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PROPERTIES OF VASOPRESSIN-ACTIVATED HUMAN PLATELET HIGH AFFINITY GTPase

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Effect of 8-arginine vasopressin (AVP) was examined on human platelet membrane GTPase activity as an index of a G-protein involvement. AVP stimulated a high-affinity GTPase activity in a dose-dependent manner (K_{act} = 1.1 ± 0.2 nM). This stimulation was blocked by a V_{1a} antagonist, thus confirming the V_{1a} nature of the platelet AVP receptor. There were important variations among individuals in the AVP-induced stimulation of GTPase activity, that were in relation with the AVP-maximal binding capacity. These data suggest a causal relationship between the binding of AVP to its receptor and transduction elicited by a G-protein, without amplification. In addition, in view of the variable AVP responsiveness observed among individuals, platelet AVP-receptor appears to be subject to regulation.

Vasopressin (AVP) has been shown to cause shape change and aggregation of human blood platelets (1). Previous studies have indicated that AVP-induced platelet activation is mediated by a stimulation of specific receptors (2, 3) and a subsequent breakdown of inositol lipids (4) followed by an increased concentration of cytosolic ionized calcium (5).

In many receptor-effector coupling systems, the link between extracellular signals and intracellular events is mediated by guanine nucleotide regulatory proteins (G-proteins) which bind GTP and display high-affinity GTPase activity (For review : 6). In human platelets, the AVP-induced responses also appear to involve G-protein(s), since the peptide, at high concentration, stimulates in a pertussis toxin insensitive fashion a GTPase activity (7).

Nonetheless, it appeared of interest to characterize more fully this AVP-receptor-effector coupling mechanism in human platelets, by examining in details the parameters of this AVP-stimulated GTPase activity. In addition, since large variations in platelet functional responsiveness to AVP have been observed between donors in the aggregatory response as well as in the transduction processes involved (8, 9) ; we have also measured AVP-induced GTPase stimulation and agonist binding parameters over a population of 12 healthy human subjects.

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MATERIALS AND METHODS

- **Chemicals** :

[^{3}H] AVP : (phenylalanine ^{3}H, 8-arginine) vasopressin, 70 Ci/mmol, was obtained from New England Nuclear (Boston, MA, USA). [γ-^{32}P] GTP : Guanosine-5'-[γ-^{32}P] triphosphate, 20-30 Ci/mmol was from Amersham France SA, Les Ulis and unlabelled AVP was purchased from Bachem (Bubendorf, Switzerland). The AVP analogue : Des-9-glycineamide-[1-(β-mercapto-$\beta\beta$' cyclopentamethylene propionic acid), 8-arginine] vasopressin : DesGly $(NH_2)^9$ $d(CH_2)_5$ AVP which exhibits vasopressor antagonistic properties (10) was kindly donated by Professor M. Manning (Toledo, OH, USA).

- **Preparation of platelet membranes** :

Blood (50 ml), withdrawn between 8-9 a.m. from the antecubital vein of recumbent healthy subjects who had not taken any medication for at least one week before sampling, was collected in 10% (v/v) ACD. Blood was centrifuged at 180 g for 20 min to obtain platelet-rich plasma (PRP). PRP was then further centrifuged at 200 g for 10 min to remove contaminating red cells. The resultant PRP was acidified to pH 6.4 with 0.15 M citric acid and centrifuged at 600 g for 15 min, to pellet platelets. All centrifugations were performed at room temperature. Membranes were prepared from this platelet pellet as in Vittet et al. (3). The final pellet was resuspended in 50 mM Tris/HCl buffer, pH 7.4, for immediate use. Protein concentration was measured according to Lowry et al. (11).

- **GTPase assay** :

GTPase activity was measured by monitoring the release of [^{32}P]P_i from [γ-^{32}P] GTP as described by Cassel & Selinger (12) with minor modifications. The incubation medium (final volume 100 μl) contained 10 mM $MgCl_2$, 5 mM phosphocreatine, 70 U/ml creatine kinase, 0.2 mM ATP, 0.1 mM EGTA, 1 mg/ml bovine serum albumin, 50 mM Tris/HCl pH 7.4 and the appropriate drugs. The reaction was initiated by adding membranes (10-20 μg of protein/tube) and conducted for 10 min at 30°C, during which time AVP binding was near equilibrium (3). Test tubes were then transferred to a water bath and incubated for 2 min at 20°C ; 0.15 μM [γ-^{32}P] GTP was added for a further 3 min incubation, unless otherwise indicated, to determinate GTPase activity. The reaction was terminated by adding 500 μl of cold 50 mM KH_2PO_4 buffer pH 7.4, containing 5% (w/v) Norit-A charcoal. The tubes were vortexed, centrifuged at 2500 g for 10 min, and 300 μl of supernatant were taken for scintillation counting. Reactions were performed in quadruplicate with blanks containing no added membranes. [^{32}P]P_i released from [γ-^{32}P] GTP in the absence of membrane represented to 0.7-2% of the added [γ-^{32}P] GTP. GTPase activity is expressed as pmol of GTP hydrolyzed per mg of protein per min.

- **Measurement of[^{3}H]AVP binding to platelet membranes** :

Binding studies were performed as described in Vittet et al. (3). Briefly, platelet membranes were incubated with the incubation medium for 15 min at 30°C. The reaction was then stopped and the preparation was filtered through GA-3 Gelman filters.

- **Presentation of data** :

Since there were considerable variations between donors with regard to platelet responses to AVP, all figures correspond to a representative experiment, and were built from means of quadruplicate determinations.

RESULTS

In human platelet membranes, a high-affinity specific GTPase (K_M ranging from 0.1 to 0.3 μM) was mainly responsible for GTP hydrolysis ; [^{32}P]P_i release was markedly reduced by adding low GTP concentrations (results not shown). However, the curvilinearity of the Lineweaver-Burk plot showed the presence of at least two enzyme activities with different

affinities for GTP, which had increased V_{max} in the presence of AVP (Fig. 1a). In all subsequent experiments, only the higher-affinity GTPase activity was considered and assays were performed in the presence of 0.15 µM GTP. The increase in GTP hydrolysis induced by AVP occurred without a lag phase and was linear for at least 10 min (Fig. 1b). After a 3 min incubation period, during which the initial rate was assessed and assays routinely carried out, the basal GTPase activity of the platelet membranes ranged from 7 to 19 pmol GTP hydrolyzed/min/mg of protein with a mean of 11 ± 1 pmol/min/mg protein (SEM, n = 17). AVP stimulated this GTPase activity in a dose-dependent manner (Fig. 2a) yielding a K_{act} value of 1.1 ± 0.2 nM (SEM, n = 5) ; maximal activation was obtained at 10 nM AVP. The V_1 antagonist DesGly $(NH_2)^9$ $d(CH_2)_5$ AVP which exhibited no partial agonistic activity, was able to inhibit the AVP-stimulated GTPase activity competitively with an inhibition constant K_i of 20 ± 10 nM (SD, n = 3) (Fig. 2b). When AVP-stimulated GTPase activity and binding parameters (Bmax, K_d) were simultaneously measured on platelet membranes from 12 different subjects,

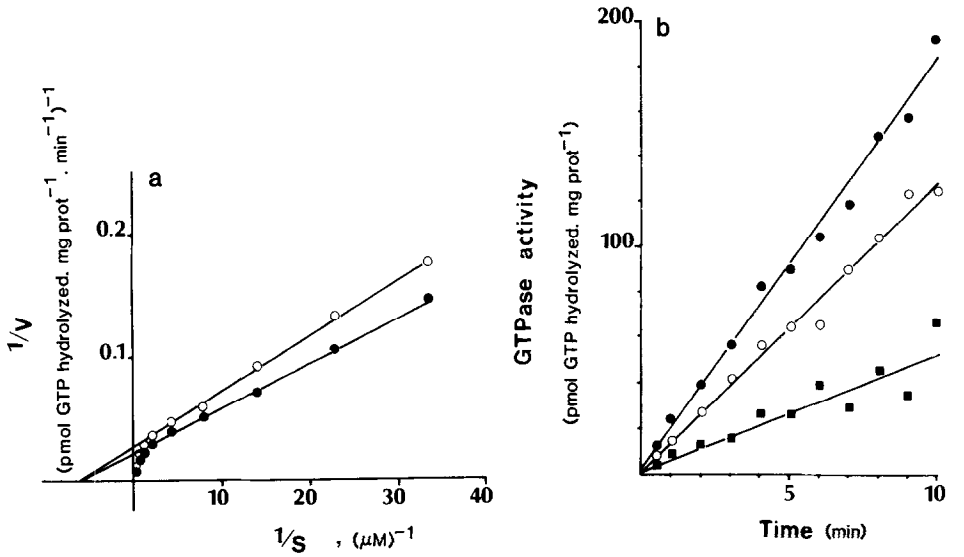

Figure 1 :Kinetic parameters of AVP-stimulated GTPase activity.

a) Lineweaver-Burk plot of GTP hydrolysis : increasing concentrations of unlabelled GTP were added to a constant amount of [γ-^{32}P] GTP to give the final concentration indicated on the abcissa. ○: basal GTPase activity ; ●: 100 nM AVP-stimulated GTPase activity. The apparent K_M value (0.15 µM) of a GTPase with high affinity for GTP was extrapolated from the linear part of the curve. In the presence of AVP, the V_{max} for high-affinity GTPase activity increased in this experiment form 32 to 41 pmol GTP hydrolyzed/min/mg of protein.

b) Time course of GTP hydrolysis : platelet membranes were incubated as described in Materials and Methods in the absence (○) or presence (●) of 100 nM AVP, for the times indicated. (■) net AVP-induced GTPase activity.

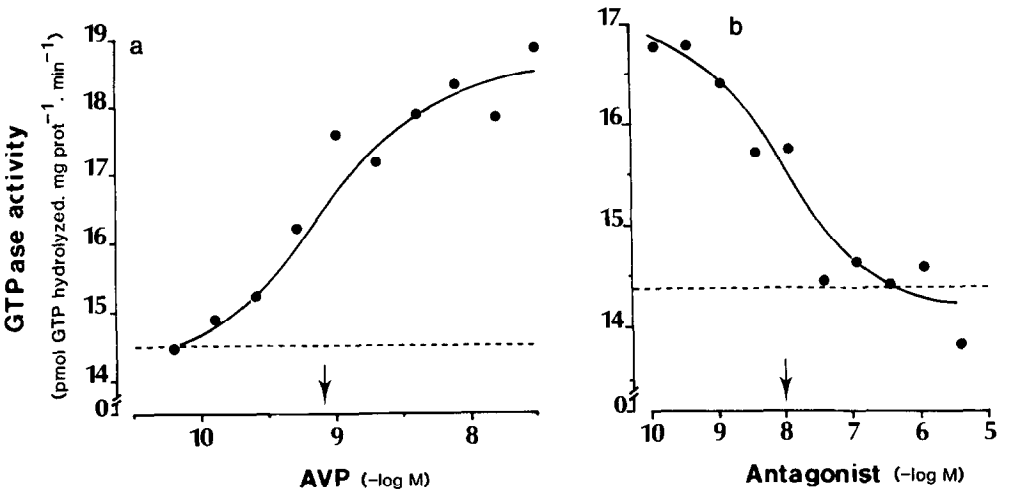

Figure 2 : Dose-response to AVP (left panel) and inhibition by DesGly$(NH_2)^9$ d$(CH_2)_5$ AVP of AVP-stimulated GTPase activity (right panel) in human platelet membranes.

GTPase assays were performed for 3 min at 20°C as described in Materials and Methods.

a) GTPase activity is plotted as a function of the AVP concentration in the medium. The pK_{act} (pK_{act} = -log K_{act}, where K_{act} is the concentration producing half-maximal stimulation) is indicated by the arrow. In this experiment pK_{act} = 9.1.

b) GTPase activity was measured in the presence of 1.5 nM AVP and increasing amounts of the antagonist added simultaneously. The pK_i (8.0) is indicated by the arrow. The K_i was calculated using the relation :

$K_i = IC_{50} \times K_{act}$ AVP / (AVP) + K_{act} AVP, with (AVP) = 1.5 nM and K_{act} AVP = 1 nM.

the AVP-dependent GTPase activity as well as the Bmax varied considerably from one subject to another, while the K_d remained stable (K_d = 1.6 ± 0.1 nM ; SEM, n = 17). These variations do not depend on the sex or age of donors. A relationship was observed between the Bmax of AVP and maximal AVP-stimulated GTPase activity (Fig. 3).

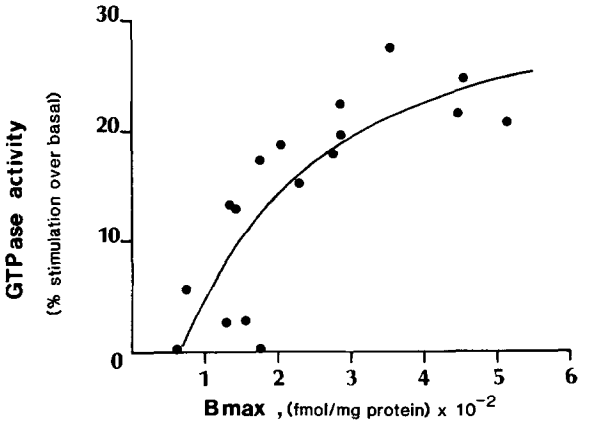

Figure 3 : Relationship between AVP-stimulated GTPase activity and maximal AVP binding capacity.

Maximal detectable AVP binding capacity (Bmax) and corresponding-maximal AVP-induced GTPase activity (expressed as % of basal value) were measured the same day on the same platelet membrane preparation ; 17 values are shown, corresponding to 12 different healthy subjects.

DISCUSSION

The present investigation confirms and extends previous results which showed that AVP stimulates a high-affinity GTPase activity in human platelet membranes subsequent to receptor activation (7). The observation of a K_M value of 0.1-0.3 μM GTP is close to those reported by others in platelets for the GTPase activities stimulated by other agonists (13, 14). The dose-dependent curve of the stimulation of the GTPase activity in platelet membranes by AVP was completely parallel with the dose-dependent curve of specific peptide binding to platelet membranes (3), suggesting a causal relationship between the two phenomena and a lack of amplification between peptide fixation to its binding sites and the first event subsequent to this activation, i.e. transduction of the signal by a G-protein. Moreover, the observation of a relationship among individuals in the ability of AVP to stimulate GTPase activity and its binding capacity also strongly suggests a direct relationship, without amplification between AVP binding to platelets and the first transduction stage.

AVP-induced human platelet activation appears to be mediated through activation of V_1-vasopressin-receptor subtype (2, 3, 4, 5, 15). Recently, two V_1 subtypes were distinguished on the basis of their ligand specificity for AVP antagonists (16) i.e. V_{1a} receptors, which correspond to the hepatic or the vascular type, and V_{1b} receptors, corresponding to the anterior pituitary type. AVP-enhanced GTPase activity was completely suppressed by the highly discriminatory AVP antagonist DesGly $(NH_2)^9$ $d(CH_2)_5$ AVP with a pK_i of 7.7, which is in the same range as the constant of this antagonist for the inhibition of AVP-induced accumulation of inositol phosphates in aortic myocytes (17). This confirms the V_{1a} nature of the platelet AVP receptor, which was previously indicated by the effects of very specific analogues on AVP-binding competition (3), on platelet shape change and aggregation (15), and by the lack of adenylate cyclase stimulation by AVP in platelet membranes (18, 19).

With regard to the nature of the G-protein involved, since AVP and adrenaline or PGE_1-stimulated GTPase activities were additive at very high concentrations of each component (7, 19), it appears different from Gi or Gs, and seems related to G_{PI} which is involved in the regulation of the phosphoinositide turnover that controls calcium sensitive processes (6). Meanwhile, as two transduction processes have been described during AVP-induced human

platelet activation ; i.e. adenylate cyclase inhibition (18, 19) and stimulation of inositol phospholipid metabolism (4), further studies are needed to determinate if more than one G-protein is involved in the receptor-effector coupling mechanism.

The observation of large variations in AVP maximal binding capacity (Bmax), in relation with variations in AVP-induced GTPase stimulation, among individuals, may represent different desensitization stages of the platelet AVP receptor. In physiological conditions, platelet AVP-receptors could appear to be subject to homologous down-regulation, since important and variable amounts of peptide are associated with platelet membranes (20, 21). Therefore, studies concerning this possible regulation are required and are now in progress in our laboratory.

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