

Human Platelet Fraction Arginine-Vasopressin

Potential Physiological Role

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Abstract

Arginine-vasopressin (AVP) immunoreactivity (Ir) has been found to be elevated in platelet-rich plasma. PlatAVP was defined as platelet-rich plasma Ir minus platelet-poor plasma Ir (Pavp). PlatAVP, Pavp, and synthetic AVP were found to have identical retention time on high performance liquid chromatography analysis and similar mobility on thin-layer chromatography. During a standard osmotic suppression-stimulation test, Pavp increased with plasma osmolality (Posm, mosmol/kg H₂O); Pavp (pg/ml) = 0.98 (Posm - 274.4), $r = 0.57$, $P < 0.001$, $n = 65$; but PlatAVP was not significantly correlated with Posm and remained at 5 pg/ml. This PlatAVP concentration was estimated to represent a true intraplatelet AVP concentration of 0.4 to 3.7×10^{-9} M. Binding studies on intact human platelets demonstrated specific binding sites for [³H]AVP ($n = 16$; BMax = 98 ± 30 binding sites/platelet; $K_d = 0.72 \pm 0.24$ nM). This in vitro affinity association constant (K_d) was close to the estimated in vivo intraplatelet AVP concentration. Measurement of PlatAVP could estimate vasopressin bound to a specific platelet receptor.

Introduction

Immunoreactive plasma arginine-vasopressin (IrAVP)¹ has been found to be elevated in platelet-rich plasma (PRP) by at least two groups of investigators using different plasma extraction procedures and different specific antisera against AVP (1, 2). It is also known that large doses of AVP aggregate platelets in vitro through V1 receptors (3) and detailed pharmacological studies of these human platelet vasopressin receptors have recently been reported (4, 5).

However, the chromatographic analysis of PlatAVP compared to Pavp or synthetic AVP have not been previously reported. This step is important in order to demonstrate that IrPlatAVP has the same chromatographic elution pattern as compared to synthetic AVP and thus possibly represents true AVP associated with platelets. Furthermore, the physiological

significance of PlatAVP is undetermined: for example, the effect of osmotic stimulation on PlatAVP is unknown. Also, in vivo studies of PlatAVP after AVP administration in central diabetes insipidus patients could shed some light on the potential physiological role of PlatAVP. Finally, the results of in vitro binding studies on intact platelets should be compared to the data obtained by radioimmunoassay (RIA) of AVP on PRP.

Our studies were designed to answer the following questions: (a) is PlatAVP chromatographically identical to Pavp? (b) What is the effect of osmotic stimulation on PlatAVP? (c) Which PlatAVP levels could be achieved after vasopressin administration in patients devoided of endogenous AVP? (d) Could we compare true in vivo intraplatelet AVP concentration and in vitro binding characteristics of [³H]AVP on intact human platelets?

Methods

Plasma AVP and platelet AVP

RIA of arginine-vasopressin. Plasma and "platelet-fraction" arginine-vasopressin were obtained according to a modification of the method described by Preibisz et al. (1). Two blood samples (A and B), of 5 ml each, were obtained at each collection period and drawn in chilled EDTA containing tubes. Sample A was centrifuged at 4°C, 110 g for 20 min; 100 µl of plasma was then removed and the platelet content of this fraction was determined (Coulter counter, model P-64, Coulter Electronics Inc., Hialeah, FL). 1 ml of plasma was then frozen at -20°C and extracted within 2 to 3 wk with the acetone-petrol-ether method (6). 100 A plasma samples were randomly obtained during these studies and their platelet content was $288,000 \pm 15,000$ µl. The plasma vasopressin concentration measured in these samples was called "platelet-rich" plasma vasopressin fraction. Sample B was centrifuged at 4°C, 950 g for 20 min; 100 plasma samples B obtained during these studies contained $31,000 \pm 2,500$ platelets/µl. The plasma vasopressin concentration measured after extraction in these samples was called "platelet-poor" plasma vasopressin fraction or plasma arginine-vasopressin. The platelet-rich vasopressin fraction minus the platelet-poor vasopressin fraction was called PlatAVP.

AVP was measured by a RIA developed in our laboratory (7, 8). In all the experiments described here, the RIA sensitivity was 0.1 pg/tube and a 50% displacement of the tracer was obtained for 1.2 pg/tube. The antiserum used did not crossreact with dDAVP up to 100 pg/tube.

High performance liquid chromatographic (HPLC) analysis of PlatAVP, Pavp, and synthetic AVP

Plasma for Pavp and PlatAVP analysis were extracted with acetone and petrol-ether (7), evaporated to dryness, reconstituted in assay buffer, and injected on the HPLC column. Quantities of 15 to 50 pg of AVP were injected. Similar amounts of synthetic AVP (400–450 IU/µg, Ferring, Malmö, Sweden) were also added to plasma from patients with central diabetes insipidus, extracted, evaporated, resuspended in assay buffer, and injected on the column.

The chromatographic material (Waters Associates, Mississauga, Ontario L4V 1M5, Canada) included two solvent delivery systems (M45G and 510) and a model 660 solvent flow programmer. The column (30 cm × 3.9 mm) was packed with 10 µm octadecyl-silica (C₁₈ µBondapak,

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1. Abbreviations used in this paper: Amm Acet, ammonium acetate; AVP, arginine vasopressin; dDAVP, 1-deamino-8-D-arginine-vasopressin; DI, diabetes insipidus; Eosm, effective osmolality; HPLC, high performance liquid chromatography; IrAVP, immunoreactive plasma arginine vasopressin; Pavp, platelet-poor plasma Ir; PlatAVP, platelet-rich plasma Ir minus platelet-poor plasma Ir; Posm, plasma osmolality; PRP, platelet-rich plasma.

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27324, Waters Associates) and run at room temperature. A Guard-Pak precolumn module with disposable silica precolumn inserts was always used.

Two elution systems were chosen (9, 10): (a) trifluoroacetic acid (TFA)-acetonitrile, pH 3.5: a linear gradient of 10–50% B was used. Solvent A was 0.1% TFA pH 3.5 and solvent B 75% acetonitrile in 0.1% TFA; (b) Ammonium acetate (Amm Acet)-methanol, pH 6.5: a linear gradient of 40–67% B was used. Solvent A was 0.05 M Amm Acet pH 6.5 and solvent B was 75% methanol in 0.05 M Amm Acet.

In control experiments we determined that, in these two elution systems, tritiated arginine-vasopressin (40 Ci/mM, New England Nuclear, Boston, MA), at a concentration range from 25 to 75 pg (2,500 to 7,500 dpm) was eluted following a constant pattern.

The elution reagents were HPLC grade (Fisher or Canlab, Montréal, Québec, Canada). All the solutions used in this HPLC analysis were filtered through a 0.45- μ m filter (Millipore, Mississauga, Ontario, Canada, or Sartorius, Palaiseau, France) and degassed. Before the injection of any sample, the column was equilibrated with the initial conditions for at least 1 h and a control gradient, i.e., without any injection, was performed. Following each gradient with an injection, a linear gradient up to 99% B was performed to "wash" the column.

The flow rate was 1 ml/min and the samples were eluted during 40 min. The samples from the control and from the experimental gradients were collected every minute and, after evaporation to dryness, resuspended in assay buffer. IrAVP was then measured in duplicate in each collected fraction. For this purpose, and to control for the possible interference in our RIA system of nonvolatile products like Amm Acet or TFA, standard curves for the AVP RIA were pipetted in the final composition of the two elutions systems used, evaporated to dryness, and reconstituted in assay buffer. Neither of the two elution systems used interfered significantly with our RIA of AVP but the sensitivity was 0.5 pg/tube of AVP for acetonitrile-TFA and 0.1 pg/tube for Amm Acet-methanol.

Thin layer chromatographic (TLC) analysis of synthetic AVP, Pavp, PlatAVP, and tritiated AVP

Synthetic AVP was added to a pool of PRP from patients with central diabetes insipidus and extracted with acetone and petrol-ether as previously described (7). Plasma AVP, i.e., AVP in platelet-poor plasma was concentrated 12 times and PlatAVP was obtained as previously described. Appropriate controls with corresponding protein concentrations were prepared by the addition of [3 H]AVP in the platelet-poor or platelet-rich pool of plasma of patients with central diabetes insipidus.

We used TLC plates from Whatman, Inc., Clifton, NJ). 50- μ l samples corresponding to 20–25 pg of the AVP compound were layered. The developing solvent was a mixture of *n*-butanol, pyridine, glacial acetic acid, and water (15 vol/10 vol/3 vol/6 vol) and the chromatographic analysis for vasopressin was developed during 7–8 h at 4°C. The migration front was carefully identified and the plate was dried. Each silica plate was then divided into 0.5 cm-width bands. The powder from each band was collected, mixed in 1 ml of 0.1 N formic acid for elution and agitated during 15 h at room temperature. Each tube was then centrifuged and the supernatant was collected and evaporated. The dry residue was resuspended in assay buffer and IrAVP was measured. Standard curves for the AVP RIA were also pipetted in 0.1 N formic acid, evaporated to dryness and resuspended in assay buffer. In these conditions, the sensitivity of the assay was found to decrease to 0.5 pg of AVP per assay tube but precise standard curves were obtained for concentrations between 0.5 and 10 pg/tube. In control experiments, we determined that [3 H]AVP migrated reliably using the above conditions. For each unknown compound, retardation factors (R_f) were calculated and compared to correspondingly concentrated tritiated controls.

Waterload and hypertonic saline infusions in 9 normal subjects

Measurement of Pavp and PlatAVP. Nine normal subjects (aged 22–28, 4 men and 5 women), after an overnight fast, received a 20 ml/kg body wt waterload administered in 30–40 min, followed 2 h later by a 3%

saline infusion at 0.1 ml/kg body wt per min. Blood and urine samples were obtained every 30 min during the test. Plasma osmolality, plasma sodium, urinary osmolality, Pavp, and PlatAVP were measured with methods previously described (7, 8). Effective osmolality (Eosm) was calculated according to the formula $Eosm = Posm - (glucose/18 \text{ mg/dl} + \text{blood urea nitrogen}/2.8 \text{ mg/dl})$.

Administration of intravenous (1 mU/kg body wt) or subcutaneous (5 U) vasopressin to four patients with neurogenic diabetes insipidus (DI)

Three women (aged 24 to 47 yr) with idiopathic complete neurogenic DI and one man (age 64) with idiopathic partial DI by indirect and direct testing (11) participated in this study. They were all treated with 1-de-amino-8-D-arginine-vasopressin (dDAVP, Ferring, Malmö, Sweden), and discontinued this treatment for 48 h before each study. They were allowed to ingest ad lib. quantities of water before and during each test but solid food was not allowed for 10 h before each study. Two studies were performed at least 1 wk apart. The first consisted in the intravenous bolus injection of 1 mU/kg body wt of an aqueous solution of synthetic vasopressin (Pitressin, Parke Davis Co., Scarborough, Ontario, Canada). Blood samples were then obtained from an indwelling line from the contralateral arm for measurements of Pavp and PlatAVP at times 0, 2.5, 5, 10, 20, and 30 min. The second study consisted in the subcutaneous injection (over the deltoid area) of 5 U of aqueous vasopressin. Blood samples were obtained from an indwelling line from the contralateral arm for measurements of Pavp and PlatAVP at 0, 1, 2, 3, 4, and 5 h.

Binding studies of [3 H]AVP on intact human platelets

We prepared a human platelet suspension according to Haslam and Rosson (12) immediately before each binding experiment. Nine normal subjects (five men, four women) participated and a total of 16 binding studies were performed. Blood samples (50–100 ml) were collected in a citrate phosphate dextrose adenine solution. Blood was centrifuged at 200 *g* (15 min, room temperature) and PRP was separated from red blood cells. The PRP was then further centrifuged at 2,000 *g* (15 min, room temperature). Platelet pellets were then washed two times with phosphate buffer solution (PBS, pH = 7.4, 290 mosmol/liter) and finally resuspended in PBS. Two 100- μ l samples of PRP and two 100- μ l samples of the final platelet suspension were used for platelet counting. The protein content of the final platelet suspension was measured in $2 \times 500 \mu$ l samples using the Lowry method. PRP had $476,000 \pm 48,000$ platelets/ μ l; the platelet suspensions contained $300,000 \pm 57,000 \mu$ l and 411 ± 77 mg protein/ml, representing a relationship of 1.42 ± 0.24 mg protein/ 10^9 platelets in accordance with previous measurements (13).

Binding assays were performed at 30°C during 30 min. Triplicate samples were incubated in 4-ml polyethylene tubes (Sarstedt, Inc., Princeton, NJ), with gentle shaking in a Dubnoff incubator in a final volume of 500 μ l. Incubation conditions were 1 mM CaCl₂, 2.6 mM KCl, 1.47 mM KH₂PO₄, 0.5 mM MgCl₂ 6H₂O, 136 mM NaCl, 8 mM Na₂HPO₄ 7H₂O, 5.5 mM glucose, 0.015 mM bovine serum albumin (BSA) and $120.3 \pm 22.7 \times 10^6$ platelets (or 0.1645 ± 0.0307 mg platelet protein). Incubations were initiated by adding 50 μ l [3 H]AVP (40 Ci/mM, New England Nuclear) to obtain increasing concentrations of 0.02 to 11 nM in the medium. Controls (without platelets) and blanks (without [3 H]AVP) were performed in each experiment. Incubations were stopped by passing each medium through a PBS presoaked filter (Whatman GF/C, 24 mm) and by rinsing with 2×5 ml of 4°C PBS. Filter associated radioactivity was measured by liquid scintillation counting (Econofluor, New England Nuclear, and LS3801 counter, Beckman Instruments, Inc., Fullerton, CA) after an overnight air dryness evaporation. Specific binding was evaluated as the differences between total (without cold AVP) and nonspecific binding (with cold 10^{-6} M AVP). Nonspecific binding did not represent > 30% of total binding. Results were analyzed by Scatchard (14) and Hill (15) methods and expressed as femtomoles [3 H]AVP bound, or number of binding sites assuming a one site-one bound [3 H]AVP molecule relationship. Dose-dependent inhibition of [3 H]AVP binding

by unlabeled AVP (Ferring, batch no. BAA 252, 1 mg = 450 IU), dDAVP (Ferring, batch no. FH 3314) and d(CH₂)₅-D-TYR(Et) desglycine VAVP (SKF 101926, SmithKline & French, Philadelphia, PA) were also performed.

In preliminary experiments, we determined that vasopressin binding to intact platelets was time dependent during a 40-min observation period, reaching an equilibrium in 20 min. The number of platelets incubated (10×10^6 to 200×10^6) was also linearly correlated with the specific [³H]AVP binding obtained. Finally, we verified that no marked inactivation of [³H]AVP occurred during the incubation in the presence of intact platelets. For this purpose, 120×10^6 platelets were incubated for 30 min at 30°C in the presence of 10 nM [³H]AVP. The incubation medium was then collected and used for binding assay on fresh platelets. Total and nonspecific [³H]AVP binding were not different from the control values determined by adding [³H]AVP to the final assay medium.

Statistical analysis and calculations. Pavp and PlatAVP results during waterload and hypertonic saline infusion were analyzed according to a multiple comparison procedure for evaluating several treatments with a control (16). A linear regression analysis program was also used to estimate the slope and the threshold of the relationship Posm/Pavp or Posm/PlatAVP. Platelet poor plasma Ir and PRP Ir concentrations during administration of AVP were compared, at each individual collection period, by a paired *t* test analysis.

The estimated true intraplatelet concentration for a PlatAVP of 5

pg/ml was calculated as follows: 5 pg of AVP in 3×10^8 platelets; volume of a single platelet: 4.2 to 33.5×10^{-12} ml (17); volume of 3×10^8 platelets = 12.6 to 100.5×10^{-4} ml; intraplatelet AVP concentration = $5 \text{ pg}/12.6$ to $100.5 \times 10^{-4} \text{ ml} = 3,968$ to 497.5 pg/ml or $0.463 \times 10^{-9} \text{ M}$ to $3.7 \times 10^{-9} \text{ M}$.

The estimated number of occupied AVP binding sites on each individual platelet, for a PlatAVP concentration of 5 pg/ml, was calculated as follows:

$$5 \text{ pg of PlatAVP} = \frac{5 \times 10^{-12} \times 6.023 \times 10^{23}}{1,080} = 2.788 \times 10^9 \text{ molecules}$$

of AVP in 3×10^8 platelets: or 9.29 molecules of AVP per platelet. Results are given as mean \pm SEM unless otherwise indicated.

These studies were approved by the Ethics Committee at Hôpital du Sacré-Cœur in Montreal and all subjects gave informed consent.

Results

HPLC and TLC of plasma and "platelet-rich" plasma AVP Ir. In the first elution system used, synthetic AVP, plasma AVP and platelet AVP eluted in a major peak in fractions 25 and 26 (24–24.75% acetonitrile) (Fig. 1). At least two experiments for each compound were performed and identical results were ob-

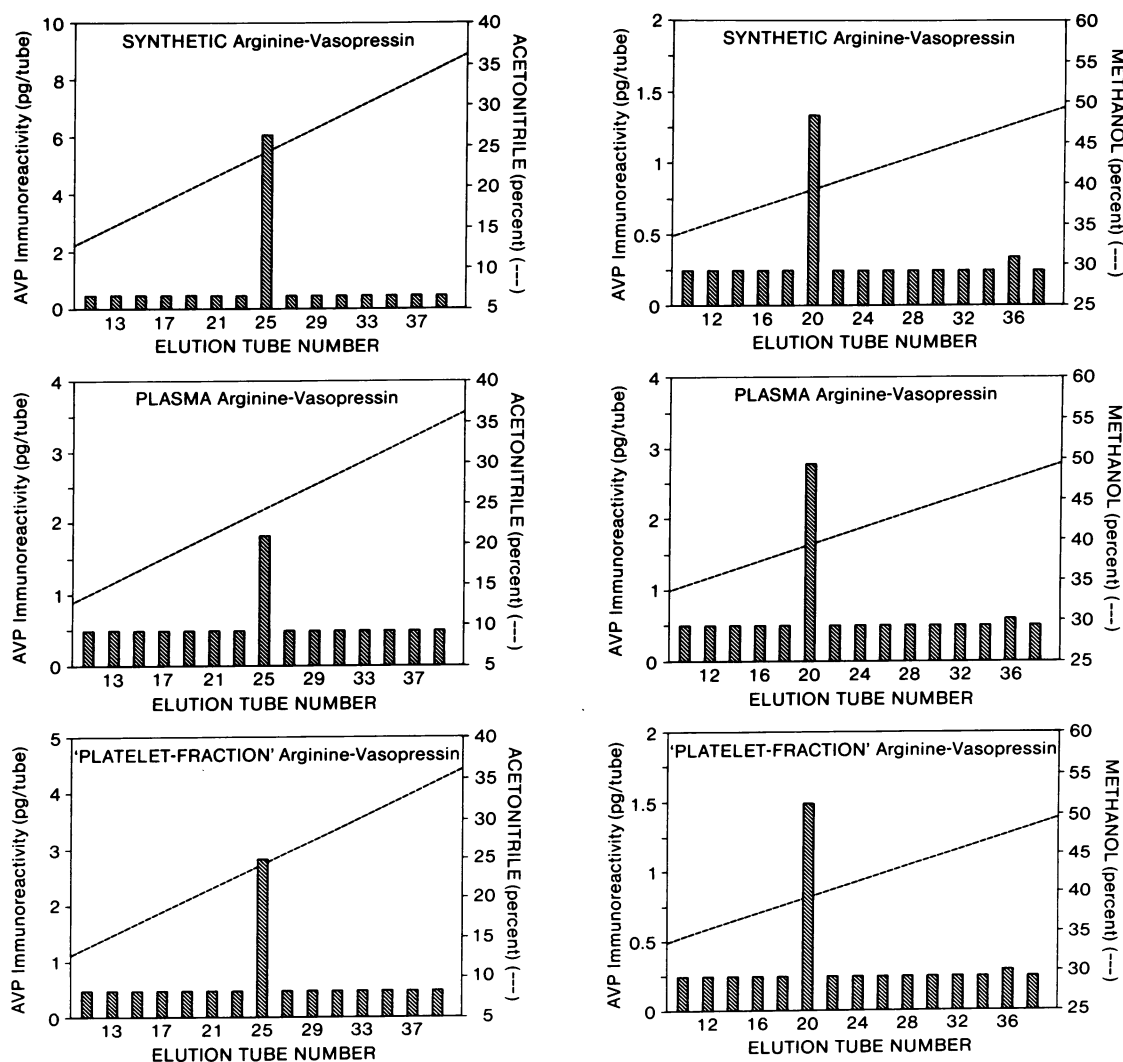


Figure 1. Platelet arginine-vasopressin, plasma arginine-vasopressin, and synthetic arginine-vasopressin have an identical retention time on HPLC analysis.

Table I. Ratio of R_f Values for Synthetic, Platelet, and Plasma AVP

| R_f unknown/ R_f control | AVP compound | Protein concentration |
|------------------------------|--------------|-----------------------|
| 0.52/0.52 = 1 | Synthetic | 1× |
| 0.51/0.52 = 0.98 | Platelet | 1× |
| 0.37/0.35 = 1.05 | Plasma | 12× |

$$R_f = \frac{\text{Distance of spot center from start point}}{\text{Distance of solvent from start point}}$$

R_f control = R_f values for [^3H]AVP placed in correspondingly concentrated plasma samples from patients with central diabetes insipidus.

The R_f value for [^3H]AVP placed in a buffer solution was 0.53.

tained; furthermore, tritiated AVP eluted similarly in the same system. In the second elution system used, a constant pattern of elution was also obtained for synthetic, plasma, platelet, or

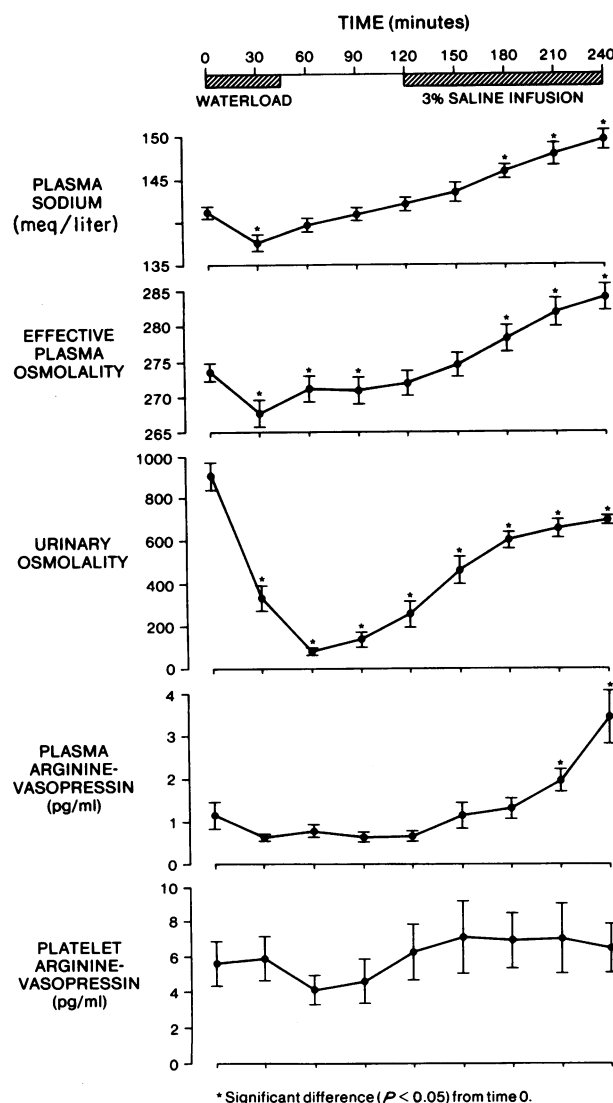


Figure 2. Effect of waterload and hypertonic saline infusion on plasma vasopressin and platelet vasopressin. Effective plasma osmolality expressed as mosmol/kg H_2O ; urinary osmolality as mosmol/kg H_2O .

tritiated AVP since a major peak was obtained in fractions 20 and 21 (38.5 to 39% methanol) and a minor peak (10% of total) was observed for each of the compounds in fraction 37 (47% methanol) (Fig. 1). At least two experiments for each compound were performed. Also, when the peak of the first HPLC analysis (acetonitrile) for tritiated, plasma, and platelet vasopressin was subjected to the second elution system (methanol), a single peak was observed in fractions 20 and 21. In TLC experiments, the mobility of plasma and platelet AVP were also found to be identical as compared to the reference compound (Table I).

Pavp and PlatAVP during waterload and hypertonic saline infusion in nine normal subjects (Fig. 2). Significant variations of Pavp were observed during the osmotic suppression-stimulation test with the consequent expected large variations in urinary osmolality values. These variations in Pavp could be described according to the linear relationship: Pavp (pg/ml) = 0.98 (Posm - 274.4) $r = 0.57$, $P < 0.001$, $n = 65$; in pg/ml: basal 1.1 ± 0.3 ; waterload 0.6 ± 0.06 , at the end of the 3% saline infusion 3.43 ± 0.7 . By contrast, PlatAVP was not correlated with Posm ($r = 0.08$): PlatAVP in pg/ml: basal: 5.7 ± 1.4 , 4.1 ± 0.8 waterload; 6.6 ± 1.4 after 3% saline). However, the individual slopes of the linear relationship Pavp/Posm were correlated with PlatAVP (Table II). A PlatAVP concentration of 5 pg/ml was estimated to represent a true platelet-AVP concentration of 500 pg to 4,000 pg/ml (i.e., 0.463×10^{-9} M to 3.7×10^{-9} M). 5 pg of PlatAVP was also estimated to represent nine molecules of AVP for each individual platelet.

Pavp and platelet-rich plasma Ir after vasopressin administration in four patients with diabetes insipidus (Fig. 3). The intravenous administration of 1 mU/kg of vasopressin resulted in identical platelet-poor plasma vasopressin Ir and PRP vasopressin Ir (upper part of Fig. 3). However, the subcutaneous administration of 5 U of Pitressin induced significantly higher PRP vasopressin Ir suggesting that a significant portion of circulating vasopressin was then associated with the platelet fraction (lower part of Fig. 3).

In vitro [^3H]AVP binding on intact platelets. After 30 min of incubation at 30°C , for a concentration of 0.754 ± 0.02 nM [^3H]AVP, specific binding values were $2.31 \pm 0.2\%$ ($n = 16$). De-

Table II. Relationship of Plasma AVP to Plasma Osmolality in Nine Normal Subjects During Waterload and Hypertonic Saline Infusion

| Subjects | Regression equation Pavp (pg/ml) = slope (Posm - threshold) | Correlation coefficient | Slope | Plat AVP pg/ml |
|--------------|---|----------------------------|-------|-------------------|
| Initials/Sex | | | | |
| SF/M | 0.065 (Posm - 277.4) | 0.91 | 0.065 | 5.96 |
| DM/M | 0.27 (Posm - 277.2) | 0.91 | 0.27 | 11.5 |
| JP/M | 0.21 (Posm - 282.8) | 0.96 | 0.21 | 2.3 |
| MP/F | 0.11 (Posm - 275) | 0.84 | 0.11 | 1.0 |
| AMG/F | 0.18 (Posm - 276.9) | 0.94 | 0.18 | 5.1 |
| AV/F | 0.41 (Posm - 276) | 0.71 | 0.41 | 9.39 |
| SM/M | 0.08 (Posm - 275.8) | 0.83 | 0.08 | 2.11 |
| FM/F | 0.08 (Posm - 280.2) | 0.88 | 0.08 | 2.37 |
| LB/F | 0.16 (Posm - 275.6) | 0.89 | 0.16 | 9.02 |

Mean value of PlatAVP during each test is given. The slope of the regression equation Pavp/Posm was found to be correlated with PlatAVP ($r = 0.639$, $P < 0.05$).

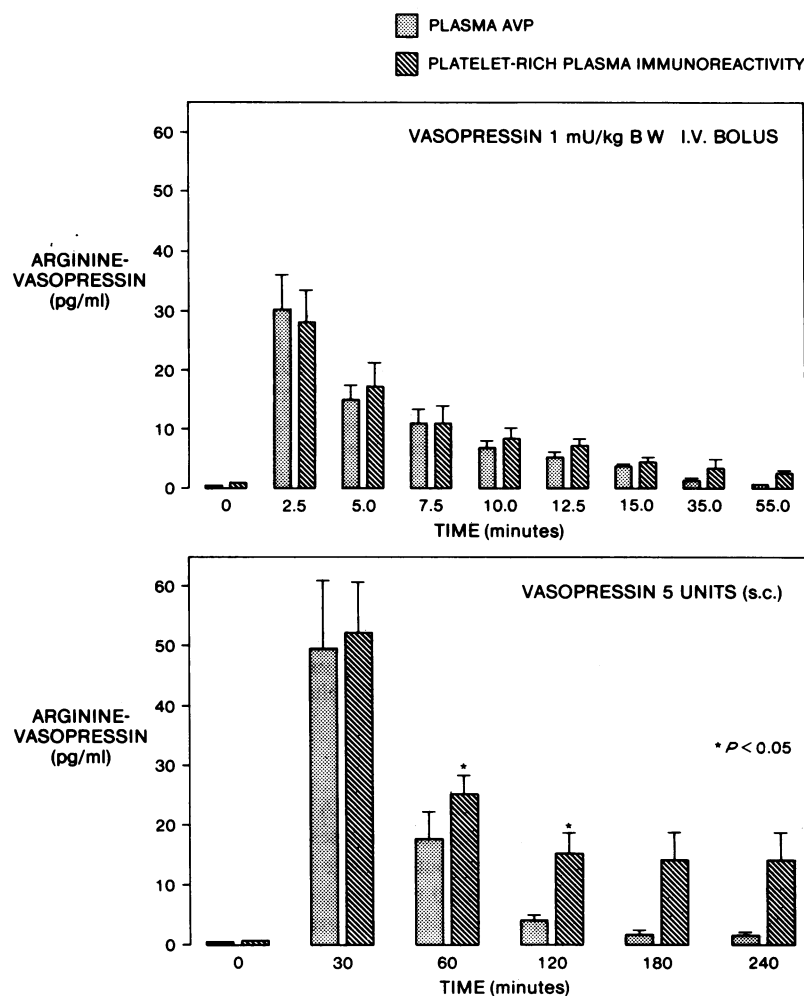


Figure 3. Plasma arginine-vasopressin and PRP immunoreactivity after vasopressin administration in four patients with DI.

termination of dose-dependent [^3H]AVP binding indicated that intact human platelets contain vasopressin binding sites displaying apparently homogeneous kinetic constants. The Scatchard plot of the binding data displayed no clear deviation from linearity (Fig. 4). In 16 experiments, the following parameters were obtained (mean \pm SD): K_d (10^{-9} M) = 0.72 ± 0.27 ; BMax (fmol/mg protein) = 109.8 ± 27 ; BMax (fmol/ 10^6 Plat) = 0.163 ± 0.05 ; number of sites per platelet = 98 ± 20 . A Hill analysis of these data gave a similar K_d result (0.70 ± 0.21 , 10^{-9}

M). Hill number was 0.968 ± 0.1 suggesting the absence of any cooperativity.

As demonstrated in Fig. 5, dDAVP and d(CH $_2$) $_5$ -D-TYR(Et) desglycine VAVP (SmithKline & French 101926) were able to inhibit [^3H]AVP binding to the same maximal extent as unlabeled AVP itself, indicating that all platelet vasopressin sites were accessible to the analogues tested. The slopes of the logit-log transforms of the displacement curves led to a linear relationship. These slopes were close to unity, suggesting that binding

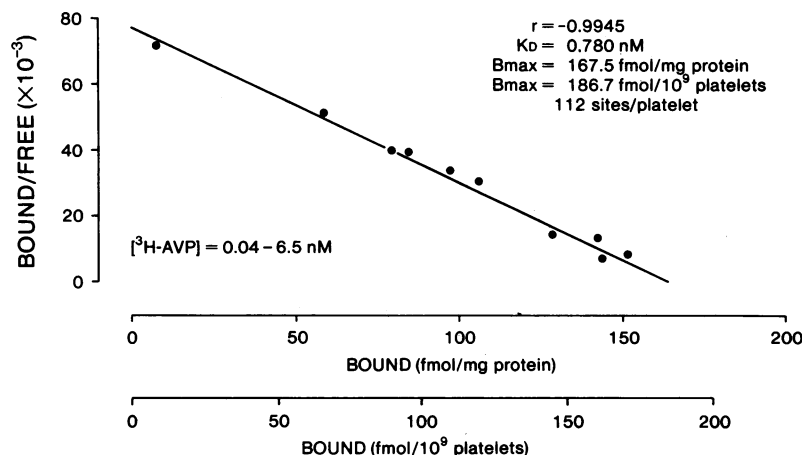


Figure 4. Scatchard type binding data for [^3H]AVP and intact platelets.

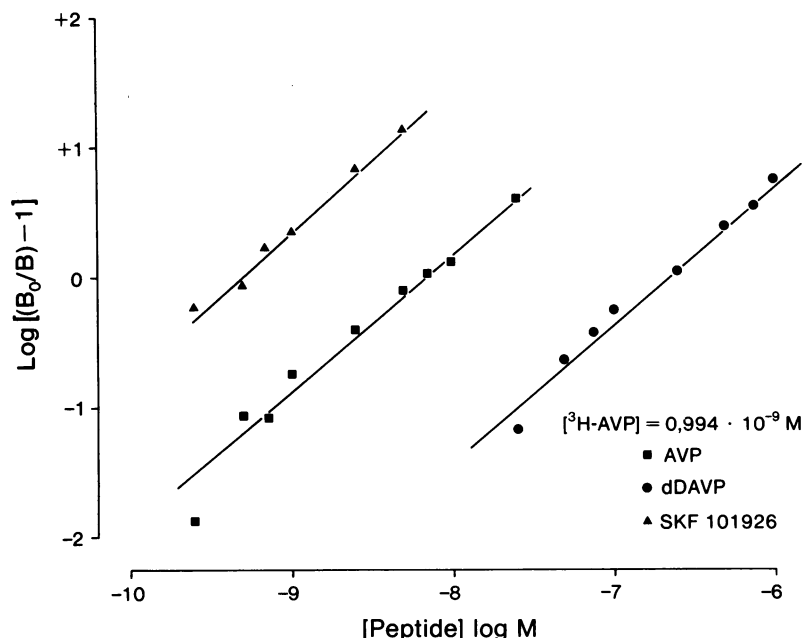


Figure 5. Dose-dependent inhibition of $[^3\text{H}]\text{AVP}$ binding by unlabeled AVP (solid squares), dDAVP (solid circles), and SK&F 101926 ($\text{d}(\text{CH}_2)_5\text{D-Tyr}(\text{Et})$ desglycine VAVP (solid triangles). Specific $[^3\text{H}]\text{AVP}$ binding was measured in presence of a constant amount of $[^3\text{H}]\text{AVP}$ (0.994 nM) and increasing amounts of unlabeled peptides. B_0 and B expressed, respectively, the specific $[^3\text{H}]\text{AVP}$ binding in the absence and in the presence of unlabeled peptide. Results are expressed as logit-log-transforms of displacement curves. The following slopes were obtained by linear regression analysis: 1.121 (solid squares), 1.1609 (solid circles) and 1.2989 (solid triangles). Computed $[\text{H}_{50}]$ were: 3.98×10^{-9} M (solid squares), 1.355×10^{-7} M (solid circles) and 0.315×10^{-9} M (solid triangles). K_i and K_d for each peptide inhibition were also calculated as

$$K_i = \frac{[\text{H}_{50}] \times K_d [^3\text{H}]\text{AVP}}{[\text{H}_{50}] + K_d [^3\text{H}]\text{AVP}} \quad \text{and}$$

$$\log K_d \left(1 + \frac{[^3\text{H}]\text{AVP}}{K_d [^3\text{H}]\text{AVP}} \right) = x \text{ intercepts}$$

$[^3\text{H}]\text{AVP}$ was the constant amount of $[^3\text{H}]\text{AVP}$ (0.994 nM) used, and $K_d [^3\text{H}]\text{AVP}$ was determined during the same experiment (0.682×10^{-9} M). Calculated K_i values were, respectively, 0.582×10^{-9} M (solid squares), 0.678×10^{-9} M (solid circles) and 0.22×10^{-9} M (solid triangles) and calculated K_d values were 1.62×10^{-9} M (solid squares), 0.551×10^{-7} M (solid circles) and 0.128×10^{-9} M (solid triangles).

was not cooperative for any of the analogues tested. Although only two analogues were tested, it is of interest that the affinities constant observed suggested that the platelet vasopressin receptor was probably of the vascular type (V1) since K_d for dDAVP was 0.55×10^{-7} M and 0.128×10^{-9} M for the V1V2 antagonist (SmithKline & French 101926).

Discussion

The present study confirm earlier findings using radioimmunoassay techniques that Ir plasma AVP concentration is higher in PRP as compared to platelet-poor plasma. This difference has been found in normal individuals, hypertensive, and congestive heart failure patients (1, 2, 7). Also, Pavp and PlatAVP have been found to be absent in four patients with central (neurogenic) DI (7).

The results of the present study extend these accumulated proofs that IrAVP in PRP is indeed true AVP associated with the platelet fraction. The fact that PlatAVP, Pavp, and synthetic AVP eluted in identical fractions from the HPLC column on two different elution systems and behave similarly on TLC analysis would suggest an identical biochemical behavior suggestive of a similar structure.

Our results also provide measurements of PlatAVP during osmotic stimulation in man as well as measurements of PlatAVP after AVP administration in patients devoided of endogenous AVP. It was hypothesized, on the basis of recent in vitro results (4, 5) that AVP could be associated to platelets through a specific V1 vascular receptor. As a consequence, acute osmotic stimulation or suppression will have little effect on PlatAVP; on the other hand, large doses of exogenous AVP could eventually bind to a specific platelet receptor. In favor of our hypothesis, we found that during a standard waterload and hypertonic saline infusion test, significant variations of Pavp were observed without

any significant variations of PlatAVP (Fig. 2). Also, as described in Fig. 3, small intravenous doses of AVP were insufficient to bind to platelets; by contrast, large doses of AVP clearly increased PlatAVP as compared to Pavp.

Analysis of the individual responses to the osmotic stimulation in normal subjects demonstrated a significant correlation between the slope of the relationship Pavp/Posm and PlatAVP (Table II). The following schematic view of the association of AVP to platelets could thus be proposed: moderate to large concentrations of plasma AVP will bind to platelets and the absolute amount called "PlatAVP" could be an index of the long term stimulation of AVP. The higher the slope of the relationship Pavp/Posm, the more AVP would be released during small increase in Posm and thus could be bound to platelets. This interpretation is also consistent with our results in a series of patients with severe congestive heart failure (7). In these patients, high Pavp levels were associated with high PlatAVP. Improvement of cardiac hemodynamics decreased Pavp and PlatAVP. Thus, both chronic osmotic and nonosmotic stimulation could determine the absolute PlatAVP concentration in man. Since PlatAVP was the subtraction of IrPRP minus Pavp, we also calculated the "true" platelet AVP concentration. Considering the very minimal amount of volume occupied by platelets in 1 ml of plasma, we calculated an intraplatelet AVP of 500 pg to 4,000 pg/ml or 0.463×10^{-9} M to 3.7×10^{-9} M (see Methods).

We then tried to compare these values to the in vitro results obtained from binding studies on intact platelets. In accordance with recent results from two other groups (4, 5), we found binding characteristics corresponding to the above in vivo values: in this regard, the association constant of $[^3\text{H}]\text{AVP}$ to intact human platelets: $K_d = 0.72 \pm 0.27$ nM was entirely compatible with the intraplatelet AVP concentration previously calculated (0.46×10^{-9} to 3.7×10^{-9} M). Our pharmacological study using two analogues also suggested, in accordance with two recent extensive

publications (4, 5), that platelet vasopressin receptors were probably of the V1 type. Our values were found to be in good agreement with those of Vittet et al. (5), who reported similar number of sites, namely a maximal binding capacity of 142 ± 48 fmol/mg of protein with a K_d of 1.3 ± 0.2 nM. Also, we estimated that for a PlatAVP of 5 pg/ml, only nine molecules of AVP were bound or associated with each individual platelet. This data was consistent with the in vitro binding characteristics obtained ($B_{\text{Max}} = 98 \pm 30$ binding sites/platelet; $K_d = 0.72 \pm 0.24$ nM).

The results of the present study raised a number of practical and theoretical new points in regard to measurements and significance of Pavp and PlatAVP. PlatAVP could be an integer over time of the long-term release (osmotic and nonosmotic) of vasopressin. Thus, a single PlatAVP measurement could eventually give some information about the long-term stimulation of the AVP system. This assertion seems to be true in chronic congestive heart failure where improvement in cardiac hemodynamics, over a 2-d period, decreased Pavp and PlatAVP (7). The effect of long-term chronic osmotic stimulation (as for example in congenital nephrogenic diabetes insipidus) have not been so far examined. Other observations and experiments will be necessary to determine the fate of PlatAVP: for example, vasopressin intraplatelet processing and possible release are completely unknown at the present time.

In summary, we recommend that measurements of plasma immunoreactive AVP should be done in platelet-poor plasma and PRP. Pavp (in platelet-poor plasma) could be considered as a measurement of circulating biologically important AVP and should be related to Uosm (with normal renal function). PlatAVP may be an index of the long term stimulation of AVP (osmotic and nonosmotic) and a direct measurement of AVP bound to a specific platelet receptor.

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