Biochimica et Biophysica Acta, 1112 (1992) 181–186

BBAMEM 75815

Vasopressin elevation of Na^+/H^+ exchange is inhibited by genistein in human blood platelets

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(Received 1 June 1992)

Key words: Sodium ion–proton exchange; Vasopressin; Genistein; Tyrosine phosphorylation; Phorbol ester; Staurosporine; Protein kinase C; Blood platelet; (Human)

The regulation of intracellular Na^+ and pH_i in human blood platelets is known to be controlled by the function of the Na^+/H^+ exchanger. The phosphorylation state of the Na^+/H^+ exchanger which determines the exchanger activity in human blood platelets is regulated by the activities of protein kinases and protein phosphatases. Observations in this study indicate that arginine vasopressin (AVP) that interacts with a V_1 receptor, activates the Na^+/H^+ exchange in human blood platelets through a genistein-inhibited mechanism. The AVP-activated Na^+/H^+ exchange is probably not regulated by protein kinase C (PKC), since this activation is not inhibited by staurosporine. The multiple ways in which platelet Na^+/H^+ exchange can be modulated may indicate the critical role played by this exchanger in the homeostasis control of pH_i in human blood platelets.

Introduction

The Na^+/H^+ exchanger plays a major role in the control of pH_i and other cell functions [1,2]. In human blood platelets, agonists such as thrombin and arginine vasopressin (AVP) are found to regulate the pH_i in addition to other platelets specific functions [3–7]. Using a specific antibody, it was demonstrated in CCL39 and A431 epithelial cells that Na^+/H^+ exchange is operated by a phosphorylated transmembrane glycoprotein of 110 kDa [8], as was also detected recently in human blood platelets [9]. Modulation of the Na^+/H^+ exchange activity in human blood platelets by thrombin, phorbol 12-myristate 13-acetate (PMA), dihexanoyl glycerol, staurosporine and okadaic acid, a protein phosphatase 1 and 2A inhibitor, indicate that the phosphorylation state of the exchanger is related to its activity and is controlled by the balance of protein kinase C (PKC) [9–11] and protein phosphatases [9,10] activities.

The physiological effects of AVP have been demonstrated both in vivo and in vitro [12], to be mediated by two functional AVP receptors, V_1 and V_2 [13]. The V_1 receptor was detected in human blood platelets, liver cells, vascular smooth muscle cells and fibroblasts [14]. The binding of AVP to the V_1 receptor activates PKC activity through a phosphatidylinositol-specific phospholipase C, GTP-binding proteins and a Ca^{2+}-mediated biochemical cascade [14,15]. V_2 receptor activates GTP-binding proteins which regulate adenylate cyclase activity, which in turn increases in kidney tubuli the cAMP-dependent Na^+ and H_2O reabsorption [16]. This V_2 type AVP receptor was not found in human blood platelets.

AVP has been shown to act synergistically with insulin to induce mitogenic effects in 3T3 fibroblasts [17], and to activate c-*myc* expression in human fibroblasts [18]. Recent observations demonstrate that AVP induces the phosphorylation of 38–44 kDa protein(s) on tyrosine residues through the V_1 receptor in human blood platelets [19] and in vascular smooth muscle cells [20]. In addition, the tyrosine phosphorylation of several proteins with molecular weight higher than 38–44 kDa was induced by AVP, bombesin, angiotensin II and endothelin in several cell types [21–23]. In line with these observations, it was interesting to test whether AVP affects the Na^+/H^+ exchange in human blood platelets due to tyrosine phosphorylation of protein(s). In this study we show that, like PMA [7,9,10], AVP stimulates the Na^+/H^+ exchange in human blood platelets [7], as was also demonstrated in mesangial cells [24,25]. But, unlike the PMA-induced Na^+/H^+ exchange, the AVP-induced one is: (1) inhibited by the tyrosine kinase inhibitor genistein [26], and (2) less sensitive than the PMA-induced one to staurosporine, a potent and relatively specific PKC inhibitor. Thus,

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the AVP-induced Na^+/H^+ exchange in human blood platelets is regulated by a genistein-sensitive, yet unidentified mechanism that does not involve PKC mediation.

Materials and Methods

Chemicals and solutions. Albumin (bovine from fraction V), hirudin, probenecid, Na propionate, AVP, staurosporine and PMA were obtained from Sigma. 2',7'-Bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF) acetoxymethyl ester was obtained from Molecular Probes. Genistein was obtained from Indofine (NJ). V_1 [desGly(NH_2)d(CH_2)$_5$Tyr (Me) AVP[b]] and V_2 [desGly(NH_2)d(CH_2)$_5$[D-Ile^2,Ile^4]AVP] receptor antagonists were kindly provided by Dr. M. Manning, Medical College of Ohio. Sepharose 2B was purchased from Pharmacia and [^{32}P]phosphoric acid (20 mCi/ml) was obtained from Rotem.

Acid-citrate-dextrose solution was composed of 65 mM citric acid, 11 mM glucose and 85 mM trisodium citrate. The standard Na^+ medium contained, in mM: 140 NaCl, 5 KCl, 0.42 NaH_2PO_4, 10 glucose and 20 Hepes (pH 7.35). The NaCl-Na propionate solution contained, in mM: 80 NaCl, 60 Na propionate, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 20 Hepes (pH 7.35). The osmolarity of all media was adjusted to 285 ± 2 mosM with distilled water or NaCl solution. Stock solution of probenecid (0.1 M) was prepared in ethanol, while stock solutions of BCECF acetoxymethyl ester (2.5 mg/ml), PMA (1.6 mM), staurosporine (0.5 mM) and genistein (20 mM), were prepared in dimethylsulfoxide (DMSO). Stock solution of AVP (20 mM) was prepared in 10 mM Na acetate (pH 4.0).

Preparation of platelet suspension. Venous blood was drawn from healthy volunteers, aged 25–50 years, who had not received any medication during the previous 14 days. The blood was anticoagulated with acid-citrate-dextrose at a volume ratio of blood/anticoagulant of 6:1. Platelet-rich plasma was obtained by centrifugation at $120 \times g$ for 10 min and had a pH of 6.5 ± 0.1. For the spectrofluorimetric measurements of pH changes, the platelets were first loaded in the dark with the parent acetoxymethyl ester (3 μM, final) for 30 min and were then gel-filtered through a Sepharose 2B column (10×0.76 cm). The solution used to equilibrate the column and to elute the platelets was the standard Na^+ medium described above but modified by adding albumin (1 mg/ml) and adjusting the pH to 6.8. The suspension of BCECF-loaded platelets was supplemented with $CaCl_2$ (1 mM), $MgCl_2$ (1 mM), hirudin (0.01 unit/ml) and probenecid (0.2 mM). Probenecid (*p*-[dipropylsulfamoyl]benzoic acid) blocks organic anion transporters that remove fluorescent dyes from the cytoplasmic matrix [27]. We found that the use of probenecid (0.2 mM) cuts down the leak of BCECF from platelets by about 3-fold. All fluorimetric measurements were conducted within 50 min after the preparation of dye-loaded platelets, with a maximal dye leak of $7.5 \pm 0.4\%$ ($n = 29$).

Determination of cytoplasmic pH changes. Changes in cytoplasmic pH were determined as described [28]. Fluorescence was measured in a Jasco FP-770 spectrofluorimeter with wavelength settings at 495 and 525 nm for excitation and emission using 5 and 10 nm slits, respectively. For measuring the Na^+/H^+ exchange, an aliquot (10–20 μl) of the gel-filtered platelets loaded with BCECF, was mixed with 1.8 ml of the NaCl-Na propionate solution ($(2–3) \cdot 10^7$ platelets/ml). AVP was added to the assay medium after dilution to the appropriate concentrations in the column elution solution. All other tested compounds were added to the assay medium dissolved in 1.8 μl DMSO, prior to the addition of the platelets. DMSO up to 0.2% did not change the control values. Fluorimetric tracings were recorded for 90 s, starting within < 3 s of adding the platelets. At termination, 9 μl of 10% Triton X-100 was added and calibration of pH vs. fluorescence was performed with increments of 3-(*N*-morpholino)propanesulfonic acid as titrant. The pH was monitored in the cuvette by GK2401C combined electrode, connected to Ion 83 Ion meter (Radiometer, Copenhagen), with a resolution of 0.001 pH unit. A predetermined factor was used to correct for the red shift of the intracellular dye. The calibration of pH_i, the Na^+/H^+ exchange rate and the pH_i set point were determined as described [28]. The Na^+/H^+ exchange rate is the ΔpH_i of the alkalinization process per 95 s at pH_i 7.

Determination of PKC in human blood platelets. Platelet-rich plasma was supplemented with prostaglandin E_1 (2.8 μM) and centrifuged at $1500 \times g$ for 10 min. The platelets were resuspended (about $(8–9) \cdot 10^8$ cells/ml) in: 140 mM NaCl, 5 mM KCl, 10 mM glucose, 20 mM Hepes (pH 6.8), 1 mg /ml albumin and were incubated for 90 min at 37°C in the presence of 0.1 mCi [^{32}P]phosphoric acid. After incubation, the platelets were gel-filtered, as described above. After 2 min stimulation at 37°C with agonists, cells were immediately mixed with an equal volume of 2× concentrated lysis buffer and frozen in liquid nitrogen (1× lysis buffer: 10% glycerol, 25 mM NaCl, 50 mM NaF, 10 mM Na pyrophosphate, 2 mM Na_2VO_4, 20 mM *p*-nitrophenyl phosphate, 25 mM Tris-HCl (pH 7.4)). The platelets' lysates were mixed with 1/4 volume of 5× sample buffer followed by heating at 100°C for 5 min (1× sample buffer: 3% SDS, 0.0015% bromophenol blue, 5% 2-mercaptoethanol, 11% glycerol, 70 mM Tris-HCl (pH 6.8)). The samples were subjected to 11% SDS-PAGE [29] and the 42 kDa protein band in each sample was identified by autoradiography, using Agfa RP2 X-ray films and DuPont lightning-Plus intensifying screens. Radioctivity in the corresponding pro-

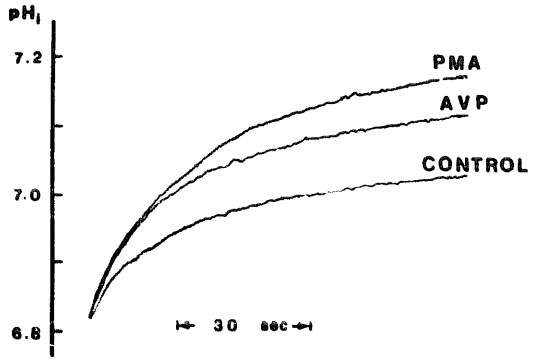

Fig. 1. Rate of alkalinization of BCECF-labeled platelets following acidification, in NaCl-Na propionate solution, as affected by $5 \cdot 10^{-7}$ M PMA or 10^{-6} M AVP. Tracings of a single experiment representative of at least ten additional experiments.

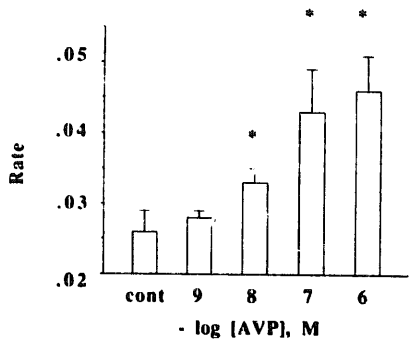

Fig. 2. Effect of AVP in the concentration range 10^{-9}–10^{-6} M on the rate of BCECF-labeled platelet alkalinization following acidification in NaCl-Na propionate solution. The data presented are averages of three experiments, each in duplicate measurements. The bars indicate S.E. and the star (*) indicates statistical differences at $P < 0.05$ according to Student's t test.

tein band was determined using liquid scintillation spectometry. Additional quantitation was obtained by scanning the films and integrating the peaks using ImageQuant Personal Densitometer, Molecular Dynamics. The two systems gave similar results.

Results and Discussion

AVP enhances Na^+/H^+ exchange in human blood platelets

When blood platelets are suspended in NaCl-Na propionate solution (pH 7.35), a rapid intracellular acidification takes place (≤ 3 s) to pH 6.83 ± 0.02, followed by a slower alkalinization process. This process represents Na^+/H^+ exchange, based on full dependency on external Na^+ and high sensitivity to amiloride and its potent analogues [28,30]. If the NaCl-Na propionate solution contains AVP (10^{-6}M) or PMA ($5 \cdot 10^{-7}$ M), the platelets respond by an increased rate of Na^+/H^+ exchange, as shown in Fig. 1. At these concentrations AVP and PMA did not change the buffering capacity. The values obtained, remained in the range of 21.4 ± 1.0 mM (pH unit)$^{-1}$ ($n = 4$), as was measured by the addition of 5 mM NH_4Cl according to previous procedure for human blood platelets [31]. Fig. 2 depicts the concentration-dependency of the AVP effect. Saturation is apparent at 10^{-7}–10^{-6} M AVP, when the Na^+/H^+ exchange rate is approximately doubled, while half-maximal effect is attained at $3 \cdot 10^{-8}$ M AVP. Intriguingly, a similar pattern of concentration dependence was found for the impact of AVP on tyrosine phosphorylation of 38 kDa protein in human blood platelets [19], and 40–44 kDa proteins in vascular smooth muscle cells [20]. As shown in Table I, the AVP effect is mediated through a V_1 receptor, since only the V_1-receptor specific antagonist [32] decreased significantly the AVP-elevated Na^+/H^+ exchange rate and pH_i-set point. These observations are in concert with the V_1 receptor existence in human blood platelets [14].

The AVP effect is inhibited by genistein

As PMA is known to activate PKC [33], and since AVP may affect both PKC [14,15], and the phosphorylation of various proteins on tyrosine residues [19–23], we compared the effect of genistein on Na^+/H^+ exchange which had been stimulated by AVP or PMA. Genistein was selected for the comparison, since this isoflavone is a relatively specific tyrosine kinase inhibitor [26], even though it interacts with the ATP binding site in the catalytic domains of tyrosine kinases that share a high degree of conserved concensus sequence with serine/threonine kinases [34]. In addition to the Na^+/H^+ exchange rate, we also examined, for this comparison, its effect on pH_i set point, which reflects regulation of the exchanger by phosphorylation and dephosphorylation [9,28].

Table II shows that both AVP and PMA increase markedly the exchange rate and cause an alkaline shift

TABLE I

The effect of V_1 and V_2 antagonists on AVP-elevated Na^+/H^+ exchange rate and pH_i set-point

Values presented are the means ± S.E. for three different experiments. The concentration of the V_1 and V_2 antagonists used was 10^{-7} M. At this concentration both antagonists did not affect the control values. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$; n.s., not significant.

Treatment	Rate ($\times 10^3$)	pH_i set-point	Statistical comparison
1. Control	16.6 ± 1.7	7.28 ± 0.02	
2. AVP, 10^{-7} M	31.7 ± 1.3 ***	7.45 ± 0.02 ***	vs. treatment 1
3. AVP + V_1 antagonist	19.8 ± 3.2 **	7.33 ± 0.04 '	vs. treatment 2
4. AVP + V_2 antagonist	26.6 ± 3.6 (n.s.)	7.42 ± 0.01 (n.s.)	vs. treatment 2

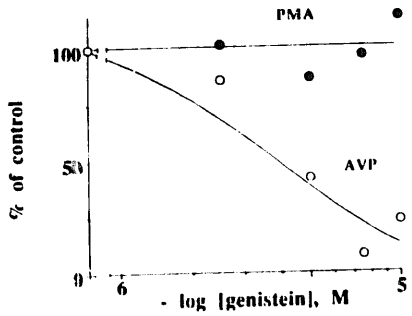

Fig. 3. Genistein-selective and -concentration dependent inhibition of the stimulation of platelet alkalinization rate by 10^{-7} M PMA (solid cycles) or 10^{-7} M AVP (open cycles). Representative of two experiments, each in duplicate measurements.

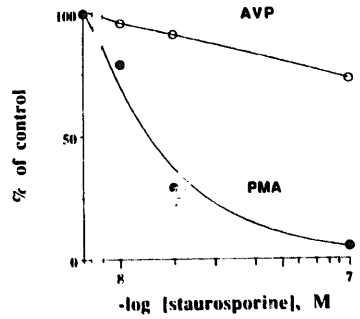

Fig. 4. Staurosporine-selective and -concentration dependent inhibition of the stimulation of platelet alkalinization rate by 10^{-7} M PMA (solid cycles) or 10^{-6} M AVP (open cycles). Representative of three experiments, each in duplicate measurements.

of the pH_i set point (treatment 2 and 3, respectively). Genistein, at $7.5 \cdot 10^{-6}$ M, nearly abolishes the impact of AVP (treatment 5), but it is selectively innocuous toward the impact of PMA (treatment 6), and toward the control platelets (treatment 4). The dose-dependency of genistein in affecting differentially the stimulation of Na^+/H^+ exchange rate by AVP and PMA (Fig. 3), indicates its high potency and selectivity. While the PMA stimulation was not affected by genistein at the concentrations indicated in the figure, almost complete inhibition of the AVP stimulation was apparent at $7.5 \cdot 10^{-6}$ M, and the concentration for half-maximal inhibition was $4 \cdot 10^{-6}$ M. A similar inhibitory potency of flavones has been reported for the inhibition of the specific tyrosine kinase activity of purified $pp60^{v\text{-}src}$ [35], preparations of EGF and insulin receptors [26]. This is the first demonstration that AVP dependent and associated tyrosine phosphorylation mediates elevation of Na^+/H^+ exchange rate and pH_i set point in human blood platelets. Furthermore, the AVP-induced Na^+/H^+ exchange is relatively less affected by increasing concentrations (10^{-6}–10^{-7} M) of staurosporine, a potent and relatively specific inhibitor for PKC [36,37], compared with the PMA-induced Na^+/H^+ exchange (Fig. 4).

Genistein was found to inhibit only slightly the PKC activity isolated from rabbit kidneys, $IC_{50} > 100$ μM, with Histone H1 as a substrate [26]. On the other hand, an additional study demonstrates the inhibitory effect of this isoflavone on PKC prepared from hog brain, $IC_{50} = 15$ μM, using the same protein substrate [38]. We therefore investigated, in the experiments illustrated in Fig. 5, the effect of genistein (20 μM) on PKC activity in intact human blood platelets. The phosphorylation of 42 kDa protein, a major substrate for PKC in human blood platelets [39,40], is markedly induced by 0.2 μM PMA (lane 2). This phosphorylation is inhibited by 0.5 μM staurosporine (lane 3), but not by 20 μM genistein (lane 4). On the other hand, the phosphorylation of the 42 kDa protein by 0.1 μM AVP (lane 6), is very low (~ 7% from the PMA treatment). Nevertheless, this low level of AVP dependent phosphorylation of the 42 kDa protein is inhibited by staurosporine (lane 7), but is not altered by genistein (lane 8). These observations confirm the differential inhibitory effect of staurosporine and genistein on the PKC activity in human blood platelets. Preliminary experiments revealed that the doses of genistein and DMSO used in this study did not affect the binding of AVP to its surface receptor in human blood platelets or in vascular smooth muscle cells (data not shown).

Thus, it is likely that AVP affects the Na^+/H^+ exchange in human blood platelets by tyrosine phosphorylation mediated pathway that is inhibited by genistein. This yet unidentified pathway may phosphorylate the Na^+/H^+ exchanger, either directly or indi-

TABLE II

The effect of genistein on AVP- and PMA-elevated Na^+/H^+ exchange rate and pH_i set-point

Values presented are means ± S.E. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; n.s., not significant.

Treatment (n)	Rate ($\times 10^3$)	pH_i set-point	Statistical comparison
1. Control (7)	22.7 ± 2.5	7.26 ± 0.01	
2. AVP, 10^{-6} M (5)	47.1 ± 3.8 ***	7.43 ± 0.02 ***	vs. treatment 1
3. PMA $5 \cdot 10^{-7}$ M (6)	54.8 ± 3.3 ***	7.61 ± 0.04 ***	vs. treatment 1
4. Genistein, $7.5 \cdot 10^{-6}$ M (5)	24.6 ± 2.7 (n.s.)	7.32 ± 0.02 (n.s.)	vs. treatment 1
5. AVP + genistein (4)	21.5 ± 2.1 **	7.34 ± 0.03 *	vs. treatment 2
6. PMA + genistein (3)	51.5 ± 1.8 (n.s.)	7.76 ± 0.1 (n.s.)	vs. treatment 3

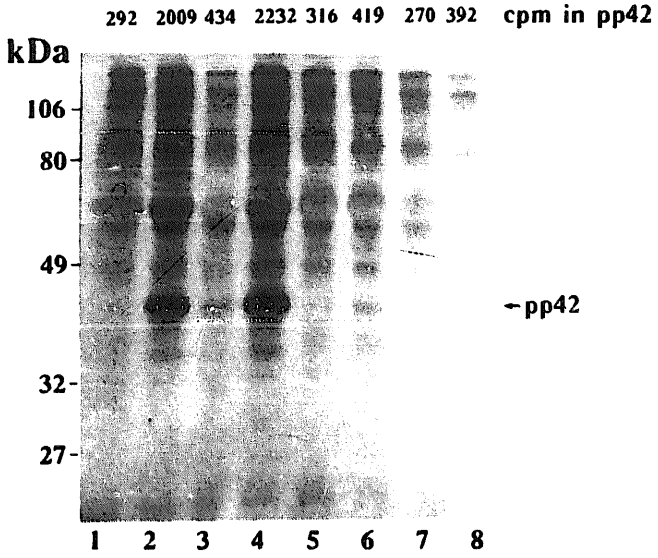

Fig. 5. PMA ($2 \cdot 10^{-7}$ M, lanes 2–4), and AVP (10^{-7} M, lanes 6–8) dependent phosphorylation of pp 42 kDa protein, a major PKC substrate in human blood platelets. Autoradiogram of a SDS-PAGE on which ^{32}P-radiolabeled platelets proteins were separated. Control (lanes 1 and 5), staurosporine $5 \cdot 10^{-7}$ M (lanes 3 and 7) or genistein $2 \cdot 10^{-5}$ M (lanes 4 and 8) treated platelets.

rectly. This suggestion is supported by several observations. First: AVP leads to phosphorylation of platelet 38 kDa protein(s) on tyrosine residues [19]. Second: the potency of genistein in inhibiting the AVP effect (Table II and Fig. 3) is similar to its effect on other tyrosine kinases [26]. Third: like PMA, AVP leads to a significant alkaline shift of the pH_i set point, as well as to an enhanced exchange rate (Table II). These expressions are attributable to phosphorylation of the Na^+/H^+ exchanger. But, unlike PMA, the possible phosphorylation of the Na^+/H^+ exchanger due to the AVP action through V_1 receptor is probably not mediated by PKC (Figs. 4 and 5), although previous studies have shown that activation of V_1 receptor mediates PKC activity [14,15].

On the basis of the data presented in this study, the following conclusions may be drawn: (1) Stimulation of platelet Na^+/H^+ exchange by PMA does not necessarily involve tyrosine kinase activity. (2) V_1 receptor-mediated stimulation of the Na^+/H^+ exchange by AVP apparently involves tyrosine kinase activity. The multiple forms in which the Na^+/H^+ exchanger can be regulated may reflect the critical role played by the exchanger in the homeostasis of pH_i in human blood platelets. The characteristics of the AVP-dependent tyrosine phosphorylation event associated with the stimulation of the Na^+/H^+ exchange in human blood platelets are under further study.

Acknowledgments

The study was carried out at the Amelia (Mimi) Rose Laboratory for Cellular Signal Transduction, at the Department of Life Sciences, Ben-Gurion University of the Negev. We thank the Oelbaum family of Toronto, Canada, the Israeli Fund for Research and Development for financial support and Ora Cohen-Cummings for editing and typing this manuscript.

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