Characterization of Human Platelet Vasopressin Receptors

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Abstract

Using tritiated arginine-8-vasopressin [^{3}H]AVP, vasopressin-specific binding sites were detected on human platelet membranes. One class of high-affinity binding sites was characterized with an equilibrium dissociation constant of 1.01 ± 0.06 nM and a maximal binding capacity of 100 ± 10 fmol/mg of protein ($n = 12$). Highly significant correlations were found between the relative agonistic ($r = 0.87$, $P = 0.002$) or antagonistic ($r = 0.99$, $P = 0.007$) vasopressor activities of a series of 13 AVP structural analogues and their relative abilities to inhibit [^{3}H]AVP binding to platelet receptors whereas no such relationship existed when antidiuretic activities were considered ($r = 0.28$, $P = 0.47$). AVP did not stimulate cyclic AMP production of human platelets; on the contrary, high AVP concentrations (10^{-6} M) inhibited cyclic AMP production measured in basal and prostaglandin E_1-stimulated conditions. AVP caused intact platelet aggregation with a half-maximal aggregation (EC_{50}) of 28 ± 2 nM. This effect was more potently reversed by the specific vascular antagonist $d(CH_2)_5Tyr(Me)AVP$ (pA2 $= 8.10\pm0.23$) than by the specific renal antagonist $d(CH_2)_5IleuAlaAVP$ (pA2 $= 6.67\pm0.12$). The pA2 values of these two antagonists in platelets are in close agreement with the pK_i values obtained in competition experiments (respectively 8.59 and 6.93) and with pA2 values reported in the literature for their in vivo antivasopressor activity (respectively 8.62 and 6.03).

The observation that human platelets bear AVP receptors belonging to the vascular class suggests that platelet receptors can be used to further explore the role of vasopressin in cardiovascular homeostasis.

Introduction

The understanding of the role of vasopressin (AVP)[1] in blood pressure regulation (1) has been aided by the development of new tools such as sensitive radioimmunoassays and, more recently, specific and potent AVP antagonists acting selectively on either the renal or vascular AVP receptors (2–4). It is now

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1. *Abbreviations used in this paper:* AVP, arginine-8-vasopressin; GppNHp, 5'-guanylylimidodiphosphate; PGE_1, prostaglandin E_1.

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well established that endogenous AVP does play a role in the maintenance of blood pressure in several experimental conditions in animals including dehydration, hemorrhage, adrenal insufficiency, or hypertension induced by desoxycorticosterone (see Reference 1 for review). In humans, the cardiovascular role of AVP is less well documented, except for the observation that high plasma and urinary AVP levels are encountered in severe forms of human hypertension (5).

Vasopressin receptors can be classified into two main types on the basis of structure activity and radioligand binding studies with AVP analogues. The first class of receptors, termed V1, appears to mediate vasoconstriction, glycogenolysis, and platelet aggregation and acts by stimulating phosphoinositide breakdown and mobilizing calcium. The second class of receptors, termed V2, activates adenylate cyclase and is responsible for the antidiuretic action of AVP. In animal preparations, smooth muscle cells, or liver extracts are used for investigating AVP specific receptors of the vascular type (6, 7), whereas kidney preparations are used to study AVP renal type receptors (8). These tissues are rarely available from man. For this reason, Block et al. (9) used human mononuclear phagocytes to characterize specific AVP receptors which are presumably of the renal type. Because Haslam and Rosson (10) showed that vasopressin 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 [^{3}H]arginine-8-AVP ([^{3}H]AVP). The pharmacologic 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 humans.

Methods

Platelet particulate preparation. Individual 50-ml units of 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 (each 63 ml contains 2 g of glucose, 1.66 g of sodium citrate anhydrous,

Compound	Abbreviation	
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-orni-thine]oxytocin	Phe2Orn8OT	h
[Deamino-dicarba, 8-argi-nine]vasopressin	dDCAVP	i
Antagonists		
[1-deaminopenicillamine, 4-valine, 8-D-arginine]vasopressin	dPVDAVP	j
[1-(β-mercapto-β, β-cyclopenta-methylenepropionic acid), 2-(O-methyl)tyrosine, 8-argi-nine]vasopressin	d(CH$_2$)$_5$Tyr(Me)AVP	k
[1-(β-mercapto-β, β-cyclopenta-methylenepropionic acid), 2-D-iso-leucine, 4-valine, 8-argi-nine]vasopressin	d(CH$_2$)$_5$DIleuValAVP	l
[1-(β-mercapto-β, β-cyclopenta-methylenepropionic acid), 2-D-iso-leucine, 4-alanine, 8-argi-nine]vasopressin	d(CH$_2$)$_5$DIleuAlaAVP	m

206 mg of citric acid, 140 mg of sodium biphosphate, and 17.3 mg of 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 1,165 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°C) was repeated three 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°C. 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 mo.

Intact platelets were also prepared for binding experiments. The platelet-rich plasma pellet (instead of being frozen for particulate preparation) was resuspended in buffer (Tris-HCl 50 mM, pH 7.4, NaCl 138 mM, EDTA 5 mM) and then centrifuged twice at 16,000 g for 15 min at 25°C. The washed intact platelets (1 × 10^6/μl) were resuspended in assay buffer and used the same day for binding experiments.

Hormone binding assays. For the binding assays, platelet particulate aliquots were thawed and diluted (0.8 to 1 mg/ml final concentration determined with Bradford's method (11) in the assay buffer containing 50 mM Tris-HCl and 4 mM MgCl$_2$, pH 7.4. Duplicate samples were incubated at 30°C in 5 ml polypropylene plastic tubes (Sarstedt, West Germany) in a final volume of 250 μl containing 1 mg/ml bovine serum albumin and different concentrations of [^{3}H]AVP ranging from 0.3 to 15 mM.

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 1–2 nM [^{3}H]AVP. Incubation was terminated by adding 5

ml of ice-cold assay buffer and free separated from bound [^{3}H]AVP by rapid (1 ml/s) filtration over Whatman GF/C glass fiber filters (Whatman Chemical Separation, Clifton, NJ) previously soaked in assay buffer plus albumin 1 mg/ml for 1 h. The filters were rinsed four times with 5 ml of buffer and transferred to vials containing 4 ml of Hydrofluor (National Diagnostics, Inc., Somerville, NJ) as scintillation fluid and the radioactivity was determined in a Packard Tricarb scintillation counter (Packard Instrument Co., Inc., Downers Grove, ILL) 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 high pressure liquid chromatography and thin-layer chromatography. In saturation experiments, unspecific binding of [^{3}H]AVP was defined as radioactivity bound to platelet particulates which was not competed by 100 nM of unlabeled 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 [^{32}P]cyclic AMP from [α-^{32}P]ATP (Amersham Corp., Des Plaines, IL, 23 Ci/mmol) in an incubation mixture (total volume 100 μl) containing 50 mM Hepes, pH 8.0, 4 mM MgCl$_2$, 0.2 mM EGTA, 100 mM NaCl, 2 mM β-mercaptoethanol, 0.1% bovine serum albumin, 10 mM creatine phosphate, 10 U/ml creatine phosphokinase, 0.4 mM ATP, 1 mM cyclic AMP, 1 μCi of [α-^{32}P]ATP, 100 μM GTP, and human platelet preparation (40 μg of protein per tube). [^{3}H]cyclic AMP (New England Nuclear, 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 E$_1$, and/or forskolin were added to the incubation mixture. After incubation for 10 min at 30°C (no time course study of cyclic AMP production was done), 2 ml of a stopping solution (1% sodium lauryl sulfate, 10 mM cyclic AMP, and 50 mM ATP) was added. [^{32}P]cyclic AMP and [^{32}P]ATP were separated according to the method of Salomon et al. (12) and the amount of [^{32}P]cyclic AMP and [^{3}H]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 [^{32}P]cyclic AMP and corrected for the recovery as determined by [^{3}H]cyclic AMP (usually 92±2%).

Platelet aggregation measurement. Venous blood (20–40 ml) was drawn on 0.01% heparin (1,000 IU/ml) by antecubital venipuncture from human volunteers who denied taking any drug for the preceding 2 wk. Platelet-rich plasma (300,000 platelets/mm^3) was prepared as described above and platelet aggregation was measured at 37°C in a Payton aggregometer (Payton Scientific, Inc., Buffalo, NY) connected to a Fisher Recordall recorder (Fisher Scientific Co., Pittsburgh, PA). 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 s before addition of AVP.

Statistical evaluation. Values given in the text, figures, and tables are mean±SEM 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 (13). For saturation experiments, data analysis was done using the following equation:

$$[PL] = \sum_{i=1}^{m} ([Pt_i] \times [Lf])/([Lf] + [K_{d_i}]),$$

where $[PL]$ = concentration of bound tracer, $[Pt_i]$ = total concentration of receptors i, Lf = concentration of unbound tracer, and K_{d_i} = dissociation constant of receptor i. Parameters were chosen which yielded the best data fit as determined by minimal variance of experimental data about the curve generated by these parameters. Parameter estimation was compared at increasing number of parameters and the number of parameters (n) chosen was that with no statistically significant difference in residual variance over (n + 1) parameters. This mathematical model was used with both total binding and specific binding determined in the presence of unlabeled AVP in excess.

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 K_i values according to the equation of Cheng and Prusoff (14):

$$K_i = IC_{50}/(1 + Lf/K_d),$$

where IC_{50} is the concentration of the competing agent that inhibits specific [³H]AVP binding by 50%; Lf is the concentration of [³H]AVP (1-2 nM); and K_d is the equilibrium dissociation constant for [³H]AVP binding determined from saturation experiments.

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

$$E/E_{max} = L/(L + EC_{50}),$$

where E = degree of platelet aggregation induced by AVP for a given concentration, E_{max} = maximum effect induced by AVP, L = corresponding AVP concentration, and EC_{50} = concentration of AVP inducing 50% of maximum aggregation.

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

$$E/E_{max} = L/(L + EC_{50} \times [1 + I/I_{50}]),$$

where I = concentration of the competitive antagonist and I_{50} = affinity constant of this antagonist.

Results

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 (Fig. 1). With a protein concentration between 0.2 and 1.5 mg/ml, a linear increase in total and specific binding of [³H]AVP (1-2 nM) was noted ($r = 0.99$, seven different concentrations, $n = 3$). Specific binding accounted for 52±2% of total binding. The binding of [³H]AVP was also dependent on the time of incubation (Fig. 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±2% of total binding within 30 min whereas non-specific binding increased linearly with time ($r = 0.95$, 11 different time points, from 2 to 120 min, $n = 3$). The rate constant for the pseudo first-order association reaction, K_{obs}, was 0.128 min⁻¹, calculated from the following formula: Ln $(B_{eq}/(B_{eq} - B))$ = $K_{obs} \times$ time. Specific binding of [³H]AVP was rapidly reversible, as shown in Fig. 3. To demonstrate reversibility, platelet particulates were incubated with 1-2 nM [³H]AVP for 30 min; thereafter a high concentration (100 nM final) of unlabeled AVP was added to the incubation mixture and specific binding was
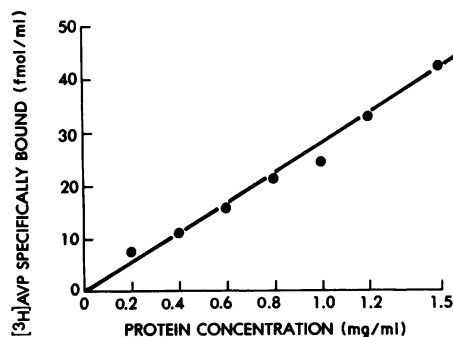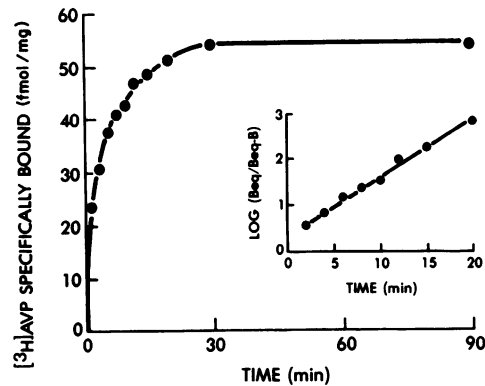
Figure 2. Specific binding of [³H]AVP to human platelet particulates as a function of time. Platelet particulate preparations (0.8-1 mg/ml) were incubated with [³H]AVP (1-2 nM) in the presence or absence of excess unlabeled AVP (100 nM) at 30°C and specific binding was determined at various time intervals between 2 and 90 min. (*Inset*) Pseudo first-order kinetic plot of [³H]AVP-specific binding versus time: Ln $(B_{eq}/(B_{eq} - B))$ = $K_{obs} \times t$, where B = amount of [³H]AVP bound at each time t, B_{eq} = amount of [³H]AVP bound at equilibrium, ($n = 3$, $K_{obs} = 0.128$ min⁻¹, $r = 0.99$).

determined at serial time intervals after AVP addition. Unlabeled 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 (B/B_{eq}) = $K_2 \times$ time. Dissociation experiments were also carried out with infinite dilution after binding (addition of 20 ml of buffer prewarmed at 30°C after the initial 30-min incubation period). The dissociation rate constant derived from this series of experiments was 0.039 min⁻¹, close to the value obtained from dissociation experiments performed with cold AVP in excess.

The second-order rate constant for the association reaction, K_1, can be calculated from the equation: K_1 = $(K_{obs} - K_2)/$
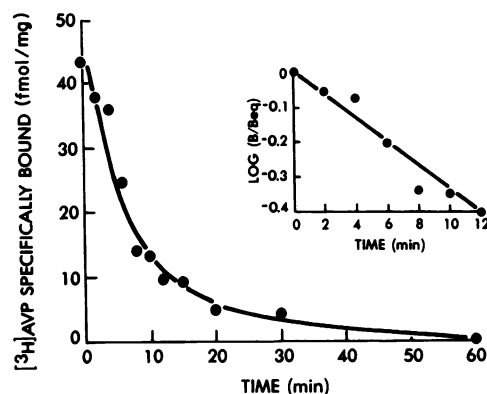
Figure 3. Reversibility of [³H]AVP-specific binding to human platelet particulates. Platelet particulate preparations (0.8-1 mg/ml) were incubated with [³H]AVP (1-2 nM) for 30 min at 30°C, after which an excess of unlabeled AVP (100 nM final concentration) was added. The time of unlabeled AVP addition 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/B_{eq}) = $K_2 \times t$, where B = amount of [³H]AVP bound at each time t after the addition of unlabeled AVP, B_{eq} = amount of [³H]AVP bound immediately prior to the addition of unlabeled AVP, ($n = 3$, $K_2 = 0.036$ min⁻¹, $r = -0.97$).

Figure 1. Specific binding of [³H]AVP as a function of human platelet particulate protein concentration. Protein concentrations between 0.2 and 1.5 mg/ml were incubated with 2 nM of [³H]AVP for 30 min at 30°C. Specific binding was measured in the presence of 100 nM unlabeled hormone ($n = 3$, $r = 0.99$).

([³H]AVP), where [³H]AVP = concentration of tritiated AVP used in the experiment. It was 0.043 × 10⁹ M⁻¹ × min⁻¹. Thus, the equilibrium dissociation constant (K_d) for [³H]AVP binding determined from the ratio K_2/K_1 was 0.83 nM.

Concentration of [³H]AVP binding sites in platelet particulates. Total binding for a series of 12 saturation experiments, expressed as a fraction of total ligand concentration, ranged from 7.8±0.2% to 1.9±0.01%, when tracer concentration was increased from 0.21±0.02 nM to 15.04±0.95 nM. Nonspecific binding increased linearly from 27±3% to 70±4% of total binding when free ligand concentration was raised from 0.21 to 15.04 nM (r = 0.99).
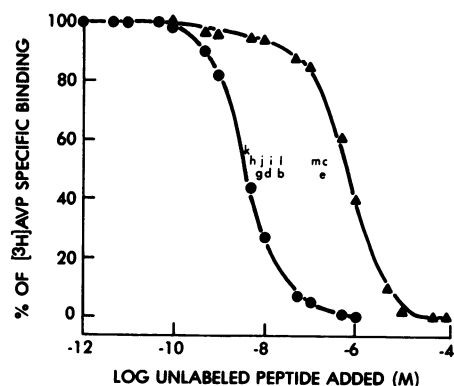
Specific binding of [³H]AVP increased with [³H]AVP concentrations reaching a steady state between 3 nM and 6 nM. A typical binding experiment is shown in Fig. 4. Computer analysis using total binding data indicated the presence of one class of high-affinity binding sites (K_d = 1.01±0.06 nM, maximal concentration of binding sites = 100±10 fmol of [³H]AVP per mg of protein, n = 12) plus nonspecific binding (0.0143±0.001). As predicted, computer analysis of "specific binding" data (in the presence of 100 nM unlabeled AVP, final concentration) also indicated the presence of one class of high-affinity binding sites with identical characteristics (K_d = 1.03±0.08 nM, maximal concentration of binding sites = 100±6 fmol of [³H]AVP per mg of protein. These K_d values are in good agreement with the value obtained from kinetic studies. GppNHp (0.1 mM) had no significant effect on saturation experiment parameters (K_d = 1.05±0.09 vs. 0.96±0.11 nM and maximal concentration of binding sites = 96±7 vs. 88±10 fmol/mg protein, n = 6). A series of six paired saturation experiments was also performed with both platelet particulates and intact platelets prepared from the same units of platelet-rich plasma. For membrane particulates, the K_d was 0.90±0.06 nM and the maximal concentration of binding sites was 73±10 fmol/mg protein. For intact platelets, the K_d was 0.42±0.01 nM and the maximal concentration of binding sites was 38±7 fmol/mg protein, which corresponds to an average of 82 fmol/10⁶ platelets.

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 homogenous class of binding sites on human platelets (Fig. 5). The dissociation constants for AVP agonists (n = 3 for each analogue tested) in human platelet particulates are shown in Table II. GppNHp did not significantly modify unlabeled AVP inhibition of [³H]AVP binding (pK_d = 8.86 vs. 8.72, n = 3 exp.). As indicated in Fig. 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 constant 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 on human platelets (n = 3 for each analogue tested). The K_i values for inhibition of [³H]AVP binding to human platelet particulates by AVP antagonists are listed in Table III. There was a significant correlation between the K_i values of these antagonists and their pA2 values for the antivasoconstrictive activity (r = 0.99, P = 0.007), whereas no such correlation was found for the pA2 value for the antidiuretic activity. Finally, serotonin, epinephrine, norepinephrine,
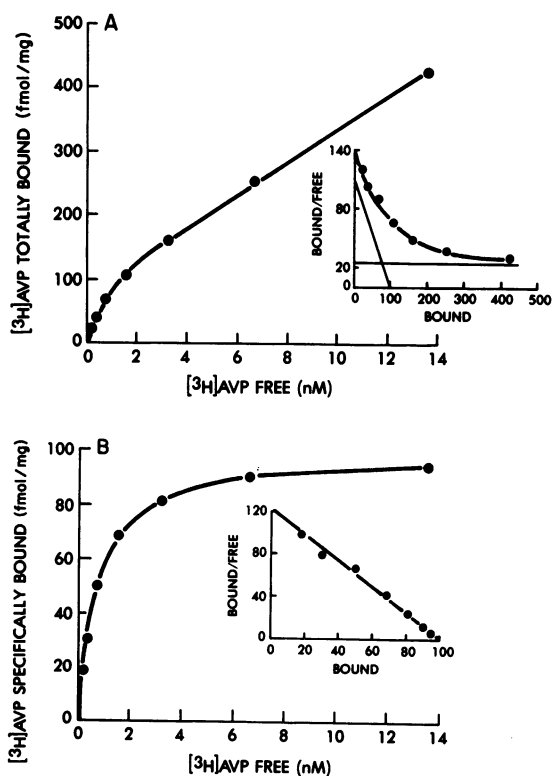
Figure 4. Binding of [³H]AVP to human platelet particulates as a function of [³H]AVP concentration. Platelet particulate preparations (0.8–1 mg/ml) were incubated for 30 min at 30°C with various concentrations of [³H]AVP ranging from 0.3 to 15 nM. The sections show a typical experiment with a protein concentration = 1 mg/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. (*A*) Total binding of [³H]AVP. In that experiment, B_{max} = 104 fmol/mg and K_d = 0.96 nM. (*B*) Specific binding of [³H]AVP in the presence of 100 nM of unlabeled AVP. In that experiment, B_{max} = 96 fmol/mg and K_d = 0.85 nM.

Figure 5. Inhibition by AVP analogues of [³H]AVP-specific binding to human platelet particulates. Platelet particulate preparations (0.3 to 1 mg/ml) were incubated with [³H]AVP (1–2 nM) for 30 min at 30°C in the absence or presence of 13 different concentrations of the competitor, (n = 3 for each analogue). Solid circles represent competition with unlabeled AVP which has the highest pK_i value (8.85), whereas solid triangles represent competition with 8DAVP, which has the lowest pK_i value (6.53). The letters stand for the pK_i values of the corresponding compounds listed in Table I. In all cases, data were best analyzed by a 2-parameter model indicating the presence of one class of binding sites without evidence of cooperativity.
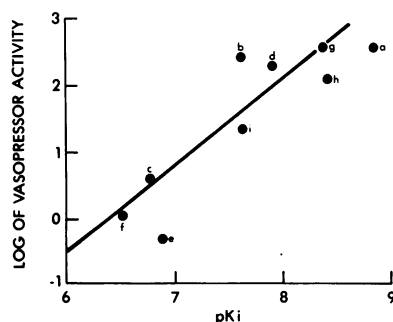
Table II. Affinity Constants for Binding of
Vasopressin and Agonists to Human Platelet Membranes:
Relation to Vasopressor and Antidiuretic Activities

Compound	pK_i	Vasopressor activity	Antidiuretic activity
		U/mg	U/mg
AVP	8.85	369	369
Phe2Orn8OT	8.54	120	0.55
dAVP	8.38	370	1390
AVT	7.92	196	274
dDCAVP	7.64	23	85
LVP	7.63	270	270
dDAVP	6.90	0.47	955
OXY	6.78	4	4.3
8DAVP	6.53	1.1	257

For abbreviations, see Table I. Values of pK_i are the mean of three independent determinations obtained as indicated in the legend to Fig. 5. $pK_i = -\log K_i$, K_i = dissociation constant (M). Pharmacologic data (vasopressor and antidiuretic activities) come from References 2–4, 6, 17, 29, and 30.

acetylcholine, and angiotensin II (at concentrations up to 10^{-3} M) did not compete for [^{3}H]AVP binding.

Effect of AVP on adenylate cyclase activity. The production of cyclic AMP in basal conditions was 28±3 pmol/mg of protein · min and was not altered by the addition of GTP (10^{-4} M) (26±3 pmol/mg of protein · min). Sodium chloride, by itself, did not significantly alter the basal production of cyclic AMP. AVP did not further stimulate cyclic AMP production with the same conditions (Fig. 7 *A*). On the contrary, AVP reduced basal cyclase activity by, respectively, 2% for 10^{-15} M AVP, 12% for 10^{-10} M AVP, and 22% for 10^{-6} M AVP. No further inhibition was observed for greater AVP concentrations (i.e., 10^{-5} and 10^{-4} M). The specific vascular antagonist, $d(CH_2)_5Tyr(Me)AVP(10^{-8}$ M), completely reversed the inhibitory effect of AVP, whereas 100 times larger doses (10^{-6} M) of the renal antagonist, $d(CH_2)_5DIleu^2Ala^4AVP$ were required to obtain the same result. Both antagonists alone had no influence by themselves on cyclic AMP levels. By comparison, 10^{-4} M epinephrine reduced basal adenylate cyclase activity by 30% ($n = 11$ for the entire series of experiments).

Figure 6. Relationship between AVP agonists binding to human platelet particulates and their vasopressor activities in vivo. The graph was constructed using data given in Table II. $pK_i = -\log K_i$, K_i = binding dissociation constant at equilibrium for the corresponding analogue, (M). For vasopressor activities references, see Table II.

Table III. Inhibition Constants for Binding of Vasopressin
Antagonists to Human Platelet Membranes:
Relation to Antivasopressor and Antiantidiuretic Activities

Command	pK_i	pA2 Antivasopressor	pA2 Antiantidiuretic
$d(CH_2)_5Tyr(Me)AVP$	8.59	8.62	agonist (0.3 U/mg)
dPVDAVP	8.02	7.82	agonist (123 U/mg)
$d(CH_2)_5DIleuValAVP$	7.33	6.94	7.98
$d(CH_2)_5DIleuAlaAVP$	6.93	6.03	7.76

For abbreviations, see Table I. Values of pK_i are the mean of three independent determinations obtained as indicated in the legend to Fig. 5. $pK_i = -\log K_i$; K_i = 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.

10^{-6} M PGE_1 increased cyclic AMP production to 465±90 pmol/mg of protein · min (Fig. 7 *B*). Only the greatest concentration of AVP (10^{-6} M) was able to reverse partially the stimulatory effect of PGE_1 (−17%) to the same extent as epinephrine 10^{-4} M (−15%), ($n = 8$ for the entire series of experiments).

In the presence of forskolin 10^{-4} M, cyclic AMP production increased to 719 pmol/mg of protein · min (Fig. 7 *C*). The addition of AVP did not significantly modify forskolin action whereas epinephrine 10^{-4} M partially blocked it (−9%).

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 $EC_{50} = 28±2$ nM ($n = 8$ exp.). Neither the vascular antagonist $d(CH_2)_5Tyr(Me)AVP$ nor the renal antagonist $d(CH_2)_5DIleu-AlaAVP$ had any effect on platelet aggregation when used alone (data not shown). The vascular antagonist (Figs. 8 and 10) potently blocked the aggregating action of AVP with a pA2 value of 8.10±0.23 ($n = 4$), whereas much greater concentrations of the renal antagonist (Fig. 9 and 10) were required in order to exert the same effect (pA2 = 6.67±0.12, $n = 4$). The I_{50} 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 K_i 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 the observations of Haslam and Rosson (10) that human platelet AVP receptors belong to the V1 or vascular class. [^{3}H]AVP specifically binds to a low-capacity, high-affinity single class of sites on human platelet particulates. [^{3}H]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 k-off values generated from our dissociation experiments with either cold AVP in excess (0.036 min^{-1}) or with infinite dilution (0.039 min^{-1}) fall within the range reported for other vasopressin receptor preparations (from 0.035 min^{-1} for pig renal medullary membrane receptors to 0.13 min^{-1} for pig LLC-PKI cells, cf. Table II of Reference 21). The same applies
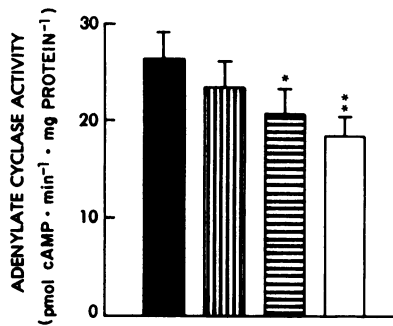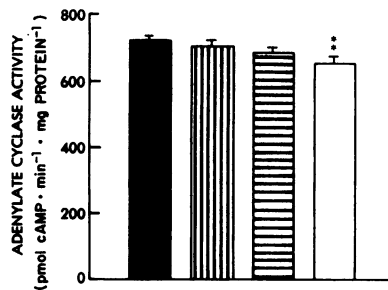
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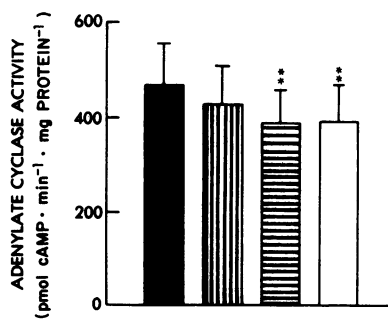
B

C

Figure 7. Effect of AVP on adenylate cyclase activity of human platelet particulates. Platelet particulate preparations (20–40 μg) were incubated for 10 min at 30°C in the presence of indicated concentrations of AVP, epinephrine, PGE_1 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, (* $= P < 0.05$, ** $= P < 0.01$). (A) Effect of AVP or epinephrine alone ($n = 11$). (B) Effect of AVP or epinephrine in the presence of PGE_1 ($n = 8$). (C) Effect of AVP or epinephrine in the presence of forskolin ($n = 8$).

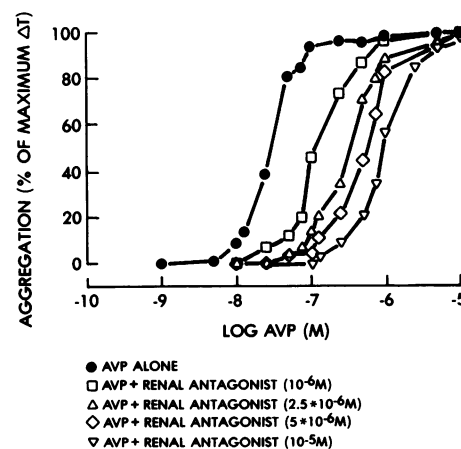Figure 8. Aggregation of platelets in human heparinized platelet-rich plasma induced by AVP alone or in the presence of four different concentrations of the specific vascular antagonist $d(CH_2)_5Tyr(Me)$ AVP.

constant value obtained from the kinetic experiments was 0.83 nM, and that value derived from the competition experiments with unlabeled 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 38 nM, varying with the type of preparation and the species investigated (6–9, 15–21). The same applies to 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 (21). It is worth noting that the binding parameters estimates that we found were identical, whether we used total binding data or so-called specific binding data in the presence of an excess of unlabeled hormone. It indicates that our mathematical model, as would be predicted, adequately fitted the binding parameters K_d and B_{max}, therefore, making it unnecessary to include experimental data points with unlabeled hormone. The traditional approach of using excess unlabeled hormone can introduce error. In addition, one may consider this alternative and practical method when characterizing a new receptor without previous information about the affinity ratio between the high-affinity binding site and nonsatu-

to our k-on value, 4.3×10^7 M$^{-1} \times$ min^{-1}, falling within the 1.1 to 6.7×10^7 M$^{-1} \times$ min^{-1} range reported by others (see Reference 21). The analysis of binding at equilibrium revealed an apparent equilibrium dissociation constant at 1.01 nM. The dissociation

Figure 9. Aggregation of platelets in human heparinized platelet rich plasma induced by AVP alone or in the presence of four different concentrations of the specific renal antagonist $d(CH_2)_5DIleuAla$ AVP.
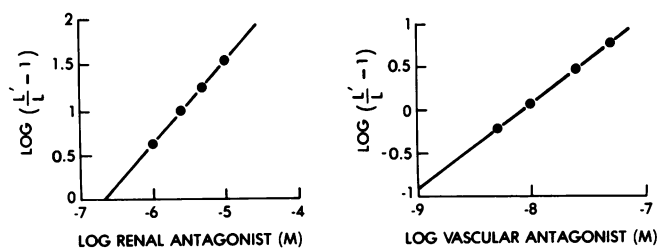
Figure 10. Shild plots of the effects of the vascular and renal antagonists on AVP-induced platelet aggregation. Log $((L'/L) - 1) = $ Log I $-$ Log I_{50}, where L and $L' = $ concentrations of AVP alone and in the presence of the antagonist causing half-maximal platelet aggregation and pA2 = Log I_{50}. Values are the mean of four separate experiments for each antagonist. pA2 values were respectively 8.10±0.23 and 6.67±0.12 for the vascular and renal antagonists. The corresponding slopes are 0.99 and 0.92.

rable binding. In that case, it is difficult to choose the appropriate concentration of the unlabeled hormone which is supposed to compete only for the high-affinity site. Saturation experiments performed with intact platelets also show the existence of one single class of low-capacity high-affinity binding sites whose capacity and affinity fit well with the values reported in the literature for other intact cells preparations (21, 22).

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 platelets 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 (6–8, 15–18). For the series of agonists 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 suggests that the binding sites on human platelets 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 [^{3}H]AVP binding, confirming the specificity of [^{3}H]AVP binding.

In competition and saturation experiments, no significant effect of a synthetic analogue of GTP, GppNHp, on the affinity and capacity of the human platelet AVP receptor was detected. Such lack of effect of this nucleotide was also found for the hepatocyte receptor and the mesenteric arteries receptor (other V1-vascular type receptors), whereas it is fully effective at the V2-renal receptor level (22). The absence of effect of GppNHp on the affinity of the platelet vasopressin receptor suggests that this receptor is not directly coupled to the adenylate cyclase system.

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 physiologic amounts of AVP and even the reduction of cyclic AMP production for a higher vasopressin concentration (known to cause a maximum increase in cAMP production by renal receptors [21, 22]) 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. (23). These authors also found that AVP concentration causing half-maximal inhibition of adenylate cyclase activity was 1.2±0.4 nM, in agreement with our results. The reduction of cAMP production by 10^{-6} M AVP was also observed in the presence of a stimulus like PGE$_1$. No increase of cAMP production by vasopressin in a preparation stimulated by forskolin is clearly distinct from the synergism of vasopressin and forskolin recently reported for renal medullary tubules (24).

However, previous observations using intact platelets (25) or cultured aortic smooth muscle cells (also bearing V1 AVP receptors [6] 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 inhibition 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 effect 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 (26). Recently, Keppens and De Wulf (27) showed that vasopressin and angiotensin enhanced the activity of liver phosphodiesterase, accounting for their cyclic AMP lowering effect. Such an effect might also explain the effect of AVP on platelet cyclic AMP levels.

We confirm that AVP causes platelet aggregation with an EC$_{50}$ value (28 nM) similar to that reported by Vanderwel et al. (23) (27 nM) using the same type of preparation. Thomas et al. (28) recently reported that a selective AVP V1 antagonist (d(CH$_2$)$_5$AVP) and a selective V2 agonist (dDAVP) potently inhibited AVP-induced platelet aggregation. This author 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 two 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 [^{3}H]AVP binding (respectively 8.59 and 6.93) implies that very similar if not identical receptors mediated both effects.

The values we found for the AVP binding dissociation constant (1 nM) and for AVP-induced platelet aggregation (28 nM) are well in agreement with data in the literature but are different from each other. Vanderwel et al. (23) also noted a difference

between AVP effect on adenylate cyclase activity of platelet particulates (K_i constant = 1.2 nM) and AVP effect on intact platelets aggregation (EC_{50} = 27 nM). This might suggest that different classes of receptors mediate the two effects. However, our data do not support this hypothesis and it is more likely that different experimental conditions (intact platelets versus platelet particulates, different temperatures of incubation, for instance) can readily explain these differences.

Acknowledgments

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