Nature and properties of human platelet vasopressin receptors

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The binding of ^3H -labelled [8-arginine]vasopressin to human platelets or crude platelet membranes was examined. Both preparations specifically bound [8-arginine]vasopressin. The binding increased linearly with protein concentration, it was temperature- and time-dependent, saturable and could be reversed to a large extent by EDTA (10 mM). In this latter case, addition of an excess of MgCl_2 (20 mM) restored the initial level of binding. Intact platelets and membranes derived from these platelets presented a single population of binding sites with a dissociation constant (K_d) of 1.3 ± 0.2 and 1.8 ± 0.3 nM and a maximal binding capacity of 142 ± 48 and 270 ± 17 fmol/mg of protein, respectively. The K_d values of various analogues correlated well with those determined on rat liver membrane V_1 vasopressin receptors but not with those determined on rat kidney membrane V_2 receptors.

INTRODUCTION

Vasopressin exerts a large variety of biological effects in mammals and in particular has antidiuretic properties, contracts the arteries and acts as a glycogenolytic agent on the liver (for review see De Wulf *et al.*, 1980). In addition to many other biological effects (Jard, 1983), vasopressin has been shown to cause the aggregation of human blood platelets (Haslam & Rosson, 1972).

Vasopressin does not exert all its biological activities with cyclic AMP as second messenger. On the basis of both functional and pharmacological criteria, two types of vasopressin receptors can be distinguished (Michell *et al.*, 1979): V_2 renal receptors, leading to the activation of adenylate cyclase (Bockaert *et al.*, 1973), and V_1 receptors, which mediate pressor and glycogenolytic actions of vasopressin, acting by a rise in the concentration of intracellular calcium (Keppens *et al.*, 1977). Vasopressin also causes enhancement of polyphosphoinositide breakdown in hepatocytes (Kirk *et al.*, 1980), and it has been suggested that this may represent an early step in the calcium-mobilizing effect of vasopressin.

Previous studies suggest that the platelet vasopressin receptor is of the V_1 subtype, since platelet aggregation induced by vasopressin is potentially inhibited by [1-(β -mercapto- $\beta\beta'$ -cyclopentamethylenepropionic acid), 8-arginine]vasopressin (Thomas *et al.*, 1983) or des-1-amino-[4-valine,8-D-arginine]vasopressin (Vanderwell *et al.*, 1983), which are V_1 antagonists, whereas des-1-amino-[8-D-arginine]vasopressin, a selective V_2 agonist, fails to induce aggregation of human platelets (Thomas *et al.*, 1983). On the other hand, Berrettini *et al.* (1982) describe a specific saturable binding of ^{125}I -AVP to human platelets and claim that pharmacological competition with analogues indicates that the platelet receptor is similar to the kidney medulla receptor (V_2 type). Meanwhile, these pharmacological data are too scarce to allow any definite conclusion on the ligand specificity of the platelet receptors.

To clarify this point, the present studies were designed

to characterize the specific binding of [^3H]AVP, a highly biologically active ligand, on human platelets and platelet membranes and to characterize directly their ligand specificity by competition studies using a series of nine vasopressin analogues.

EXPERIMENTAL

Chemicals

[^3H]AVP ([phenylalanine- ^3H ,8-arginine]vasopressin, 90 Ci/mmol), obtained from New England Nuclear (Boston, MA, U.S.A.), was purified by h.p.l.c. and stored in liquid N_2 until use; we have checked that the labelled vasopressin preparation displayed the same biological activity as the unlabelled peptide in its potency to activate adenylate cyclase of membranes from rat kidney medulla.

Unlabelled AVP was purchased from Bachem (Bubendorf, Switzerland) and the AVP analogues were kindly donated by Professor M. Manning (Toledo, OH, U.S.A.).

Biological material

Platelet-rich plasma was obtained from the Centre de Transfusion Sanguine, Montpellier (France). It contained 3.63 g of glucose, 3.26 g of anhydrous sodium citrate and 1.19 g of citric acid/100 ml.

Preparation of washed human platelets. Citrated platelet-rich plasma (40 ml) was centrifuged firstly at 200 g for 10 min at 18 °C, to remove contaminating red cells, and secondly at 2450 g for 15 min at 4 °C, to pellet the platelets. The platelet plug was washed three times at 4 °C by resuspension and centrifugation in an iso-osmotic buffer solution (NaCl, 100 mM; EDTA, 5 mM; MgCl_2 , 15 mM; Tris/HCl, pH 7.4, 50 mM). The washed platelets were finally resuspended in 10–20 ml of the same buffer.

Preparation of partially purified platelet membranes. Human platelets were isolated and washed as above; they were then lysed in a hypo-osmotic buffer (EGTA, 5 mM;

Abbreviation used: AVP, [8-arginine]vasopressin.

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Tris/HCl, pH 7.4, 10 mM). After freezing and thawing three times of this suspension, the particulate fraction was isolated by centrifugation at 30000 *g* for 15 min at 4 °C. This centrifugation step was repeated twice and the membrane pellet was finally resuspended in 3–5 ml of the washing iso-osmotic buffer. The preparation was either used immediately or stored in liquid N₂ for 1 week.

[³H]AVP binding assays

All experiments were done in triplicate. Protein concentration was measured (Lowry *et al.*, 1951) from an aliquot of washed platelets or platelet membranes.

Measurement of [³H]AVP binding to intact platelets.

The incubation medium (final volume 200 μ l) contained: iso-osmotic buffer (NaCl, 100 mM; EDTA, 5 mM; MgCl₂, 15 mM; Tris/HCl, pH 7.4, 50 mM), bovine serum albumin (2 mg/ml), bacitracin (1 mg/ml), and various amounts of [³H]AVP (except when otherwise stated). The reaction was initiated by the addition of 100 μ l of platelet suspension (2×10^8 cells). Incubation was carried out at 30 °C with gentle shaking; it was stopped by the addition of cold iso-osmotic solution (NaCl, 150 mM; MgCl₂, 10 mM; Tris/HCl, pH 7.4, 50 mM), and the content of the tubes was immediately filtered through Whatman GF/C filters presoaked with 4 ml of the same buffer. Filters were washed four times with 4 ml of the stopping solution. Radioactivity of filters was measured by liquid-scintillation counting. Non-specific binding was determined in the presence of 10 μ M unlabelled AVP. At a [³H]AVP concentration close to the equilibrium dissociation constant (2 nM), non-specific binding represented less than 30% of total binding.

To show the importance of magnesium in promoting the specific binding of [³H]AVP to platelets, dissociation studies were carried out after preincubation of platelets at 30 °C with 2.5 nM-[³H]AVP. EDTA (10 mM) was added to the medium, and specific residual binding measured after different periods of incubation. An excess of MgCl₂ (20 mM) was then added to induce a reassociation.

Dissociation constants for the binding of vasopressin analogues listed in Table 1 were determined from competition experiments; specific [³H]AVP binding was measured in the presence of 1.5 nM-[³H]AVP and of increasing amounts of the unlabelled peptides to be tested.

Measurement of [³H]AVP binding to platelet membrane.

The incubation medium (final volume 100 μ l) contained MgCl₂, 10 mM; Tris/HCl, pH 7.4, 50 mM; bovine serum albumin, 1 mg/ml; bacitracin, 1 mg/ml, and various amounts of [³H]AVP. The reaction was initiated by the addition of membranes (50 μ g of protein). Incubation was carried out at 30 °C with gentle shaking for various periods and was stopped by the addition of cold buffer (MgCl₂, 10 mM; Tris/HCl, pH 7.4, 50 mM). The preparation was filtered through GA-3 Gelman filters. Filters were washed three times with 4 ml of the same cold solution. Non-specific binding was determined in the presence of 10 μ M unlabelled AVP; for 2 nM-[³H]AVP (a concentration close to the apparent dissociation constant, *K*_d) non-specific binding represented less than 12% of total binding. Radioactivity measurements were performed by liquid-scintillation counting.

The dissociation constants of the vasopressin analogues were deduced from competition experiments with

1.5 nM-[³H]AVP (see the experiments with intact platelets).

Stability experiments

In order to examine the stability of [³H]AVP in binding experiments, [³H]AVP (2 nM final concentration) was incubated with intact platelets (4×10^8 cells) for 15 min or 30 min at 30 °C. After incubation, the platelets were pelleted by centrifugation at 0 °C and aliquots of the supernatant were used for a binding assay with fresh platelets. The specific binding of [³H]AVP after preincubation was similar to control values obtained without preincubation of [³H]AVP. Moreover, kinetic studies of [³H]AVP binding were performed in the presence of phenylalanine (1 mM) added to the incubation medium to prevent any incorporation into platelets of [³H]phenylalanine-labelled peptide which could have been released from hydrolysis of the [³H]AVP.

In another set of control experiments, platelet suspensions were preincubated without [³H]AVP at 30 °C for various periods (0–60 min). Specific [³H]AVP binding was then measured as described above, after incubating platelets at 30 °C for 15 min. A 15 min preincubation period had little effect on specific binding (12% reduction in binding was observed), and after a 60 min preincubation period there was a 45% loss of binding.

RESULTS

[³H]AVP specific binding to intact platelets or platelet membranes was linear with the amount of protein (0–400 and 0–100 μ g of protein/assay) for platelets and platelet membranes respectively (results not shown); it was temperature- and time-dependent (Fig. 1). The amount of specifically bound hormone increased progressively up to a plateau value; the level of this plateau and the duration required to reach it were clearly dependent on the hormone concentration used. The observed time course of [³H]AVP binding could be accounted for by a pseudo-first-order type of reaction. At 37 °C, specific binding of [³H]AVP reached an equilibrium after 15 min, but this plateau value remained lower than the 30 °C binding level. At 4 °C, [³H]AVP binding increased slowly, and at 120 min, there was 75% of the binding measured at 30 °C. All further experiments were performed at 30 °C and the incubation period was 15 min; this was consistent with the results of the stability experiments (see above).

EDTA (10 mM) dissociated 75% or 100% of bound [³H]AVP from platelets and on platelet membranes, respectively (results not shown); the dissociation of bound hormone induced by EDTA could be abolished by the addition of an excess of Mg²⁺, which restored the initial level of binding (Fig. 2).

Specific [³H]AVP binding was saturable. The dose-binding curve for [³H]AVP at equilibrium (Fig. 3) did not reveal any marked heterogeneity in the population of vasopressin binding sites as indicated by a linear Scatchard (1949) plot with an apparent dissociation constant (*K*_d) of binding of 1.3 ± 0.2 and 1.8 ± 0.3 nM for intact platelets and platelet membranes, respectively. The following maximal binding capacities (*B*_{max}) were calculated from the regression lines: 142 ± 48 fmol/mg of protein (intact platelets) which corresponds to 95 receptor sites per platelet, and 270 ± 17 fmol/mg of protein (platelet membranes). The *K*_d deduced from the association (*k*₊₁) and dissociation (*k*₋₁) rate constants,

Fig. 1 Time course of specific $[^3\text{H}]\text{AVP}$ binding to human intact platelets

(a) Effect of temperature on $[^3\text{H}]\text{AVP}$ binding to intact platelets. $[^3\text{H}]\text{AVP}$ (1.5 nM) was incubated for varying periods with 400 μg of protein (4×10^8 cells) at the temperatures indicated. (b) Kinetic study of $[^3\text{H}]\text{AVP}$ association to platelets. Intact platelets (235 μg of protein) were incubated at 30 °C in the presence of the indicated concentrations of $[^3\text{H}]\text{AVP}$. Each point represents the mean of triplicate determinations from a representative experiment. Inset: logarithmic transform of the association curves for binding values below equilibrium level. $[^3\text{H}]\text{AVP}$ binding is expressed as the natural logarithm of the proportion of binding at various periods of time (B) relative to binding at equilibrium (B_{eq}). The computed rate constant for the formation (k_{+1}) and dissociation (k_{-1}) of the hormone-receptor complexes, deduced from logarithmic transform of the association curves for 5 nM- and 2 nM- $[^3\text{H}]\text{AVP}$, were $k_{+1} = 4.46 \times 10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$; $k_{-1} = 0.022 \text{ min}^{-1}$.

estimated from the association curves ($k_{+1} = 4.46 \times 10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$; $k_{-1} = 0.022 \text{ min}^{-1}$; $K_d = 0.5 \text{ nM}$), was in good agreement with that derived from dose-dependent binding on platelets at equilibrium ($K_d = 1.3 \pm 0.2 \text{ nM}$).

Potencies of a series of AVP analogues in competing with $[^3\text{H}]\text{AVP}$ for binding sites on intact platelets and on platelet membranes are listed in Table 1. All the peptides tested inhibited $[^3\text{H}]\text{AVP}$ binding to the same maximal extent as did unlabelled vasopressin, indicating that they interact with the same population of binding sites. The

Fig. 2. Effect of divalent ions on the specific binding of $[^3\text{H}]\text{AVP}$ to intact platelets

After 5 min of preincubation of platelets with 2 nM- $[^3\text{H}]\text{AVP}$, EDTA (10 mM final concentration) was added and total residual binding was measured as a function of time after addition of EDTA. At $t = 5 \text{ min}$, reassociation was then induced by addition of MgCl_2 (20 mM final concentration). Experimental values are expressed as fractions of the corresponding control values.

experimental results obtained show a rather good correlation ($r = 0.94$) between K_d of analogues for platelets and platelet membranes. A fair correlation also occurs between respective relative affinities of analogues for human platelet and rat liver membranes ($r = 0.79$), whereas no correlation is observed between the respective K_d for human platelet and rat kidney membranes. (Table 1).

DISCUSSION

The present studies, using $[^3\text{H}]\text{AVP}$ as a ligand for platelet vasopressin receptors, confirm and extend the observations of Berrettini *et al.* (1982) on the existence of vasopressin binding sites on human platelets. The analysis of binding at equilibrium on platelet membranes reveals an apparent K_d of 1.8 nM, not significantly different from that observed in liver (3.2 nM; Cantau *et al.*, 1980) or kidney (0.4 nM; Rajerison *et al.*, 1974). The comparison of K_d for platelets and platelet membranes (1.3 and 1.8 nM, respectively), indicates a comparable affinity of the ^3H -labelled ligand for both systems and suggests that the different manipulations needed to obtain crude membranes did not modify to an important extent the properties of the binding sites. The merely doubled total maximal binding capacity observed in going from platelets to platelet membranes might be expected from the preparation of low purity crude particulate fractions of cells.

The population of vasopressin binding sites on human platelets appeared to be homogeneous, as indicated by a linear Scatchard plot; this suggests that there is no co-operativity in hormonal binding.

Fig. 3. Dose-dependency for specific $[^3\text{H}]\text{AVP}$ binding to intact platelets (*a* and *b*) and platelet membranes (*c* and *d*)

(*a*, *c*) Specific $[^3\text{H}]\text{AVP}$ binding is plotted as a function of the $[^3\text{H}]\text{AVP}$ concentration in the incubation medium; incubation period was 15 min at 30 °C. (*b*, *d*) Scatchard plots of dose-binding curves shown in (*a*) and (*c*). K_d values and maximal binding capacities estimated from the regression lines are respectively: intact platelets, 1.3 nM and 142 fmol/mg of protein, i.e. about 95 binding sites per platelet; platelet membranes, 1.8 nM and 270 fmol/mg of protein.

The characteristics of the specific binding of $[^3\text{H}]\text{AVP}$ to platelets at 4 °C indicate that the $[^3\text{H}]\text{AVP}$ binding observed at 30 °C is most likely an equilibrium reaction. Indeed, 75% of total binding measured at 30 °C is obtained at 4 °C within 2 h. Dissociation of the $[^3\text{H}]\text{AVP}$ binding on platelets by an excess of unlabelled hormone yielded a partial reversibility: 20–40% of bound hormone is dissociated within 30 min (results not shown); that supports the observations of Berrettini *et al.* (1982). The critical role of Mg^{2+} is clearly shown by both the dissociation of specifically bound hormone after addition of EDTA, and the ability for re-binding when an excess of MgCl_2 was added. This corroborates the prominent role of Mg^{2+} in vasopressin-induced activation of human blood platelets (Erne & Pletscher, 1985).

The relative affinities of a series of vasopressin analogues for the $[^3\text{H}]\text{AVP}$ binding sites in human platelets, determined from competition experiments, reveal a high degree of specificity (for details see Table 1). The K_d values determined correlate rather well with those found in rat liver membranes, but no correlation appears with those observed in rat kidney membranes. These results, using nine vasopressin analogues, support previous partial pharmacological results (Thomas *et al.*, 1983; Vanderwell *et al.*, 1983). Although we had compared affinities of vasopressin analogues which correspond to different species (rat and human), they suggest that the platelet vasopressin receptor is of the V_1 subtype, according to the classification of Michell *et al.* (1979), and corroborate the lack of stimulation of platelet

Table 1. Dissociation constants for the binding of vasopressin structural analogues to intact platelets and platelet membranes; comparison with pK_d for binding to rat liver and kidney membranes

The dissociation constant for the unlabelled peptide (K_d) was deduced by fitting the experimental data to the expected linear relationship:

$$\log[(B_0/B) - 1] \{ ([^3H]AVP/K_d[^3H]AVP) + 1 \} = \log[I] - \log K_d$$

in which $[I]$ is the concentration of unlabelled peptide, $[^3H]AVP = 1.5$ nM and $K_d[^3H]AVP = 1.3$ nM (platelets) or 1.8 nM (platelet membranes). K_d values deduced from calculated regression lines are expressed in terms of pK_d ($-\log K_d$). pK_d values for liver and kidney membranes are from: ^aS. Jard (personal communication), ^bJard (1983), ^cCantau *et al.* (1980), ^dBarth *et al.* (1983), ^eButlen *et al.* (1983).

Analogue	pK_d			
	Intact platelet (human)	Platelet membrane (human)	Liver membrane (rat)	Kidney membrane (rat)
[8-Arginine]vasopressin (AVP)	8.6	8.55	8.5 ^b	9.4 ^b
Des-9-glycine-[8-arginine]vasopressin	6.9	7.05	6.7 ^a	7.2 ^a
Des-9-glycineamide-[8-arginine]vasopressin	6.4	6.1	5.8 ^a	9.15 ^a
Des-9-glycine-[1-(β -mercapto- $\beta\beta'$ -cyclopentamethylenepropionic acid), 8-arginine]vasopressin	8.3	8	9.13 ^a	7.3 ^a
Des-9-glycineamide-[1-(β -mercapto- $\beta\beta'$ -cyclopentamethylenepropionic acid), 8-arginine]vasopressin	8.2	7.7	8.4 ^a	6.5 ^a
Vasopressinoic acid	6.4	6.65	5.7 ^a	7.2 ^a
Des-1-amino-[4-valine, 8-D-arginine]vasopressin	8.45	7.75	6.5 ^c	9.6 ^c
[1-L-2-Hydroxy-3-mercaptopropanoic acid, 4-valine, 8-D-arginine]vasopressin	7.4	7.4	6.35 ^b	9.1 ^b
Oxytocin	7.1	6.9	7.11 ^d	7.45 ^e

membrane adenylate cyclase activity by AVP (Haslam *et al.*, 1978). Elsewhere, previous studies on the relationship between the primary signal in vasopressin action and the final biological response had indicated that this was so: Drummond *et al.* (1985) have shown that vasopressin (100 nM) stimulated Ca^{2+} mobilization in platelets, and induced changes in inositol phospholipid metabolism monitored as [^{32}P]phosphatidic acid formation in platelets prelabelled with [^{32}P]P_i. Meanwhile an extensive study on the existence of a causal relationship between hormone-induced increase in polyphosphoinositide breakdown and hormone binding must be investigated to definitely prove that the platelet vasopressin receptor belongs to the V₁ subtype.

In preliminary experiments, AVP was found to induce platelet shape change with a potency ($pK_a = 8.2$) which fits rather well with the binding parameters ($pK_d = 8.6$), suggesting that the binding of AVP is responsible for the shape change, and indicating that no marked amplification exists between the primary signal in vasopressin action on platelets and the final functional effects, i.e. shape change or platelet aggregation. The concentration of AVP required to lead to the saturation of binding sites is far higher than the human blood levels of AVP (Haslam & Rosson, 1972). Thus only a small fraction of platelet vasopressin receptors may be activated in normal conditions, and only unusually high concentrations of blood AVP, such as those observed in haemorrhage (Arnoud *et al.*, 1977) or water deprivation (Landgraf & Gunther, 1983) may lead to an important occupation or saturation of the platelet vasopressin binding sites. A correlation between the relative affinities of the tested analogues for the platelet vasopressin-receptor and the relative potencies of these analogues in inducing shape change, aggregation or serotonin release remains to be demonstrated. Accordingly, work is needed to connect

the affinities of analogues for platelets to their biological effects.

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