston, Mass, 32 Ci/mmol) was added (30,000 cpm) to determine recovery. For certain experiments (as noted in the results section of this paper), appropriate concentrations of AVP, epinephrine, prostaglandin E1 and/or forskolin were added to the incubation mixture. After incubation for 10 min at 30 degrees, 2 ml of a stopping solution (1% sodium lauryl sulfate, 10 mM cyclic AMP and 50 mM ATP) was added. [32P]cyclic AMP and

[32P]ATP were separated according to the method of Salomon et al (15) and the amount of [32P]cyclic AMP and [3H]cyclic AMP was measured in a Packard scintillation counter. Incubations were done in triplicate and the picomoles of cyclic AMP formed were calculated from the amount of [32P]cyclic AMP corrected for the recovery as determined by [3H]cyclic AMP (usually 92 ± 2%).

- Platelet aggregation measurement

Venous blood (20-40 ml) was drawn on 0.01% heparin (1000 IU/ml) by antecubital venepuncture from human volunteers who denied taking any drug for the previous 2 weeks. Platelet-rich plasma (300,000 platelets/mm3) was prepared as described above and platelet aggregation was measured at 37 degrees in a Payton aggregometer connected to a Fisher Recordall recorder. The maximal change in light transmittance after addition of AVP or analogues was determined. When antagonism of the action of AVP by specific antagonists was studied, platelets were incubated with the antagonist for 30 seconds before the addition of AVP.

- Statistical evaluation

Values given in the text, figures and tables are mean ± S.E.M. of n experiments. Data from saturation and competition analysis were analyzed using an iterative nonlinear least squares curve fitting program. The program and the mathematical models upon which the program is based are described elsewhere (16). For the saturation experiments, data analysis was done using the following equation :

$$[PL] = \Sigma \ ([Pt_i]*[Lf])/([Lf] + [Kd_i])$$

where [PL] = concentration of bound tracer, $[Pt_i]$ = total concentration of receptors i, Lf = concentration of unbound tracer and Kd_i = dissociation constant of receptor i.

Parameters estimation was compared with an increasing number of parameters and the number of parameters (n) chosen was that which resulted in a statistically significant improvement in residual variance over (n-1) parameters. This mathematical model was used with both total binding and specific binding determined in the presence of a large amount of unlabelled AVP. IC 50 values for inhibition of ^{3}H AVP binding by different agonists and antagonists were determined from dose-response curves as described and converted into Ki values according to the equation of Cheng and Prusoff (17) :

$$Ki = IC\ 50/(1+L/Kd)$$

where IC 50 is the concentration of the competing agent which inhibits specific ^{3}H AVP binding by 50%, L is the concentration of [^{3}H]AVP (1-2 nM) and Kd is the equilibrium dissociation constant for [^{3}H]AVP binding determined from saturation experiments.

For platelet aggregation experiments, the effect of AVP was analyzed using the following equation:

$$E/Emax = L/(L+EC50)$$

where E = degree of platelet aggregation induced by AVP for a given concentration, Emax = maximum effect induced by AVP, L = corresponding AVP concentration and EC50 = concentration of AVP

inducing 50% of maximum aggregation.

In the presence of a vasopressin competitive antagonist, the following equation was used:

$E/Emax = L/(L+EC50*(1+I/I50))$

where I = concentration of the competitive antagonist and I50 = affinity constant of this antagonist.

RESULTS

1) Kinetics of [3H]AVP binding to human platelet particulates: The amount of [3H]AVP that binds to human platelet particulate varies as a function of the protein concentration (figure 1). With a protein concentration between .2 and 1.5 mg/ml, a linear increase in specific binding of [3H]AVP (2 nM) was noted (r = 0.99, n = 3 exp.). The binding of [3H]AVP was also dependent on the time of incubation (figure 2). At a concentration of 2 nM [3H]AVP, the amount of specifically bound hormone increased with time and reached an equilibrium value of 78 $\pm$ 2% of total binding within 30 min. The rate constant for the pseudo first order association reaction , kobs, was 0.128 min-1, calculated from the following formula : Ln (Peq/(Peq-P)) = kobs*time. Specific binding of [3H]AVP was rapidly reversible, as shown in figure 3. In order to demonstrate reversibility, platelet particulates were incubated with 1 to 2 nM [3H]AVP for 30 min; thereafter a high concentration (100 nM final) of AVP was added to the incubation mixture and specific binding was determined at serial time intervals after AVP addition. Unlabelled AVP rapidly replaced [3H]AVP from the binding sites. After 60 min almost no [3H]AVP was specifically bound. The dissociation rate constant was 0.036 min-1, calculated from the formula : Ln (P/Peq) = k2*time.

The second order rate constant for the association reaction, k1, can be calculated from the equation : k1 =

(kobs-k2)/([3H]AVP) where [3H]AVP = concentration of tritiated AVP used in the experiment. It was 0.043*10 9 M-1*min-1. Thus, the equilibrium dissociation constant (Kd) for [3H]AVP binding determined from the ratio k2/k1 was 0.83 nM.

2) Concentration of [3H]AVP binding sites in platelet particulates:

Specfic binding of [3H]AVP augmented with increasing [3H]AVP concentrations reaching a steady state between 3 nM and 6 nM. A typical binding experiment is shown in figures 4. Computer analysis using total binding data indicated the presence of one class of high affinity binding sites (Kd = 1.01 $\pm$ 0.06 nM, maximal concentration of binding sites = 100 $\pm$ 10 fmoles of [3H]AVP per mg of protein, n = 12 exp.) plus non specific binding (0.0143 $\pm$ 0.001). Computer analysis using specific binding data (in the presence of 100 nM unlabelled AVP, final concentration) also indicated the presence of one class of high affinity binding sites with identical characteristics (Kd = 1.03 $\pm$ 0.08 nM, maximal concentration of binding sites = 100 $\pm$ 6 fmoles of [3H]AVP per mg of protein. These Kd values are in good agreement with the value obtained from kinetic studies. GppNHp (.1mM) had no significant effect on saturation experiment parameters (Kd = 1.05 $\pm$ 0.09 vs 0.96 $\pm$ 0.11 nM and maximal concentration of binding sites = 96 $\pm$ 7 vs 88 $\pm$ 10 fmol/mg protein, n = 6 exp.). Sodium chloride (150 mM) reduced the binding capacity by 50% but it did not modify Kd value (n =

6 exp.).

3) Characterization of [3H]AVP binding sites to human platelet particulates:

- Several AVP agonists were tested for their ability to compete for specific [3H]AVP binding. Analysis of the agonists' competition for [3H]AVP binding indicated the presence of a single and homogeneous class of binding sites on human platelets (figure 5). The dissociation constants for AVP agonists (n = 3 exp. for each analogue tested) in human platelet particulates are shown in table 2. Cppt4p did not modify significantly the inhibition by unlabelled AVP of [3H]AVP binding (pKd = 8.86 vs 8.72, n = 3 exp.) As indicated in figure 6, there was a significant correlation between the binding dissociation constant values of these agonists and their corresponding vasopressor activities (r = 0.87, p = 0.002), whereas there was no correlation between the same binding dissociation constants and their antidiuretic potency (r = 0.28, p = 0.47).

- Vasopressin antagonists were also tested for their ability to compete for specific [3H]AVP binding. Analysis of antagonists competition for [3H]AVP binding sites indicates a single and homogeneous class of binding sites on human platelets (n = 3 exp. for each analogue tested). The Ki values for inhibition of [3H]AVP binding to human platelet particulates by AVP antagonists are listed in table 3. There was a significant

correlation between the Ki values of these antagonists and their pA2 values for vasoconstrictive activity (r = 0.99, p = 0.007) whereas no such correlation was found for the pA2 value for antidiuretic activity. Finally, serotonin, epinephrine, norepinephrine, acetylcholine and angiotensin II (at concentrations up to 10-3 M) did not compete for [3H]AVP binding.

4) Effect of AVP on adenylate cyclase activity:

The production of cyclic AMP in platelet preparations in the presence of GTP alone was 26.35 ± 2.76 pmol/mg protein/min (figure 7a). AVP did not further stimulate cyclic AMP production with the same conditions. On the contrary, AVP reduced basal cyclase activity by respectively 2% for AVP 10-15M, 12% for AVP 10-10M and 22% for AVP 10-6M whereas epinephrine 10-4M reduced basal adenylate cyclase activity by 30% (n = 11 for the entire series of experiments).

Prostaglandin E1 10-6M increased cyclic AMP production to 465.27 ± 90.02 pmol/mg protein/min (figure 7b). Only the high concentration of AVP (10-6M) was able to reverse partially the stimulatory effect of prostaglandin E1 (-17%) to the same extent as epinephrine 10-4M (-15%), (n = 8 for the entire series of experiments).

In the presence of forskolin 10-4M, cyclic AMP production increased to 718.56 pmol/mg of protein/min (figure 7c). The addition of AVP did not significantly modify forskolin action

whereas epinephrine 10-4M partially blocked it (-9%).

5) Effect of AVP on platelet aggregation with or without specific antagonists:

AVP-induced aggregation of platelets in heparinized platelet-rich plasma in a dose-dependent fashion with EC50 = 28 ± 2 nM (n = 8 exp.). Neither the vascular antagonist d(CH2)5Tyr(Me)AVP nor the renal antagonist d(CH2)5DIleuAlaAVP had any effect on platelet aggregation when used alone (data not shown). The vascular antagonist (figures 8 and 10) potently blocked the aggregating action of AVP with a pA2 value of 8.10 ± 0.23 (n = 4 exp.), whereas much greater concentrations of the renal antagonist (figures 9 and 10) were required in order to exert the same effect (pA2 = 6.67 ± 0.12, n = 4 exp.). The I50 values for the vascular and renal antagonists in these experiments were respectively 8 ± 2 and 232 ± 4 nM, in the same order of magnitude of their corresponding Ki values derived from the competition experiments of AVP binding to platelet particulates.

DISCUSSION

This study shows that blood platelets may prove a convenient biological tool for exploration of AVP receptors in man. It also further supports Haslam's observations that human platelet AVP receptors belong to the V1 or vascular class (13). [3H]AVP specifically binds to a low capacity high affinity single class of sites on human platelet particulates. [3H]AVP specific binding is saturable with time, it is dependent on the concentration of both the ligand and protein preparation and it is reversible. The analysis of binding at equilibrium revealed an apparent dissociation constant of 1.01 nM. The dissociation constant value obtained from the kinetic experiments was 0.83 nM and that value derived from the competition experiments with unlabelled AVP was 1.22 nM. These values are consistent and are in agreement with the dissociation constant values reported in the literature for AVP specific receptors, which range from 0.4 to 38nM, varying with the type of preparation and the species investigated (9-12, 18-24). The same is true for the maximum binding capacity of our preparation (100 fmol/mg), which is within the range reported in the literature for AVP receptor concentrations in different tissues (24).

It is worth noting that the binding parameters estimates we found were identical, whether we used total binding data or specific binding data in the presence of an excess of

unlabelled hormone. It indicates that our mathematical model adequately fitted the binding parameters Kd and Bmax, therefore making it unecessary to include experimental data points with unlabelled hormone. One may consider this alternative approach when characterizing a new receptor without previous information about the affinity ratio between the high affinity binding site and non saturable binding. In that case it is difficult to choose the appropriate concentration of the unlabelled hormone which is supposed to compete only for the high affinity site.

The V1 (vascular type) and V2 (renal type) AVP receptors clearly differ with respect to the specificity of their respective binding sites and to their coupling with the adenylate cyclase system. In our study, we have used several approaches to show that AVP receptors present on human platelet membranes are of the V1 vascular type. Previous studies have shown that AVP receptors from several species discriminate among neurohypophyseal peptides and closely related synthetic structural analogues (9-11, 18-21). For the series of analogues we tested in competition experiments, we found a highly significant correlation between their relative affinities for binding to the preparations and their relative vasopressor potencies in vivo whereas no correlation between binding affinities and antidiuretic potencies was disclosed. Such specificity for the hormonal binding-effect relationship suggest that the binding sites on human platelet are of the V1

(vascular) type.

This notion is supported by the significant correlation found between the apparent affinities of AVP antagonists for binding and their corresponding vascular pA2 values and by the lack of correlation between their binding affinities and their renal pA2 values. Finally, other potent vasoactive agents such as epinephrine, norepinephrine, serotonin, acetylcholine and angiotensin II did not interfere with [3H]AVP binding, confirming the specificity of [3]AVP binding.

The effect of AVP on cyclic AMP production is a valuable tool for differentiating renal type receptors (stimulating cyclic AMP production) from vascular type receptors (acting through calcium mobilization). In this study, the absence of increased cyclic AMP production in the presence of physiological amounts of AVP and even the reduction of cyclic AMP production for higher vasopessin concentration argue against a renal type of receptor on human platelets. This inhibitory effect of AVP on platelet particulate production of cyclic AMP recently has been reported by Vanderwel et al (25). These authors also found that AVP concentration causing half maximal inhibition of adenylate cyclase activity was 1.2 $\pm$ 0.4 nM. However, previous observations using intact platelets (26) or cultured aortic smooth muscle cells (also bearing V1 AVP receptors, (9)) showed that AVP had no detectable effect on cyclic AMP production in these intact cells. Despite the fact that in both intact and broken cell preparations AVP did not stimulate cyclic AMP

production (therefore ruling out a renal type receptor) one might wonder why no cyclic AMP production is encountered with intact cells whereas cyclic AMP inhibition is reported with broken cell preparations. This suggests that unidentified factors may exist that modulate the effector system with which the AVP-V1 receptor complex interacts. In addition, further investigations are needed to find out whether the efect of AVP on cyclic AMP production is directly linked to the adenylate cyclase system or whether it is secondary to the activation of cyclic GMP-dependent protein kinase G and calcium dependent protein kinase C (27).

We confirm that AVP causes platelet aggregation with an EC50 value (28 nM) similar to that reported by Vanderwel (27 nM) using the same type of preparation (25). Thomas (28) recently reported that a selective AVP V1 antagonist (d((CH2)5AVP) and a selective V2 agonist (DDAVP) potently inhibited AVP-induced platelet aggregation. Thomas also showed that the response to partial agonists (Oxytocin, 1deaminoAVP) was enhanced by increasing the cytosolic calcium concentration and therefore concluded that platelet AVP receptors were of the V1-vascular type. We confirm this conclusion by demonstrating that AVP-induced aggregation was inhibited more efficiently by a specific vascular antagonist (pA2 = 8.10) than by a specific renal antagonist (pA2 = 6.67). Moreover, the respective pA2 values of these 2 antagonists were in close agreement with previously reported pA2 values of these agents for in vivo

antivasopressor effects (respectively 8.62 and 6.03). The close correspondence between the pA2 values of these antagonists in platelet aggregation experiments and their pKi to compete for [3H]AVP binding (respectively 8.59 and 6.93) implies that very similar if not identical receptors mediated both effects.

The values we found for AVP binding dissociation constant (1nM) and for AVP-induced platelet aggregation (28nM) are well in agreement with literature data but are different from each other. Vandenvel et al (25) also noted a difference between AVP effect on adenylate cyclase activity of platelet particulates (Ki = 1.2 nM) and AVP effect on intact platelets aggregation (EC 50 = 27 nM). This might suggest that different classes of receptors mediate both effects. However, diffrent experimental conditions (intact platelets versus platelet particulates and different temperatures of incubation, for instance) can readily explain these differences.

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TABLE 1 Vasopressin structural analogues used

Compound	Abbrevation	id
Agonists		
[8-Arginine]vasopressin	AVP	a
[8-Lysine]vasopressin	LVP	b
Oxytocin	OXY	c
Arginine-Vasotocin	AVT	d
[1-Deamino, 8-D-Arginine]vasopressin	dDAVP	e
[8-D-Arginine]vasopressin	8DAVP	f
[1-Deamino, 8-Arginine]vasopressin	dAVP	g
[2-Phenylalanine, 8-Ornithine]oxytocin	Phe2Orn8OT	h
[Deamino-Dicarba, 8-Arginine]vasopressin	dDCAVP	i
Antagonists		
[1-deaminopenicillamine, 4-Valine, 8-D-Arginine]vasopressin: dPVDAVP		j
[1-(beta-Mercapto-beta, beta-cyclopentamethylenepropionic acid), 2-(O-Methyl)Tyrosine, 8-Arginine]vasopressin: d(CH2)5Tyr(Me)AVP		k
[1-(beta-Mercapto-beta, beta-cyclopentamethylenepropionic acid), 2-D-Isoleucine, 4-Valine, 8-Arginine]vasopressin: d(CH2)5DIleuValAVP		l
[1-(beta-Mercapto-beta, beta-cyclopentamethylenepropionic acid), 2-D-Isoleucine, 4-Alanine, 8-Arginine]vasopressin: d(CH2)5DIleuAlaAVP		m

TABLE 2 Affinity constants for binding of vasopressin and agonists to human platelet membranes : relation to vasopressor and antidiuretic activities

Compound	pKi	Vasopressor Activity (U/mg)	Antidiuretic Activity (U/mg)
AVP	8.85	369	369
Phe2Orn8OT	8.54	120	.55
1dAVP	8.38	370	1390
AVT	7.92	196	274
dDAVP	7.64	23	85
LVP	7.63	270	270
dDAVP	6.90	.47	955
OXY	6.78	4	4.3
8DAVP	6.53	1.1	257

For abbrevations, see table 1. Values of pKi are the mean of 3 independent determinations obtained as indicated in the legend to figure 5. pKi = - logKi, Ki = dissociation constant (M). Pharmacological data (vasopressor and antidiuretic activities) come from references 2-4, 6, 17, 26 and 27.

TABLE 3 Inhibition constants for binding of vasopressin antagonists to human platelet membranes : relation to antivasopressor and antiantidiuretic activities

Compound	pKi	pA2 anti vasopressor	pA2 anti antidiuretic
d((CH2)5Tyr(Me)AVP	8.59	8.62	agonist (0.3 U/mg)
dPVDAVP	8.02	7.82	agonist (123 U/mg)
d((CH2)5DIleuValAVP	7.33	6.94	7.98
d((CH2)5DIleuAlaAVP	6.93	6.03	7.76

For abbreviations, see table 1. Values of pKi are the mean of 3 independent determinations obtained as indicated in the legend to figure 5 . pKi = - log Ki; Ki = dissociation constant (M). pA2 is the negative logarithm of the molar concentration of antagonist that reduces the response to 2* units of agonist to equal the response to 1* units in the absence of antagonist. Values in the table are in vivo pA2 values from references 2-4. They were estimated by assuming a volume of distribution of the antagonist equal to that of vasopressin.

^{3}H-AVP SPECIFICALLY BOUND

Figure 1. Specific binding of [3H]AVP as a function of human platelet particulate protein concentration.

Protein concentrations between .2 and 1.5 mg/ml were incubated with 2nM of [3H]AVP for 30 min at 30 degrees C. Specific binding was measured in the presence of 100 nM unlabelled hormone (n = 3 exp., r = 0.99)

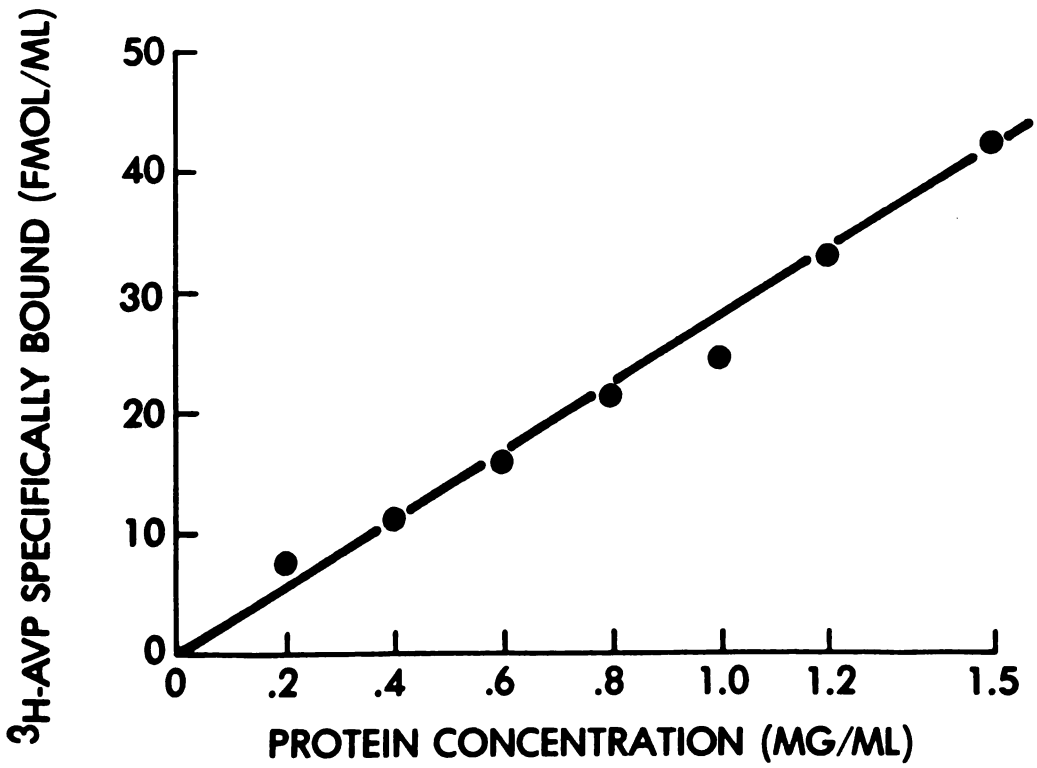


Figure 2. Specific binding of [3H]AVP to human platelet particulates as a function of time.

Platelet particulate preparations (.8 to 1mg/ml) were incubated with [3H]AVP (1-2 nM) in the presence of excess unlabelled AVP (100 nM) at 30 degrees C. and specific binding was determined at various time intervals between 2 and 90 min.

Inset: Pseudo-first order kinetic plot of [3H]AVP specific binding versus time:

Ln (Beq/(Beq-B)) = Kobs*t, where B = amount of [3H]AVP bound at each time t, Beq = amount of [3H]AVP bound at equilibrium, (n = 3 exp., Kobs = 0.128 min-1, r = 0.99).

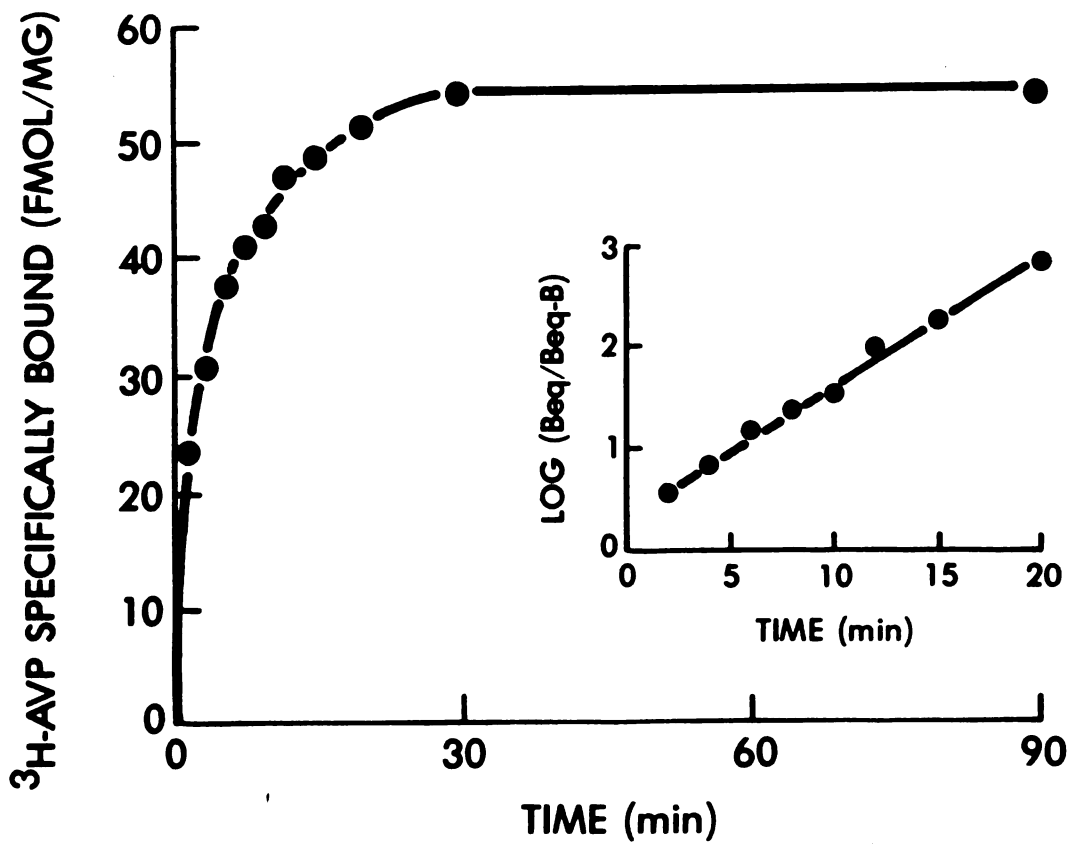
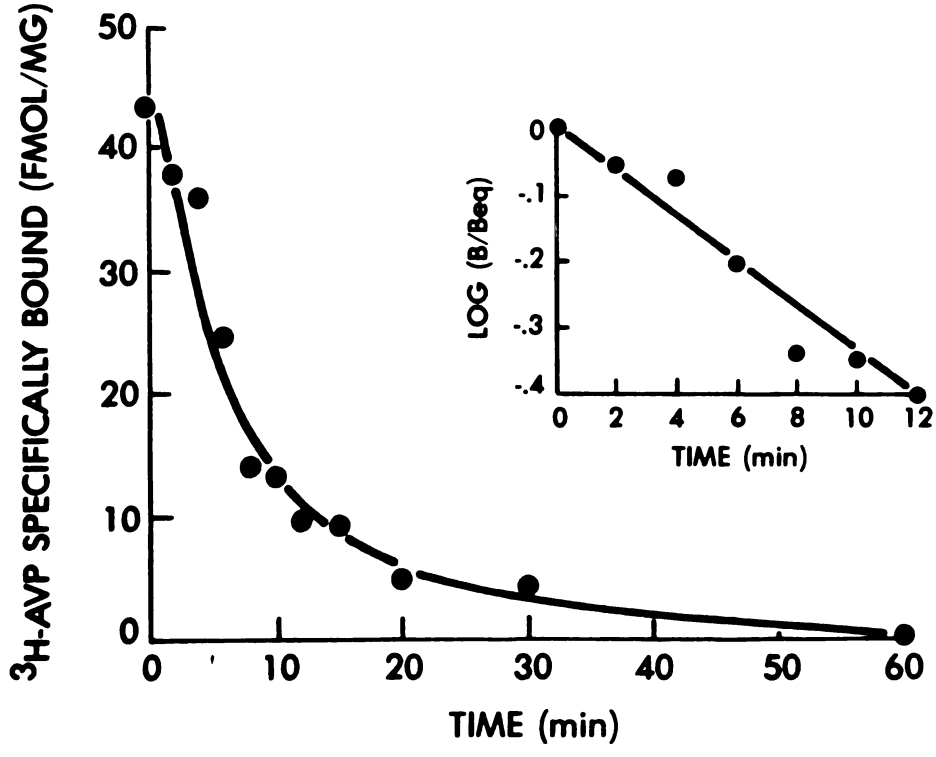


Figure 3. Reversibility of [3H]AVP specific binding to human platelet particulates.

Platelet particulate preparations (.8 to 1 mg/ml) were incubated with [3H]AVP (1-2 nM) for 30 min at 30 degrees C., after which an excess of unlabelled AVP (100nM final concentration) was added. The time of unlabelled AVP addition was defined as t = 0 and [3H]AVP specific binding was determined at the indicated subsequent time intervals.

Inset: First order kinetic plot of the dissociation of [3H]AVP binding versus time: Ln (B/Beq) = k2*t, where B = amount of [3H]AVP bound at each time t after the addition of unlabelled AVP, Beq = amount of [3H]AVP bound immediately prior to the addition of unlabelled AVP,(n = 3 exp., k2 = 0.036 min-1, r = -0.97).



Figures 4. Binding of [3H]AVP to human platelet particulates as a function of [3H]AVP concentration.

Platelet particulate preparations (.8 to 1 mg/ml) were incubated for 30 min at 30 degrees C. with various concentrations of [3H]AVP ranging from .3 to 12 nM. The figures show a typical experiment with a protein concentration = 1mg/ml.

Insets: Scatchard arrays of [3H]AVP binding. The ratio B/F of bound [3H]AVP to free [3H]AVP is plotted as function of B = bound 3H-AVP.

Figure 4a: total binding of [3H]AVP.

In that experiment, Bmax = 104 fmol/mg and Kd = 0.96 nM.

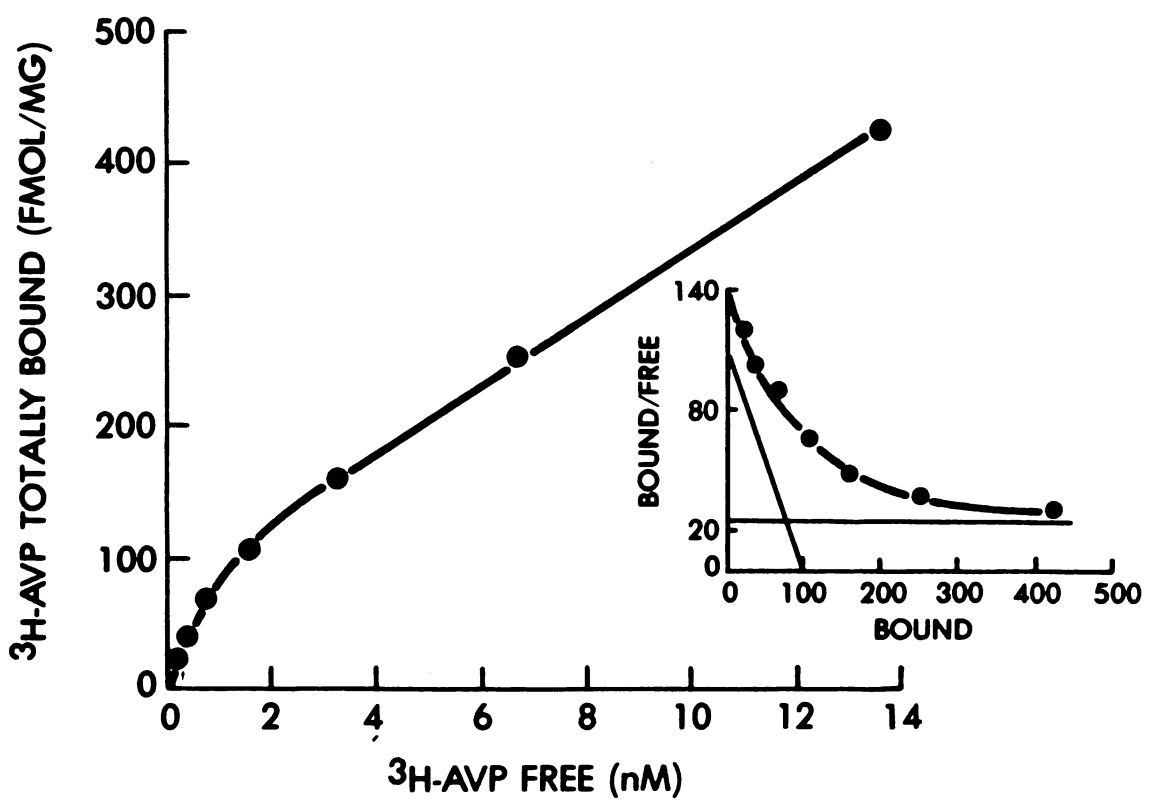


Figure 4b: specific binding of [3H]AVP in the presence of 100nM of unlabelled AVP.

In that experiment, Bmax = 96 fmol/mg and Kd = 0.85 nM.

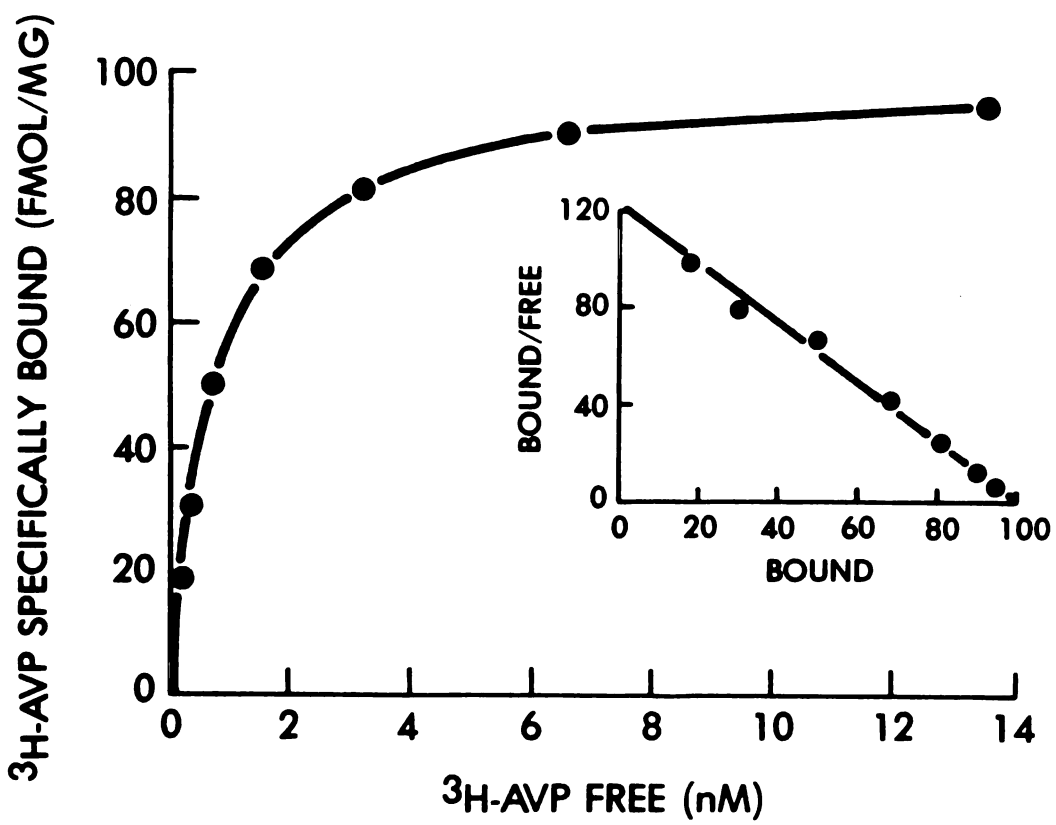
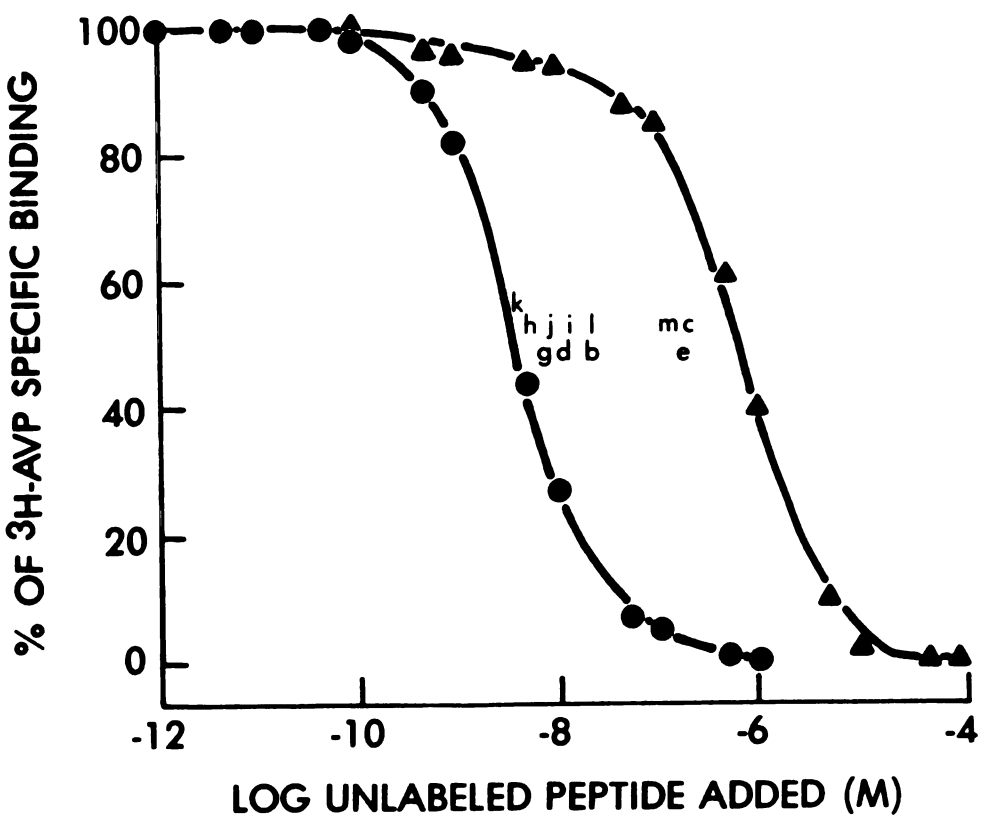


Figure 5. Inhibition by AVP analogues of [3H]AVP specific binding to human platelet particulates.

Platelet particulate preparations (.8 to 1 mg/ml) were incubated with [3H]AVP (1-2 nM) for 30 min at 30 degrees C. in the absence or presence of 13 different concentrations of the competitor, (n = 3 exp. for each analogue).

Full circles represent competition with unlabelled AVP which has the highest pKi value (8.85) whereas full triangles represent competition with dDAVP which has the lowest pKi value (6.53).

The letters stand for the pKi values of the corresponding compounds listed in table 1.



Figures 6. Relationships between AVP agonists binding to human platelet particulates and their vasopressor or antidiuretic activities in vivo.

Graphs were constructed using data given in table 2. pKi = - log Ki, Ki = binding dissociation constant at equilibrium for the corresponding analogue, (8). For vasopressor and antidiuretic activities references, see table 2.

Figure 6a: relationship between binding dissociation constant and vasopressor activity (r = 0.87, p = 0.002)

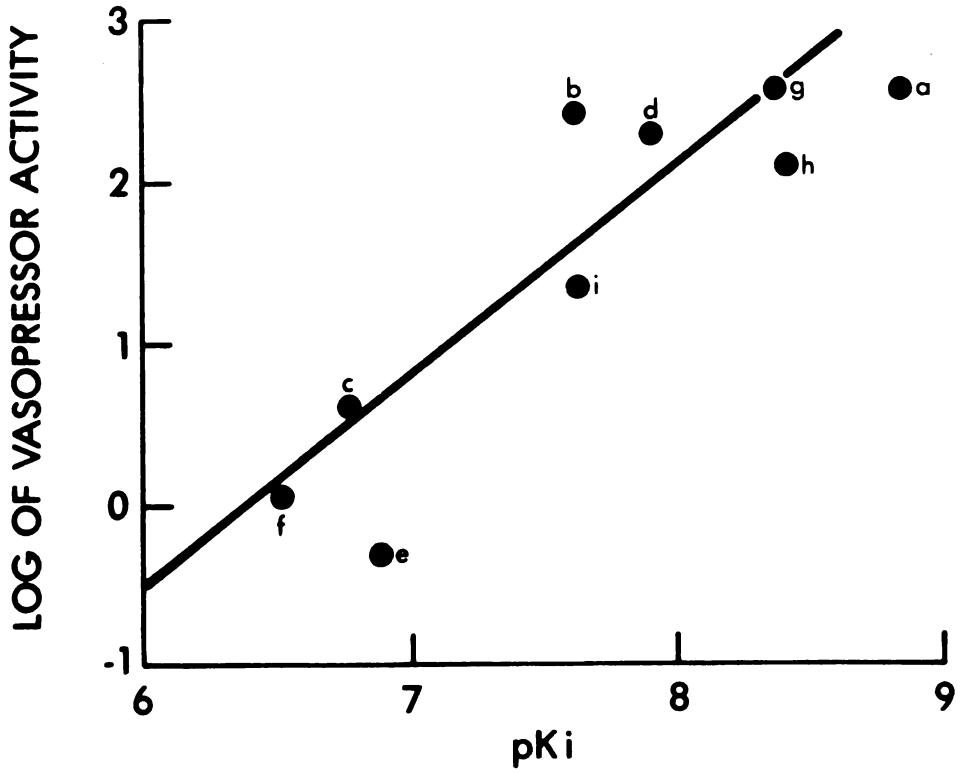
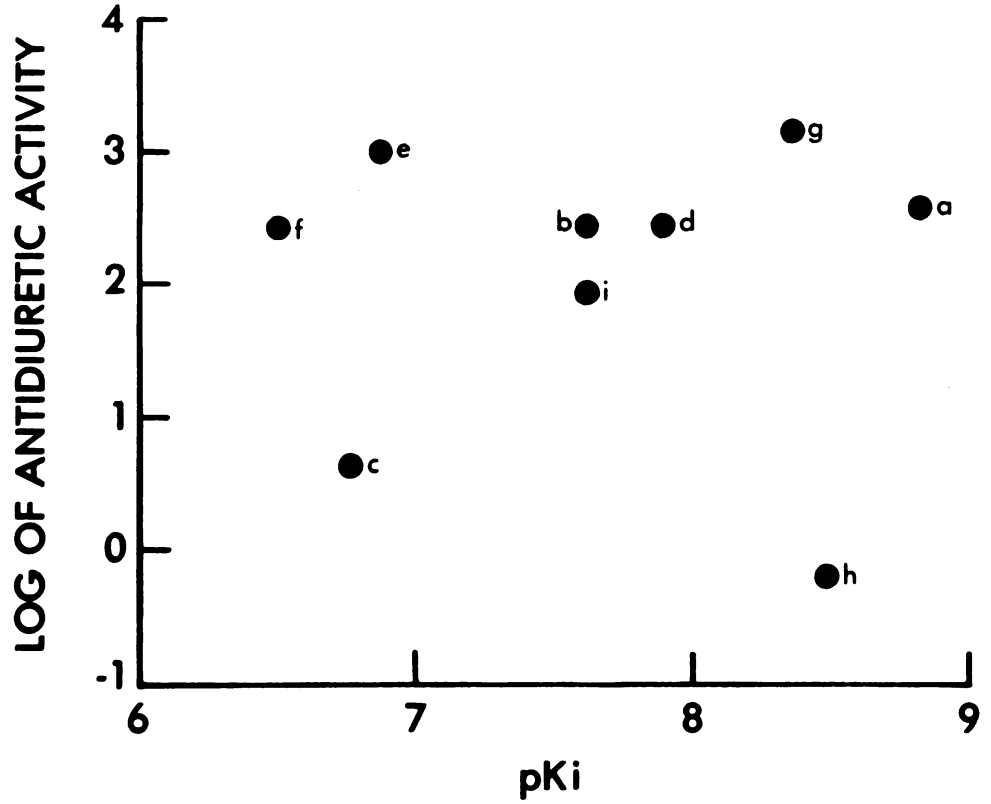


Figure 6b: relationship between binding dissociation constant and antidiuretic activity ($r = 0.28$, $p = 0.47$)



Figures 7. Effect of AVP on adenylate cyclase activity of human platelet particulates.

Platelet particulate preparations (20 to 40 ug) were incubated for 10 min at 30 degrees C. in the presence of indicated concentrations of AVP, epinephrine, prostaglandin E1 and/or forskolin.

After testing homogeneity of variance of the data with Levene's test, statistical analysis used two-way analysis of variance on blocked data and Dunnett's procedure for multiple comparisons, (* = $p < 0.05$, ** = $p < 0.01$).

Figure 7a: effect of AVP alone (n = 11).

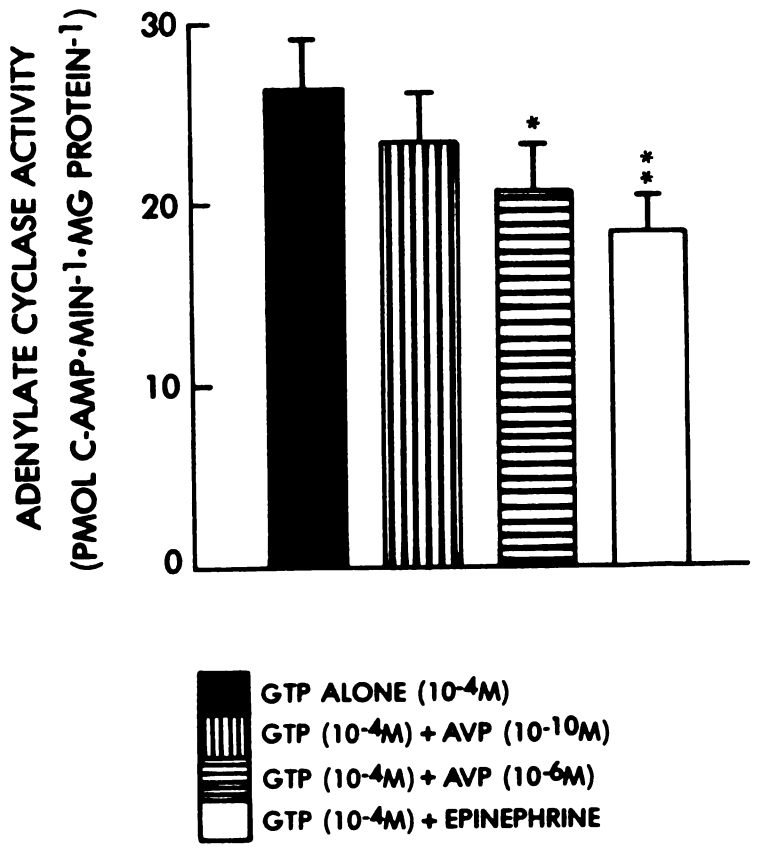


Figure 7b: effect of AVP in the presence of prostaglandin E1 (n = 8).

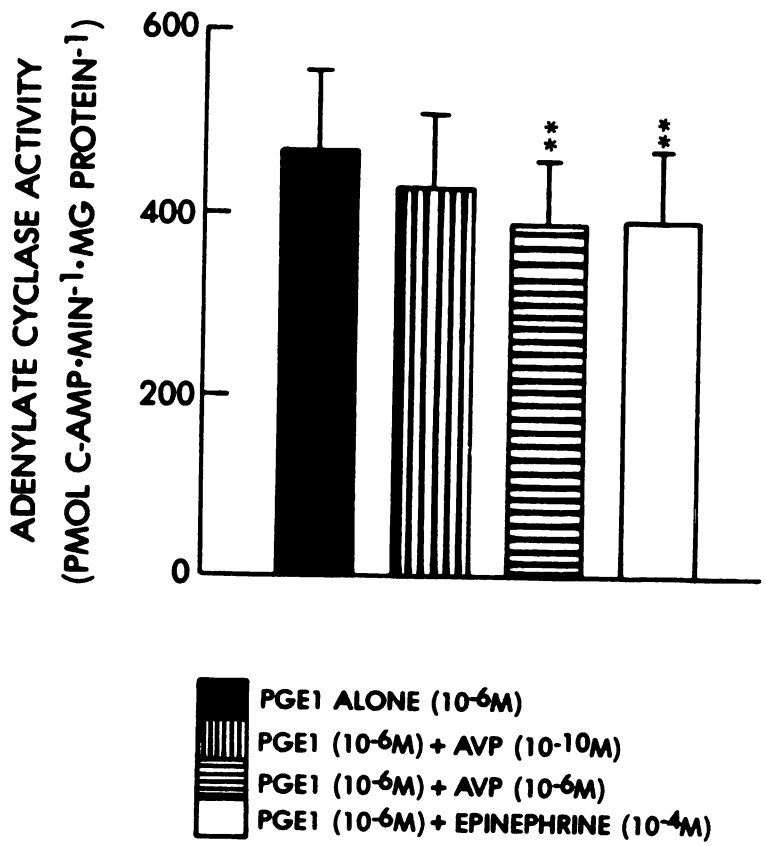


Figure 7c: effect of AVP in the presence of forskolin (n = 8).

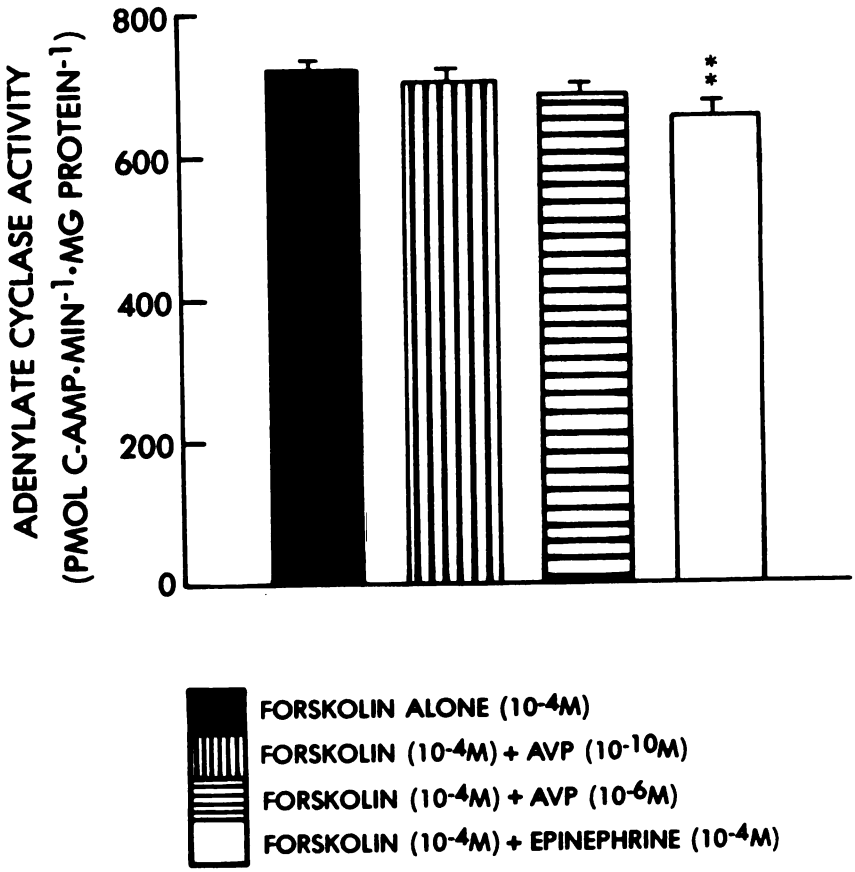


Figure 8. Aggregation of platelets in human heparinized PRP induced by AVP alone or in the presence of 4 different concentrations of the specific renal antagonist d((CH2)5DIleuValAVP.

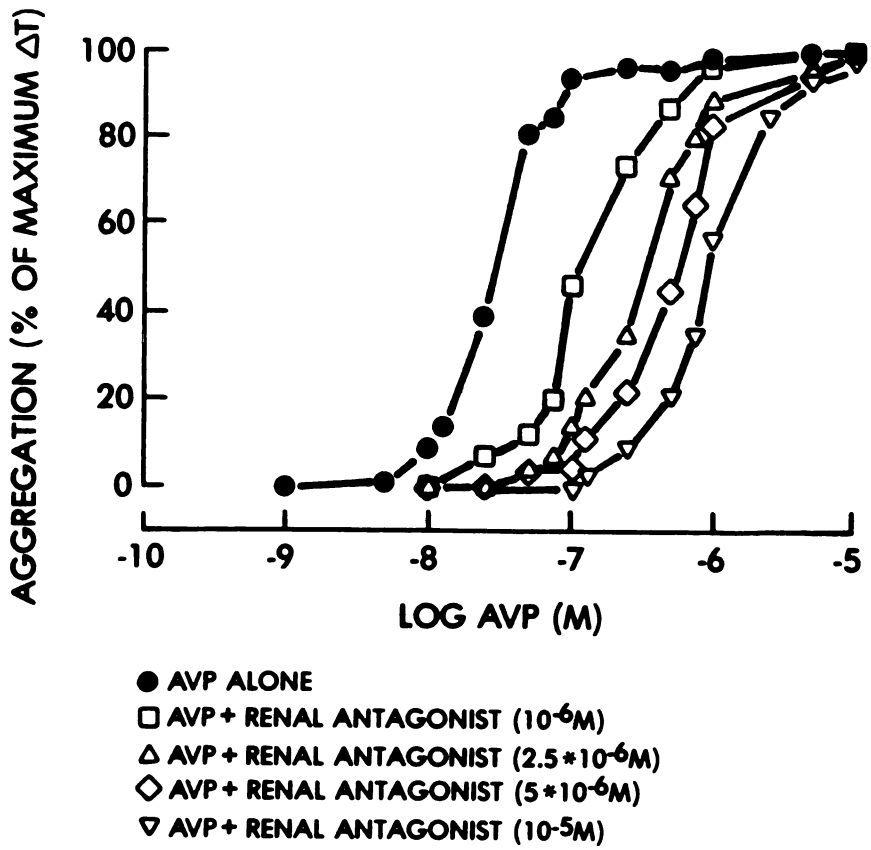


Figure 9. Aggregation of platelets in human heparinized PRP induced by AVP alone or in the presence of 4 different concentrations of the specific vascular antagonist d((CH2)5Tyr(Me)AVP.

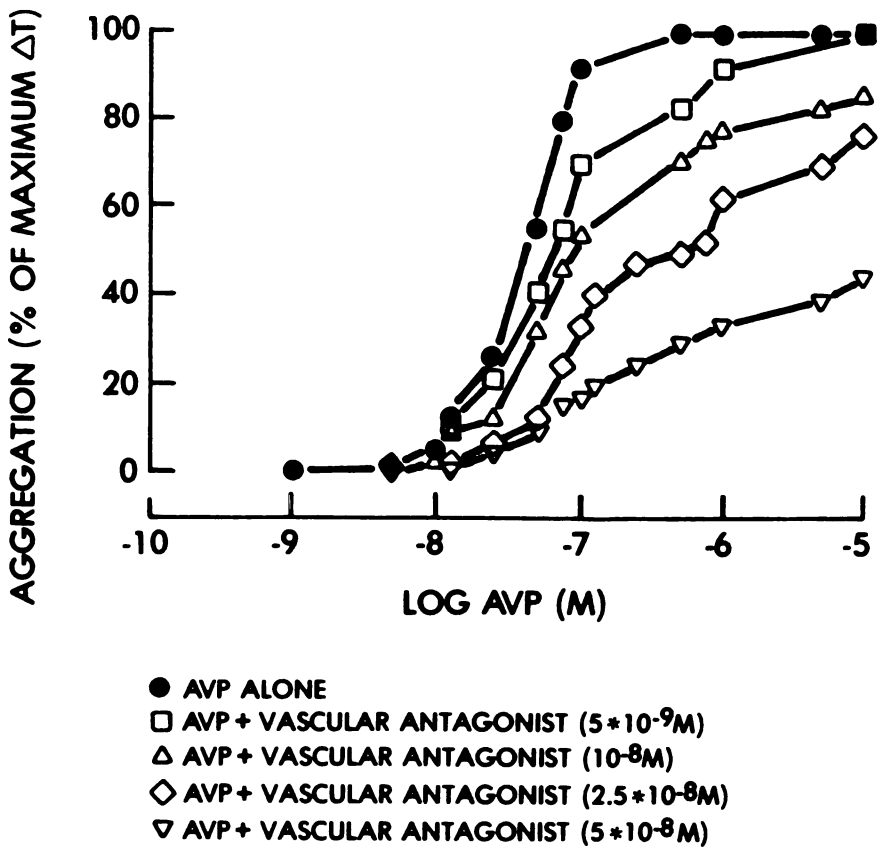
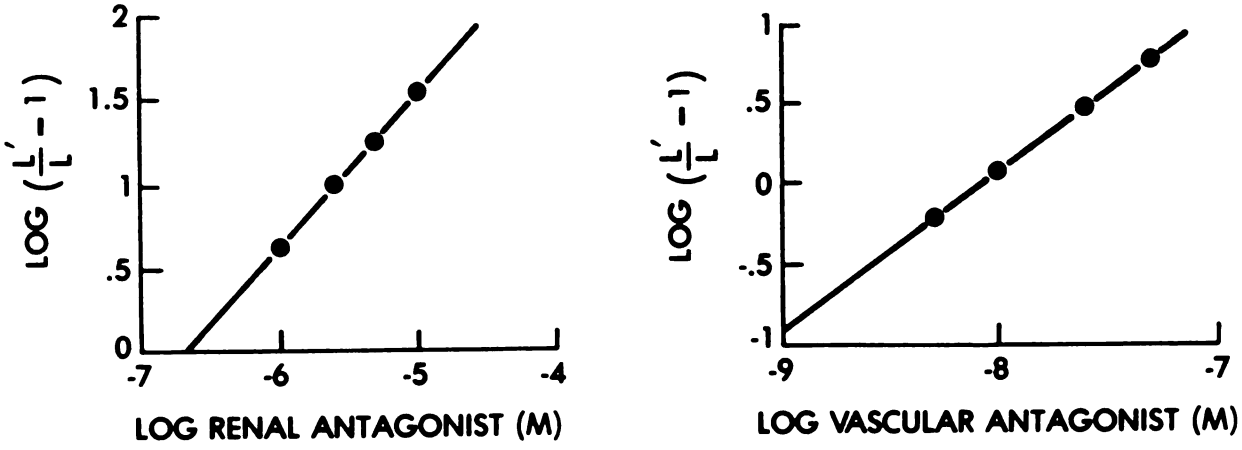


Figure 10. Shild plots of the effects of the vascular and renal antagonists on AVP-induced platelet aggregation. Log ((L'/L) - 1) = Log I - Log I50, where L and L' = concentrations of AVP alone and in the presence of the antagonist causing half-maximum platelet aggregation and pA2 = - Log I50. Values are the mean of 4 separate experiments for each antagonist. pA2 values were respectively 8.10 $\pm$ 0.23 and 6.67 $\pm$ 0.12 for the vascular and renal antagonists. The corresponding slopes are 0.99 and 0.92



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