Desensitization and antagonism of vasopressin-induced phosphoinositide metabolism and elevation of cytosolic free calcium concentration in human platelets

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1. The receptor mechanisms underlying vasopressin-induced human platelet activation were investigated with respect to stimulation of phosphoinositide metabolism and changes in the cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). 2. Vasopressin stimulated phosphoinositide metabolism, as indicated by the early formation of [^{32}P]phosphatidic acid ([^{32}P]PtdA) and later accumulation of [^{32}P]phosphatidylinositol ([^{32}P]PtdIns). In addition, vasopressin elicited a transient depletion of [^3H]PtdIns and accumulation of [^3H]PtdA. The effects of vasopressin on phosphoinositide metabolism were concentration-dependent, with half maximal [^{32}P]PtdA formation occurring at 30 ± 15 nM-vasopressin. 3. In the presence of 1 mM extracellular free Ca^{2+} , vasopressin induced a rapid, concentration-dependent elevation of $[\text{Ca}^{2+}]_i$ in quin2-loaded platelets: half-maximal stimulation was observed at 53 ± 20 nM-vasopressin. 4. The V_1 -receptor antagonist [1-(β -mercapto- β , β -cyclopentamethylenepropionic acid),2-(O -methyl)tyrosine,8-arginine]-vasopressin selectively inhibited vasopressin (100 nM)-induced [^{32}P]PtdA formation [I_{50} (concn. giving 50% inhibition) = 5.7 ± 2.4 nM] and elevation of $[\text{Ca}^{2+}]_i$ ($I_{50} = 3 \pm 1.5$ nM). 5. Prior exposure of platelets to vasopressin rendered them unresponsive, in terms of [^{32}P]PtdA formation and elevation of $[\text{Ca}^{2+}]_i$, to a subsequent challenge with vasopressin, but responsive to a subsequent challenge with U44069, a thromboxane- A_2 mimetic. 6. These results indicate that vasopressin-induced human platelet activation is initiated by combination with specific V_1 receptors on the platelet, and that the sequelae of receptor occupancy (stimulation of phosphoinositide metabolism and elevation of $[\text{Ca}^{2+}]_i$) are equally susceptible to inhibition by receptor antagonists and by receptor desensitization.

INTRODUCTION

The polypeptide hormone vasopressin exerts numerous pharmacological actions, including activation of human platelets, vasoconstriction and anti-diuresis. These effects are initiated via combination with distinct receptors that have been classified, on the basis of the putative transduction processes to which they are linked, into two major sub-types, V_1 and V_2 : the former are coupled to phosphoinositide hydrolysis and elevation of cytosolic free $[\text{Ca}^{2+}]$, whereas the latter are coupled to adenylate cyclase and cyclic AMP formation (Michell *et al.*, 1979).

The major biochemical intermediaries that regulate the reactivity of blood platelets are cyclic AMP, Ca^{2+} and 1,2-diacylglycerol (DG). Increased cyclic AMP is generally considered to be an inhibitory modulator (Haslam *et al.*, 1978), whereas Ca^{2+} and DG are proposed to be stimulatory modulators in platelets (Nishizuka, 1983; Rink *et al.*, 1983). More recent evidence would suggest that DG may also function as a negative feedback regulator of platelet reactivity (MacIntyre *et al.*, 1985a).

Elevated $[\text{Ca}^{2+}]$ and DG formation may be derived from a common receptor-operated transduction process,

the phospholipase-C-catalysed hydrolysis of membrane phosphoinositides. Hydrolysis of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5) P_2] yields DG and inositol 1,4,5-trisphosphate [Ins(1,4,5) P_3] (Berridge, 1984). Ins(1,4,5) P_3 can mobilize Ca^{2+} from internal, non-mitochondrial stores in pancreatic cells (Streb *et al.*, 1983), hepatocytes (Burgess *et al.*, 1984) and platelets (O'Rourke *et al.*, 1985), among others: its involvement, if any, in Ca^{2+} influx remains to be ascertained (Berridge & Irvine, 1984).

Phosphatidylinositol (PtdIns) and phosphatidylinositol 4-phosphate (PtdIns4P) may merely function to replenish the pool of PtdIns(4,5) P_2 that is degraded, or may themselves be hydrolysed by phospholipase C to yield DG and the corresponding inositol phosphate(s) (Wilson *et al.*, 1985).

Although vasopressin is a potent agonist on human platelets, little has been documented on the transduction processes activated by occupancy of platelet vasopressin receptors. Vasopressin receptors in different tissues may be categorized by using specific antagonists (Sawyer & Manning, 1984). For example, the specific V_1 receptor antagonist $\text{d}(\text{CH}_2)_5\text{Tyr}(\text{Me})\text{AVP}$ selectively inhibits

Abbreviations used: DG, 1,2-diacylglycerol; PtdIns(4,5) P_2 , phosphatidylinositol 4,5-bisphosphate; PtdIns4P, phosphatidylinositol 4-phosphate; PtdIns, phosphatidylinositol; PtdA, phosphatidic acid; Ins(1,4,5) P_3 , inositol 1,4,5-trisphosphate; $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concentrations; $\text{d}(\text{CH}_2)_5\text{Tyr}(\text{Me})\text{AVP}$, [1-(β -mercapto- β , β -cyclopentamethylenepropionic acid),2-(O -methyl)tyrosine,8-arginine]vasopressin.

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vasopressin-induced phosphoinositide metabolism and Ca^{2+} flux in hepatocytes (Kirk *et al.*, 1981; Creba *et al.*, 1983; Thomas *et al.*, 1983; Joseph & Williamson, 1983; Mauger *et al.*, 1984) and vascular smooth muscle (Nabika *et al.*, 1985). Pharmacological studies using $\text{d}(\text{CH}_2)_5\text{Tyr}(\text{Me})\text{AVP}$ have shown the human platelet vasopressin receptor to be of the V_1 sub-type (Thomas *et al.*, 1982). In accordance with this classification, studies with quin2 (Brydon *et al.*, 1983; Hallam *et al.*, 1984) indicate that vasopressin evokes elevation of $[\text{Ca}^{2+}]_i$ in human platelets, and Vanderwel *et al.* (1983) have reported that vasopressin receptors are not coupled to inhibition of adenylate cyclase, at least in intact platelets.

To characterize further vasopressin receptor-effector coupling in human platelets, we examined the concentration-response relationships between elevation of $[\text{Ca}^{2+}]_i$ and phosphoinositide hydrolysis, and the effects of the V_1 receptor antagonist $\text{d}(\text{CH}_2)_5\text{Tyr}(\text{Me})\text{AVP}$ and of prior exposure to vasopressin on these biochemical sequelae of receptor occupancy.

EXPERIMENTAL

Preparation of platelet-rich plasma

Blood (60–80 ml) was obtained from the antecubital vein of healthy human volunteers, who denied having taken drugs known to affect platelet function within the previous 7 days, and collected into plastic tubes containing 10% (v/v) trisodium citrate (0.13 M). After centrifugation (1000 *g* for 4 min, at 15–20 °C), the supernatant platelet-rich plasma was aspirated into plastic tubes.

Phospholipid studies

Stimulation of phosphoinositide metabolism was monitored as changes in radiolabelled PtdA and PtdIns. Those studies were carried out essentially as previously described (MacIntyre & Pollock, 1983; Pollock *et al.*, 1984). Briefly, platelet-rich plasma was centrifuged (800 *g* for 10 min at 15–20 °C) in the presence of prostaglandin I_2 (0.3 μM), and the resultant pellet resuspended in one-fifth the platelet-rich-plasma volume of Ca^{2+} -free, phosphate-free, Hepes buffer, pH 7.4, and incubated (37 °C; 90 min) with either carrier-free $[\text{P}^{32}]\text{P}_i$ (30 $\mu\text{Ci}/\text{ml}$) or $[\text{H}^3]\text{glycerol}$ (40 $\mu\text{Ci}/\text{ml}$). Platelets were then pelleted by centrifugation (800 *g* for 10 min at 15–20 °C) in the presence of prostaglandin I_2 (0.3 μM). Pellets were resuspended in 1.5-fold the labelling volume of fresh buffer, and portions (0.4 ml; containing 0.5–1 mg of protein) were dispensed into plastic tubes at 37 °C. Reactions were initiated by the addition of the appropriate concentration of vasopressin (4 μl) or vehicle (4 μl) in control samples. For antagonist studies, $\text{d}(\text{CH}_2)_5\text{Tyr}(\text{Me})\text{AVP}$ (4 μl) or vehicle (4 μl) was added to platelets 30 s before the addition of vasopressin. For desensitization studies, platelets were exposed to vasopressin or vehicle, and 5 min later the platelets were re-challenged with vasopressin, the thromboxane A_2 mimetic, U44069, a heterologous agonist that combines with a distinct receptor (Pollock *et al.*, 1984), or vehicle. Reactions were terminated by transferring the entire platelet sample into a glass test tube containing 2 ml of chloroform/methanol/10 M-HCl (25:50:4, by vol.) at room temperature (Lloyd *et al.*, 1972). Lipids were extracted essentially by method B of Lloyd *et al.* (1972), dried at 40 °C under N_2 , separated by two-dimensional

t.l.c. (Yavin & Zutra, 1977), and detected by exposure to iodine vapour and/or autoradiography. Carrier PtdA (5 μg) was added to all extracts to facilitate its identification.

To examine the effects of vasopressin on PtdIns4P and PtdIns(4,5) P_2 , portions (0.4 ml) of ^{32}P -labelled platelets were treated with vasopressin or vehicle, as appropriate. Reactions were terminated by transferring the entire platelet sample into a glass test tube containing 1.2 ml of chloroform/methanol (1:2, v/v) on ice. Lipids were extracted essentially as described by Schacht (1981), dried at 40 °C under N_2 and separated by t.l.c. on oxalate-impregnated high-performance-t.l.c. plates (Jolles *et al.*, 1981). Individual phospholipids, identified by using authentic standards, were detected by exposure to iodine vapour and/or autoradiography.

Measurement of platelet cytosolic free Ca^{2+}

Platelet cytosolic free Ca^{2+} was monitored by using the fluorescent quinoline dye, Quin2 (Tsien *et al.*, 1982). Platelet-rich plasma was incubated (37 °C; 30 min) with quin2 acetoxymethyl ester, which is membrane-permeant and crosses the plasma membrane, where it is rapidly hydrolysed by cytoplasmic esterases to the membrane-impermeant polycarboxylate anion that is quin2. The quin2 is thus trapped within the cytosol of intact platelets and can report the resting concentration of $[\text{Ca}^{2+}]$ and changes that result from exposure to agonists. The quin2-labelled platelets were separated from plasma containing extraneous dye by gel filtration on columns of Sepharose 2B equilibrated with a modified Hepes-buffered Tyrode solution (Pollock *et al.*, 1984). The quin2 content of platelets was around 1 mmol/litre of cells. Gel-filtered platelets were stored at 37 °C in stoppered plastic tubes, and, to compensate for chelation of Ca^{2+} by citrate in the buffer, the external free Ca^{2+} concentration was adjusted to 1 mM by addition of CaCl_2 immediately before use. Platelets (2 ml) were placed in 1 cm-square quartz cuvettes at 37 °C, and fluorescence was monitored in a Perkin-Elmer LS3 fluorescence spectrometer with standard monochromator settings of 339 nm excitation and 492 nm emission. Quantification of quin2 fluorescence as the concentration of cytosolic free Ca^{2+} was performed after lysis of the platelet sample with digitonin (30 μM) to give F_{max} , and addition of excess EGTA to give F_{min} , essentially as described by Tsien *et al.* (1982).

Platelet function tests

Platelet aggregation was monitored photometrically in 0.17 ml samples of washed platelet suspension containing purified human fibrinogen (50 $\mu\text{g}/\text{ml}$).

Other assays

Protein was measured by the method of Lowry *et al.* (1951).

Materials

Carrier-free $[\text{P}^{32}]\text{P}_i$ was obtained from the West of Scotland Regional Isotope Dispensary, Glasgow, U.K. $[\text{H}^3]\text{Glycerol}$ (500 Ci/mol) was obtained from Amersham International, Amersham, Bucks, U.K. Prostaglandin I_2 and U44069 (9 α ,11 α -epoxymethanoprostano-5-*cis*,13-*trans*-dienoic acid) were given by Dr. J. E. Pike, Upjohn Co., Kalamazoo, MI, U.S.A. and were dissolved as described previously (Pollock *et al.*,

1984). Quin2 acetoxymethyl ester, purchased from Lancaster Synthesis, Morecambe, U.K., was dissolved in dimethyl sulphoxide. [8-Arginine]vasopressin, purchased from Sigma, Poole, Dorset, U.K., and $\text{d}(\text{CH}_2)_5\text{Tyr}(\text{Me})\text{AVP}$, a gift from Dr. M. Manning, Medical College of Ohio, Toledo, OH, U.S.A., were dissolved in iso-osmotic saline. Phospholipid carriers and standards were obtained from Sigma. All organic solvents and other laboratory reagents were analytical grade. Macherey–Nagel Polygram Sil G pre-coated glass t.l.c. plates and Merck silica gel 60 high-performance t.l.c. plates were purchased from Camlab, Cambridge, U.K., and Macfarlane Robson Ltd., Glasgow, U.K., respectively.

RESULTS

Preliminary studies established that addition of vasopressin (10–1000 nM) to quin2-labelled platelets or to platelets prepared under conditions for radiolabelling resulted in concentration-dependent platelet shape-change and aggregation (results not shown). Thus, under conditions for monitoring phosphoinositide hydrolysis and $[\text{Ca}^{2+}]_i$, platelet functional responsiveness to vasopressin is maintained.

Phosphoinositide metabolism and $[\text{Ca}^{2+}]_i$

The time course of vasopressin-induced phosphoinositide metabolism was monitored by using a supra-maximal concentration of agonist. Fig. 1(a) depicts the effects of vasopressin on platelet $[\text{P}^{32}]\text{PtdA}$ and $[\text{P}^{32}]\text{PtdIns}$. In these experiments, the control amounts of $[\text{P}^{32}]\text{PtdA}$ did not change during the course of the experiment, whereas $[\text{P}^{32}]\text{PtdIns}$ showed some fluctuation. Vasopressin (1 μM) elicited a rapid increase in $[\text{P}^{32}]\text{PtdA}$, which was maximal $[627 \pm 147\%$ (mean $\pm$ S.D., $n = 4$) of the zero-time control] within 30 s of agonist addition. The

amount of $[\text{P}^{32}]\text{PtdA}$ did not change between 30 and 60 s, but thereafter declined to $185 \pm 44\%$ (mean $\pm$ S.D., $n = 3$) of the control value at 300 s. The corresponding amounts of $[\text{P}^{32}]\text{PtdIns}$ did not change significantly within 60 s of agonist addition. Although in the experiment shown there is an apparent decrease in $[\text{P}^{32}]\text{PtdIns}$ at 30 s and 60 s after addition of vasopressin, this pattern was not reproduced in three other experiments. In contrast, 300 s after the addition of vasopressin, $[\text{P}^{32}]\text{PtdIns}$ increased significantly, to $136 \pm 14\%$ (mean $\pm$ S.D., $n = 4$) of the control value. Increased $[\text{P}^{32}]\text{PtdA}$ soon after agonist addition most probably reflects the initial receptor-activated hydrolysis of phosphoinositides, whereas the subsequent decline in $[\text{P}^{32}]\text{PtdA}$ and increase in $[\text{P}^{32}]\text{PtdIns}$ most probably reflect PtdA catabolism. Fig. 1(b) depicts similar experiments performed with platelets incubated with $[\text{H}^3]\text{glycerol}$ to label the 'backbone' of the phospholipids. Within 30–120 s of addition of vasopressin (1 μM) there was a significant increase in $[\text{H}^3]\text{PtdA}$, which later declined to regain the control value at 300 s. The increase in $[\text{H}^3]\text{PtdA}$ after addition of vasopressin was never greater than 200% of the zero-time control. The corresponding amounts of $[\text{H}^3]\text{PtdIns}$ fell significantly within 30 s of agonist addition. The maximum depletion of $[\text{H}^3]\text{PtdIns}$, $38 \pm 4\%$ (mean $\pm$ range, $n = 2$) of the zero-time control, was observed at 60 s after agonist addition, and thereafter the amount of $[\text{H}^3]\text{PtdIns}$ returned towards control values. Resting amounts of $[\text{P}^{32}]\text{PtdIns}$ (4,5) P_2 and $[\text{H}^3]\text{PtdIns}$ (4,5) P_2 were not altered after the addition of vasopressin (1 μM) for up to 30 s (results not shown).

Fig. 1 shows that, of the two procedures utilized, formation of $[\text{P}^{32}]\text{PtdA}$ is the more sensitive and rapidly occurring index of vasopressin-stimulated phosphoinositide metabolism in human platelets. The concentration–response relationships for vasopressin-induced $[\text{P}^{32}]\text{PtdA}$ formation and elevation of $[\text{Ca}^{2+}]_i$ are

Fig. 1. Time-course of $[\text{P}^{32}]\text{PtdA}$ and $[\text{H}^3]\text{PtdA}$ changes induced by vasopressin

Samples (0.4 ml) of $[\text{P}^{32}]\text{PtdA}$ -prelabelled (a) or $[\text{H}^3]\text{PtdA}$ -prelabelled (b) platelets were incubated at 37°C in the presence of vasopressin (1 μM) (■, ●) or saline, in control samples (□, ○), for the times indicated. Reactions were quenched, phospholipids extracted and separated, and the amounts of $[\text{P}^{32}]\text{PtdA}$ or $[\text{H}^3]\text{PtdA}$ (●, ○) and $[\text{P}^{32}]\text{PtdIns}$ or $[\text{H}^3]\text{PtdIns}$ (■, □) were monitored. Results are mean values $\pm$ S.E.M. for triplicate determinations. The experiments shown are typical of three similar experiments for (a) and two similar experiments for (b), performed with platelets obtained from different donors. Statistical analysis (control versus exposure to vasopressin) was by Student's t test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Fig. 2. Concentration-dependence of vasopressin-induced $[^{32}\text{P}]\text{PtdA}$ formation and elevation of $[\text{Ca}^{2+}]_i$

Samples (0.4 ml) of ^{32}P -prelabelled platelets were incubated at 37°C for 30 s in the presence of vasopressin, at the concentrations indicated, or saline in control samples. Reactions were quenched, phospholipids extracted and separated, and the amounts of $[^{32}\text{P}]\text{PtdA}$ monitored (●). Samples (2 ml) of quin2-labelled platelets suspended in a medium of external free $[\text{Ca}^{2+}] = 1\text{ mM}$ were challenged with vasopressin at the concentrations indicated. Changes in $[\text{Ca}^{2+}]_i$ were calculated from the observed changes in dye fluorescence (○). The results are mean values $\pm$ S.E.M. for three to six determinations on platelets from different donors. The data are expressed as a percentage of the maximum response, respectively increased $[^{32}\text{P}]\text{PtdA}$ formation (●), range 200–1628 c.p.m./mg of protein, and increase in $[\text{Ca}^{2+}]_i$ (○), range 105–653 nM.

depicted in Fig. 2. For ease of comparison the data are expressed as a percentage of the maximum response. The threshold concentration for stimulation of $[^{32}\text{P}]\text{PtdA}$ formation is around 10 nM, and maximum responses were observed at 0.1–1 μM -vasopressin, depending on the platelet donor. The calculated EC_{50} (the concentration of agonist that elicits 50% of the maximum response) for $[^{32}\text{P}]\text{PtdA}$ formation was $30 \pm 15\text{ nM}$ -vasopressin (mean $\pm$ S.D., $n = 5$). As measured with quin2-labelled platelets suspended in a medium of extracellular free Ca^{2+} concentration of 1 mM, the resting $[\text{Ca}^{2+}]_i$ was $90 \pm 3\text{ nM}$ (mean $\pm$ S.E.M., $n = 42$). After addition of vasopressin, there was a rapid concentration-dependent increase in $[\text{Ca}^{2+}]_i$ to around 400–500 nM. The threshold concentration for vasopressin-induced elevation of $[\text{Ca}^{2+}]_i$ was around 10 nM, and the calculated EC_{50} was $53 \pm 20\text{ nM}$ (mean $\pm$ S.E.M., $n = 7$).

Effects of $\text{d}(\text{CH}_2)_5\text{Tyr}(\text{Me})\text{AVP}$

Modifications of the basic structure of [8-arginine]-vasopressin give rise to compounds of different affinity for V_1 and V_2 receptors. The compound $\text{d}(\text{CH}_2)_5\text{Tyr}(\text{Me})\text{AVP}$ is a specific V_1 -receptor antagonist (Kruszynski *et al.*, 1980) and an inhibitor of vasopressin-induced human platelet activation (Thomas *et al.*, 1982). Preincubation of platelets with $\text{d}(\text{CH}_2)_5\text{Tyr}(\text{Me})\text{AVP}$ resulted in the concentration-dependent inhibition of vasopressin-in-

Fig. 3. Effect of $\text{d}(\text{CH}_2)_5\text{Tyr}(\text{Me})\text{AVP}$ on vasopressin-induced $[^{32}\text{P}]\text{PtdA}$ formation and elevation of $[\text{Ca}^{2+}]_i$

Platelets pre-labelled with quin2 or ^{32}P were incubated (30–60 s; 37°C) with $\text{d}(\text{CH}_2)_5\text{Tyr}(\text{Me})\text{AVP}$, at the concentrations indicated, or saline, in control samples, before addition of vasopressin (100–300 nM) and measurement of $[^{32}\text{P}]\text{PtdA}$ formation and elevation of $[\text{Ca}^{2+}]_i$ as described in Fig. 2. Results (mean values $\pm$ S.E.M. of duplicate or triplicate determinations in three different platelet preparations) are expressed as percentage inhibition of the control (saline + vasopressin) response, respectively increased $[^{32}\text{P}]\text{PtdA}$ formation (●), range 343–440 c.p.m./mg of protein, and increase in $[\text{Ca}^{2+}]_i$ (○), range 100–330 nM.

duced $[^{32}\text{P}]\text{PtdA}$ formation and increased $[\text{Ca}^{2+}]_i$ (Fig. 3). The I_{50} value (concentration of antagonist that decreases the response to the agonist by 50%) was $5.7 \pm 2.4\text{ nM}$ for $[^{32}\text{P}]\text{PtdA}$ formation and $3 \pm 1.5\text{ nM}$ for elevation of $[\text{Ca}^{2+}]_i$ (results are mean values $\pm$ S.D., $n = 3$). The effects of $\text{d}(\text{CH}_2)_5\text{Tyr}(\text{Me})\text{AVP}$ were specific for vasopressin, as even when tested at 100 nM, the compound did not alter changes in $[\text{Ca}^{2+}]_i$ evoked by other agonists such as platelet-activating factor or U44069 (results not shown).

Effects of prior exposure to vasopressin

Platelet functional responses (aggregation) display the phenomenon of agonist-specific (or homologous) desensitization (Raff, 1976), whereby prior exposure of the cells to one agonist renders them hypo-responsive (refractory) to a subsequent addition of agonists that act on the same receptor, but normo-responsive or hyper-responsive to a subsequent addition of agonists that act at a different receptor (Evans & Gordon, 1974; Ruggles & Scrutton, 1979). As vasopressin-induced $[^{32}\text{P}]\text{PtdA}$ formation and elevation of $[\text{Ca}^{2+}]_i$ are reversible, we sought to examine whether desensitization of functional responsiveness was mediated, at least in part, by desensitization of the putative receptor-activated transduction processes.

Fig. 4 depicts the effects of prior exposure of platelets to vasopressin on $[^{32}\text{P}]\text{PtdA}$ formation induced by subsequent challenge with vasopressin or a heterologous agonist, U44069. As expected, vasopressin (1 μM) elicited a marked stimulation of $[^{32}\text{P}]\text{PtdA}$ formation within 30 s, which returned towards control values at 300 s. At this point a second addition of vasopressin (1 μM) for 30 s elicited no further increase in $[^{32}\text{P}]\text{PtdA}$. In contrast,

Fig. 4. Desensitization of vasopressin-induced $[^{32}\text{P}]\text{PtdA}$ formation

Samples (0.4 ml) of ^{32}P -prelabelled platelets were exposed to $1\ \mu\text{M}$ -vasopressin ($\blacktriangle$), $1\ \mu\text{M}$ -U44069 ($\blacksquare$) or saline ($\triangle$, $\square$) as a first addition (at A_1). At 300 s, the platelets were then exposed to a second addition (at A_2) of agonists or saline. Reactions were quenched at the times indicated and $[^{32}\text{P}]\text{PtdA}$ was measured as described in Fig. 1. Results are mean values $\pm$ S.E.M. of triplicate determinations. The experiment shown is typical of two similar experiments with platelets obtained from different donors.

Fig. 5. Desensitization of vasopressin-induced elevation of $[\text{Ca}^{2+}]_i$

Samples (2 ml) of quin2-labelled human platelets were exposed to vasopressin (VP; 500 nM) or saline, in control samples; 4 min later, when the vasopressin-induced elevation of $[\text{Ca}^{2+}]_i$ had returned to control values, the platelets were re-challenged with vasopressin (a) or with U44069 (100 nM) (b). Changes in $[\text{Ca}^{2+}]_i$ were calculated from the observed changes in dye fluorescence. The data shown are representative of three experiments performed with platelets obtained from different donors.

within 30 s of addition of U44069 ($1\ \mu\text{M}$) to vasopressin-treated platelets, $[^{32}\text{P}]\text{PtdA}$ was elevated to an amount comparable with that observed after addition of U44069 ($1\ \mu\text{M}$) to untreated platelets. This pattern of desensitization was also evident for vasopressin-induced elevation of $[\text{Ca}^{2+}]_i$ (Fig. 5).

DISCUSSION

The results of the present study indicate that, after combination with specific receptors on the human platelet plasma membrane, vasopressin elicits phosphoinositide metabolism and elevation of $[\text{Ca}^{2+}]_i$; both responses may be of importance in vasopressin-induced platelet activation.

Vasopressin-induced elevation of $[\text{Ca}^{2+}]_i$ is maximal within 10–15 s of agonist addition, and, depending on the donor, is evident at concentrations around 10 nM-vasopressin and is maximal around 100–300 nM-vasopressin. The maximum extent of the elevation of $[\text{Ca}^{2+}]_i$ after addition of vasopressin is 500 nM, a value in reasonable accord with that reported by Hallam *et al.* (1984).

Vasopressin-induced phosphoinositide hydrolysis was not monitored directly as inositol phosphate(s) accumulation, because human platelets do not readily accumulate $[^3\text{H}]\text{inositol}$. Rather, phosphoinositide hydrolysis was monitored indirectly as PtdA formation by using platelets prelabelled with $[^{32}\text{P}]\text{P}_i$ or $[^3\text{H}]\text{glycerol}$. In the former situation, it is known that $[^{32}\text{P}]\text{PtdA}$ is in isotopic equilibrium with the $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ of the platelet pool of metabolic adenine nucleotides. Thus changes in $[^{32}\text{P}]\text{PtdA}$ truly reflect changes in PtdA concentration (Holmsen *et al.*, 1984). Moreover, the DG precursor of $[^{32}\text{P}]\text{PtdA}$ is known to derive predominantly via hydrolysis of phosphoinositides rather than via synthesis *de novo*, hydrolysis of other phospholipids or deacylation of triacylglycerol (Pollock *et al.*, 1984). $[^3\text{H}]\text{Glycerol}$ labelling and measurement of $[^3\text{H}]\text{PtdA}$ proved to be a less sensitive index of vasopressin-induced phosphoinositide metabolism than was $[^{32}\text{P}]\text{PtdA}$. The most likely explanation of this discrepancy is that the specific radioactivity of $[^3\text{H}]\text{DG}$ formed as a consequence of phosphoinositide metabolism is lower than that of resting $[^3\text{H}]\text{PtdA}$.

Unlike $[^{32}\text{P}]\text{PtdA}$, under the conditions of these experiments, $[^{32}\text{P}]\text{PtdIns}$ does not attain isotopic equilibrium with the metabolic $[^{32}\text{P}]\text{ATP}$. Consequently, PtdIns hydrolysis is compensated by an increase in the specific radioactivity of $[^{32}\text{P}]\text{PtdIns}$. This would explain the inconsistent effects of vasopressin on $[^{32}\text{P}]\text{PtdIns}$ at early time points, and the accumulation of $[^{32}\text{P}]\text{PtdIns}$ at later time points, after vasopressin addition, when the cycle has turned over many times. $[^3\text{H}]\text{Glycerol}$ labelling circumvents these rapid changes in PtdIns specific radioactivity, to reveal a substantial depletion of $[^3\text{H}]\text{PtdIns}$ within 30–60 s of vasopressin addition. This depletion of PtdIns may result from flux of PtdIns via $\text{PtdIns}4\text{P}$ to $\text{PtdIns}(4,5)\text{P}_2$ to replenish $\text{PtdIns}(4,5)\text{P}_2$ hydrolysed after receptor occupancy. In this context it is noteworthy that, under conditions, where thrombin (1 unit/ml) evokes a 25% loss of $[^{32}\text{P}]\text{PtdIns}(4,5)\text{P}_2$ within 10 s of agonist addition, we could detect no decrease in $[^{32}\text{P}]\text{PtdIns}(4,5)\text{P}_2$ in response to vasopressin ($1\ \mu\text{M}$). This would suggest that, if $\text{PtdIns}(4,5)\text{P}_2$ is the inositol lipid species degraded after receptor occupancy, then with

'weak' agonists (e.g. vasopressin), unlike 'strong' agonists (e.g. thrombin), the rate of [32 P]PtdIns(4,5) P_2 re-synthesis must equal that of [32 P]PtdIns(4,5) P_2 hydrolysis. Alternatively, depletion of [3 H]PtdIns may reflect hydrolysis of PtdIns by phospholipase C, known to occur with thrombin as agonist (Wilson *et al.*, 1985), or by phospholipase A $_2$ (Lapetina, 1982).

That vasopressin-induced phosphoinositide hydrolysis and elevation of [Ca^{2+}] $_i$ are sequelae of receptor occupancy is indicated by desensitization studies and by the use of the vasopressin antagonist d(CH $_2$) $_5$ Tyr(Me)AVP. Vasopressin-induced [32 P]PtdA formation and elevation of [Ca^{2+}] $_i$ are reversible processes, suggesting that the receptors undergo a rapid desensitization and that cellular metabolism and the normal homeostatic mechanisms return the [32 P]PtdA content and elevated [Ca^{2+}] $_i$ to or near control values. That receptor desensitization can occur is evident, as subsequent addition of vasopressin, but not the thromboxane A $_2$ mimetic U44069, fails to stimulate either [32 P]PtdA formation or elevation of [Ca^{2+}] $_i$, in platelets previously exposed to vasopressin.

Since vasopressin elicits elevation of [Ca^{2+}] $_i$ and phosphoinositide hydrolysis, the transduction processes initiated by occupancy of vasopressin receptors on platelets are similar to those on hepatocytes (Creba *et al.*, 1983; Joseph & Williamson, 1983) and vascular smooth muscle (Nabika *et al.*, 1985), and would suggest that vasopressin receptors on platelets are of the V $_1$ type (Michell *et al.*, 1979). This is confirmed by the effects of the specific V $_1$ -receptor antagonist d(CH $_2$) $_5$ Tyr(Me)AVP, which selectively inhibits, with equal potency, vasopressin-induced phosphoinositide hydrolysis and Ca^{2+} flux in platelets.

Vasopressin induces platelet activation (shape change and/or aggregation) over the same concentration range in which it induces phosphoinositide metabolism and elevation of [Ca^{2+}] $_i$. The increase in [Ca^{2+}] $_i$ elicited by vasopressin, and other platelet agonists, comprises 10–20% mobilization of intracellular Ca^{2+} and 80–90% influx of extracellular Ca^{2+} (Brydon *et al.*, 1983; Rink & Hallam, 1984; MacIntyre *et al.*, 1985b). We can only speculate that, by analogy with other cell types (Berridge & Irvine, 1984), Ins P_3 formation precedes and may evoke the mobilization of internal Ca^{2+} . As platelets are electrically non-excitable (MacIntyre & Rink, 1982; Doyle & Ruegg, 1985), Ca^{2+} influx must occur via some form of receptor-operated Ca^{2+} channel in the plasma membrane. The involvement, if any, of phosphoinositide metabolism in Ca^{2+} influx remains to be established (Berridge, 1984).

Were [Ca^{2+}] $_i$ to be the only mediator of platelet responsiveness, for agonists to elicit shape-change, secretion and aggregation, [Ca^{2+}] $_i$ would require to be elevated to 500 nM, 800 nM and 2000 nM respectively (Rink *et al.*, 1982). Platelet functional responses are observed at concentrations of vasopressin (> 10 nM, depending on the donor) that induce but small changes in [Ca^{2+}] $_i$: even maximally effective concentrations of vasopressin elevate [Ca^{2+}] $_i$ to an extent that is insufficient for [Ca^{2+}] $_i$ alone to mediate the gamut of platelet responsiveness. However, at these same concentrations, vasopressin elicits [32 P]PtdA formation, indicative of phosphoinositide hydrolysis and DG formation. Consequently vasopressin-induced platelet activation most probably arises, at least in part, from the synergistic

interactions of [Ca^{2+}] $_i$ and DG (Nishizuka, 1983; Rink *et al.*, 1983).

There is evidence that inhibition of adenylate cyclase may also be a transduction process by which agonists (e.g. α_2 -adrenoceptor agonists) can elicit platelet activation (Steer & Salzman, 1980). Vanderwel *et al.* (1983) have shown that vasopressin, by combining with V $_1$ receptors, can inhibit adenylate cyclase in human platelet particulate fractions. However, this effect was not observed in intact cells, and thus may not be of relevance to the platelet activation process. It is of interest that GTP-binding proteins are known to link receptor occupancy to inhibition of adenylate cyclase and to activation of phospholipase C (Gilman, 1984; Haslam & Davidson, 1985; Cockcroft & Gomperts, 1985). Fractionation of human platelets may alter the conditions, or the components, of V $_1$ -receptor-effector coupling such that the 'normal' process in intact cells (i.e. activation of phospholipase C) is replaced by inhibition of adenylate cyclase.

Having considered the mechanisms underlying vasopressin-induced platelet activation, it is intriguing to consider that these receptors may have no physiological role, as the concentrations of vasopressin that circulate (Ahmed *et al.*, 1967) are too low to activate platelets (Haslam & Rosson, 1972). Hanley *et al.* (1984) have identified a vasopressin-like peptide as a co-transmitter in sympathetic nerves. Perhaps this, or other as yet unidentified vasopressin-like peptides, is the endogenous agonist at the platelet V $_1$ receptor.

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