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Involvement of Na^+/H^+ exchanger in desmopressin-induced platelet procoagulant response

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Desmopressin (DDAVP) action on platelets is associated with the development of procoagulant response but the underlying mechanism of this phenomenon is not known. We investigated whether this effect of DDAVP might be due to activation of plasma membrane Na^+/H^+ exchanger. The DDAVP-induced platelet procoagulant response, measured as phospholipid-dependent thrombin generation, was dose dependent and significantly weaker than that produced by collagen or monensin (mimics Na^+/H^+ antiport). Both the DDAVP- and collagen-produced procoagulant responses were less pronounced in the presence of EIPA, an Na^+/H^+ exchanger inhibitor. Flow cytometry studies revealed that *in vitro* treatment of platelets with DDAVP or collagen was associated with the appearance of both degranulated (and fragmented) and swollen cells. The DDAVP-evoked rise in size and granularity heterogeneity was similar to that produced by collagen or monensin and was not observed in the presence of EIPA. Using flow cytometry and annexin V-FITC as a probe for phosphatidylserine (PS) we demonstrated increased and uniform binding of this marker to all subsets of DDAVP-treated platelet population. The DDAVP-evoked PS expression was dose dependent, strongly reduced by EIPA and weaker than that caused by monensin or collagen. As judged by optical swelling assay, DDAVP in a dose dependent manner produced a rise in platelet volume. The swelling was inhibited by EIPA and its kinetics was similar to that observed in the presence of monensin. Electronic cell-sizing measurements showed an increase in mean platelet

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Abbreviations: DDAVP, desmopressin ([deamino-Cys[1],D-Arg]-vasopressin); EIPA, 5-(*N*-ethyl-*N*-isopropyl) amiloride; FITC, fluorescein isothiocyanate; PS, phosphatidylserine.

volume and a decrease in platelet count and platelet crit upon treatment with DDAVP. DDAVP elicited a slow (much slower than collagen) alkalinization of platelet cytosol. Altogether the data indicate an involvement of Na^+/H^+ exchanger in the generation of procoagulant activity in DDAVP-treated platelets.

Desmopressin ([deamino-Cys^1,D-Arg]-vasopressin, abbreviated DDAVP), is a synthetic analog of arginine vasopressin used for the treatment of patients with hemophilia A, von Willebrand disease, several platelet disorders and uremic bleeding (Mannucci, 1997; Lethagen, 2003). DDAVP induces an increase in the plasma levels of von Willebrand factor, coagulation factor VIII and tissue plasminogen activator (Mannucci, 1997). DDAVP can reduce the bleeding time prolongation caused by heparin (Johnstone *et al.*, 1990) and aspirin (Schulman & Johnsson, 1991). The compound is also useful in the treatment of bleeding disorders not associated with a deficiency or dysfunction of factor VIII and von Willebrand factor, including congenital and acquired defects of platelet function (Cattaneo *et al.*, 1989; Dimichele & Hathaway, 1990; Greinacher *et al.*, 1993; Mannucci, 1997).

The mechanism of improved hemostasis in patients with platelet dysfunction remains incompletely understood. It has been observed that DDAVP is able to accelerate normalization of the *in vitro* platelet dysfunction induced by GPIIb/IIIa inhibitors and aspirin (Reiter *et al.*, 2003). DDAVP has no observable effect on platelet aggregation and secretion (Yang *et al.*, 1990; Ghosh & Rao, 1993). It has been reported, however, to be able to potentiate aggregation induced by ADP and collagen (Balduini *et al.*, 1999) and at pharmacological concentrations produce P-selectin expression on the platelet surface (Wun *et al.*, 1995; Jy *et al.*, 1998). The DDAVP effects on platelets were suggested to be mediated through the activation of vasopressin receptor V1 (Wun *et al.*, 1995). It was also suggested that DDAVP may affect fluidity of platelet plasma membrane (Giorgi *et al.*, 1996).

Horstman *et al.* (1995) found that DDAVP acts on platelets to generate platelet microparticles and an enhanced procoagulant activity. In fact DDAVP was proposed to act on platelets as a weak inducer of procoagulant response. The mechanism underlying this phenomenon remains unknown.

We have found recently that generation of procoagulant activity in platelets may be evoked by the rise in the intracellular Na^+ ($[Na^+]_{cyt}$) produced by activated Na^+/H^+ exchanger (Samson *et al.*, 2001; Stelmach *et al.*, 2002). Na^+/H^+ exchangers are ubiquitous integral plasma membrane proteins that exchange extracellular Na^+ for intracellular H^+ with the stoichiometry of one per one (Orlowski & Grinstein, 1997). DDAVP as an analog of vasopressin with antidiuretic properties (Lethagen, 2003) is expected to activate Na^+/H^+ exchanger in platelets. Vasopressin is able to activate Na^+/H^+ exchanger in human platelets (Aharonovitz & Granot, 1996). It seems therefore reasonable to test the hypothesis assuming an involvement of Na^+/H^+ exchanger in the generation of procoagulant activity in DDAVP-treated platelets. As it is discussed further such a hypothesis may explain not only the DDAVP-evoked platelet procoagulant response but also the platelet volume changes and the transient thrombocytopenia observed in patients receiving this drug. Some of the results have appeared in a preliminary form (Tomasiak *et al.*, 2003).

MATERIALS AND METHODS

Chemicals. Desmopressin ([deamino-Cys^1, D-Arg]-vasopressin (DDAVP), Hepes, EGTA, apyrase, monensin, 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA), prostaglandin E_1 (PgE_1), bovine serum albumin (BSA), Russel's viper

venom (RVV), 2,7-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF/AM), *p*-nitrophenylphosphate were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Collagen (fibrillar, from equine tendon) was from Hormon Chemie (Munich, Germany). Thrombin (human) was from La Roche (Basel, Switzerland). Chromogenic substrate for thrombin was obtained from Chromogenix AB (Mölndal, Sweden). Fluorescein isothiocyanate-labelled annexin V (annexin-FITC), phycoerythrin (PE)-labelled anti GPIIb/IIIa MoAb (PE-CD41a) and PE-labelled isotypic mouse MoAb were from BD Biosciences-PharMingen.

DDAVP was dissolved at 0.1 mg/ml in phosphate-buffered saline (PBS). This stock solution was stored frozen at –80°C in small aliquots; a fresh aliquot was taken for each experiment. Monensin and EIPA were dissolved in ethanol and methanol, respectively.

Blood collection. Venous blood was collected with minimum trauma and stasis *via* a 21-gauge needle (0.8 × 40 mm) into 10 ml polypropylene tubes containing 1 ml of 130 mM trisodium citrate. All blood donors gave their informed consent. The study protocol was approved by the Ethics Committee at the Medical University of Białystok.

Preparation of washed platelets. Platelet-rich plasma (PRP) and washed platelets were prepared as described previously (Bruzgo *et al.*, 2004).

Platelet activation and flow cytometry. For PS exposure and morphology experiments 300 μl samples of PRP were placed in a cuvette of an aggregometer (Elvi Logos, Milan, Italy) and incubated at 37°C for two minutes without stirring. DDAVP or monensin (or appropriate vehiculum in control) was added and after initial mixing (30 s) incubation was continued without stirring at 37°C for 10 to 60 min. To stop the incubation 60 μl aliquots of the incubation mixture were transferred to polystyrene tubes (12 × 74 mm) containing 240 μl of Tyrode-Hepes buffer supplemented with BSA (3.5 mg/ml), $CaCl_2$ (3 mM f.c.) and hirudin (2 ATU/ml). Samples of 30 μl of the diluted suspension were combined with 20 μl of annexin-FITC (marker of PS expression on the surface of platelets) and 10 μl of PE-CD41a (marker of platelets) and incubated for 20 min in the dark. To stop the incubation the samples were diluted with 1 ml of Tyrode/Hepes buffer supplemented with BSA (3.5 mg/ml) and 2 mM $CaCl_2$. The Tyrode-Hepes buffers used in this study were prepared "particle-free". Preparation involved filtration through a 0.2-μm filter (Millipore). Flow cytometry analysis was performed within 45 min after final dilution.

Flow cytometry analysis. Flow cytometry analysis was performed using a Coulter EPICS XL (argon laser) flow cytometer. Ten thousand events were acquired for each sample and analyzed for forward light scatter (forward scatter), right angle light scatter (side scatter) and for two colour fluorescent signals. The light scatter and the fluorescence signals were set in a logarithmic gain and were stored in list mode data files. The obtained data were further analyzed using the Win MDI software program. The events were counted by triggering on a preset threshold of PE fluorescence of the platelet marker CD41a. The threshold was set above the background fluorescence with PE-labelled isotypic mouse MoAb. The CD41a positive particle populations were separated by bitmaps (dotplots) where log forward scatter is the X-axis and log side scatter is the Y-axis.

Measurement of platelet procoagulant activity. The assay system used was similar to that described by Rota *et al.* (1996). It is based on Russell's viper venom, which induces thrombin generation by activation of factors V and X, and in the presence of Ca^{2+} ions is dependent on the availability of PS. Preparation of defibrinated plasma, activation procedure and the assay of phospholipid dependent thrombin generation were performed as described previously (Stelmach *et al.*, 2002). The amidolytic activity of thrombin was expressed in international units (U) as

the number of micromoles of *p*-nitroaniline liberated in 1 min and was calculated for 1 ml of platelet suspension.

Measurement of changes in platelet volume. Changes in platelet volume (swelling) were followed spectrophotometrically by recording absorbance at 680 nm in diluted cell suspensions essentially as described by Rosskopf *et al.* (1991). A drop in absorbance reflects a rise in cell volume. The changes in absorbance were measured at 37°C in plastic cuvettes (light path 10 mm) without stirring. Aliquots of PRP (140 μl) were incubated for 2 min at 37°C in the cuvette of the instrument, then 860 μl of Tyrode-Hepes buffer (pH 7.4, osmolality 300 mOsm, previously prewarmed to 37°C) and 1–5 μl of the tested substances (or vehiculum) were added quickly. The suspension was carefully mixed for exactly 10 s and the changes in absorbance were recorded for 2 min at 10 s intervals using a sensitive spectrophotometer (Helios gamma, Unicam) connected with a personal computer.

Measurement of mean platelet volume (MPV) and platelet number. Changes in MPV and platelet number were measured by electronic cell sizing using a hematologic analyzer (Coulter Electronic GmbH).

Measurement of intracellular pH. Cytosolic pH (pH_{cyt}) was determined using an intracellularly trappable fluorescent indicator, BCECF/AM (Siffert *et al.*, 1989).

Data analysis. Data reported in this paper are the mean (± S.D.) of the number of determinations indicated (n). Statistical analysis was performed by the Student's *t*-test, elaborating experimental data by means of Slide Write plus (Advanced Graphics Software, Inc, Carlsbad, CA, U.S.A.)

RESULTS

As shown in Table 1, DDAVP induced in a dose dependent manner a procoagulant response in platelets. Compared to control, a 60-min incubation of platelets with DDAVP (50–200 nM) produced a 2.3- to 5.9-fold higher procoagulant activity. This is a relatively weak procoagulant response since platelets treated for 10 min with collagen (15 μg/ml) express a 19.7-fold rise in proco-

Table 1. Platelet procoagulant response in the presence of various substances

Additions	Time of incubation (min)	Phospholipid-dependent thrombin generation (nU/ml)
none	60	4.2 ± 1
Collagen 15 μg/ml	10	83.1 ± 8***
Collagen 15 μg/ml +EIPA 200 μM	10	7.0 ± 2
Monensin 10 μM	10	73.0 ± 7***
DDAVP 50 nM	60	10.0 ± 2*
DDAVP 100 nM	60	15.0 ± 2**
DDAVP 200 nM	60	25.0 ± 1.9***
DDAVP 300 nM	60	27.0 ± 2.2 ***
DDAVP 200 nM + EIPA 200 μM	60	6.0 ± 1.2

Procoagulant activity of washed platelets (7.5×10^8 cells/ml) measured as phospholipid-dependent thrombin generation, after incubation at 37°C without and with the tested substances added to the final concentration as indicated. Phospholipid-dependent thrombin generation was evaluated as under Materials and Methods. The amidolytic activity of thrombin in international units (U) is reported. Mean values ± S.D. are reported (n = 16). *$P < 0.5$, **$P < 0.01$, ***$P < 0.005$.

agulant activity. Collagen is the strongest physiological inducer of platelet procoagulant response. Both the DDAVP- and collagen-evoked procoagulant responses were strongly reduced after the inhibition of Na^+/H^+ exchanger by EIPA (200 μM), a selective exchanger inhibitor (Sweatt *et al.*, 1985).

Monensin was used to mimic the function of the Na^+/H^+ exchanger in platelet plasma membrane. In human platelets this ionophore immediately raises both pH_i and $[Na^+]_{cyt}$ (Siffert & Akkerman, 1988; Borin & Siffert, 1991). As it is seen in Table 1, monensin evoked a procoagulant response in platelets. In comparison to control, a ten minute incubation of platelets with monensin (10 μM) produced a 17.3-fold increase in procoagulant activity.

Figure 1 shows the results of flow cytometry studies performed to determine how DDAVP affects platelet morphology (i.e., their size and granularity). To analyze the changes in platelet morphology the bi-variate scatterplot (forward scatter *versus* side scatter dot plot) was arbitrarily split into four regions (R1–R4, see Fig. 1 as an example). The splitting of the scatterplot, defining normal untreated platelets, was performed in such a way that one of the regions (here R2) shows the majority (i.e., at least 80%) of the aquisited events. The percentage of events of the 10 000 total found (whole analyzed platelet population) is shown in each region. Assuming that forward scatter and side scatter are the criteria of platelet size and granularity, respectively (Bode & Hickerson, 2000), in the population of normal platelets R1 comprises the subpopulation of small platelets, R2 comprises the platelets with mean and large volume, R3 comprises smaller degranulated platelets and/or cells with changed surface and R4 defines degranulated, mean-volume and large platelets.

As seen from Fig. 1, a one-hour incubation of platelets with 100 nM (panel B) and 200 nM DDAVP (panel C) produces a much broader light scatter profile than in control.

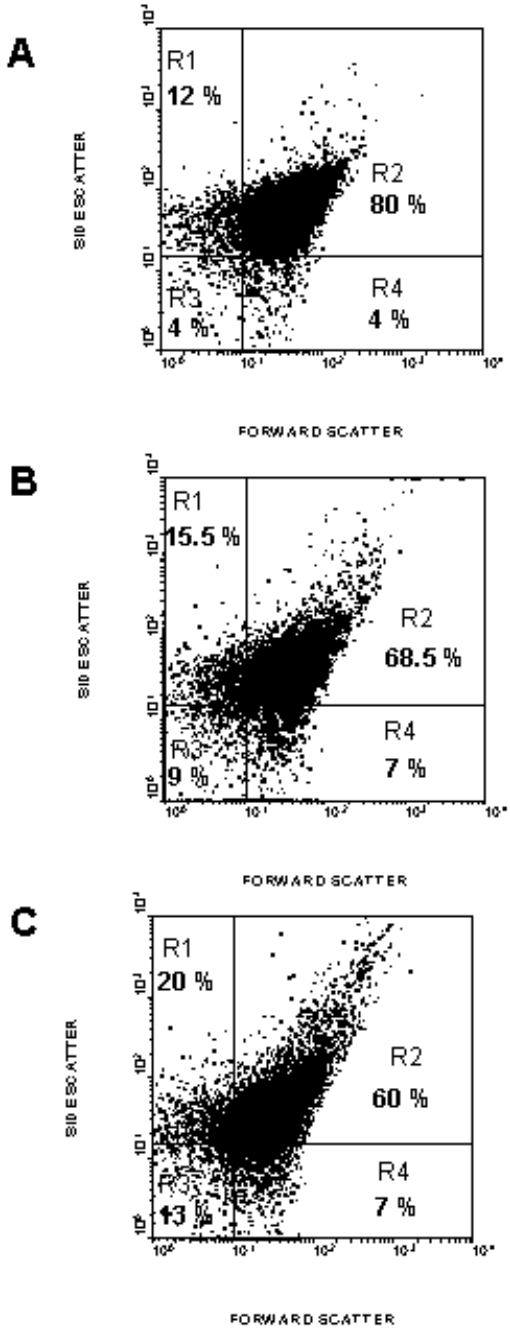

Figure 1. The effect of DDAVP on the morphology (size and granularity) of platelets.

Dot plots of forward scatter *versus* side scatter of a platelet suspension (PRP) untreated (panel A) and treated for one hour at 37°C with 100 nM DDAVP (panel B) and 200 nM DDAVP (panel C). The percentage of events of the 10 000 total found in each region is shown. Each dot plot is representative of 12 determinations performed on four different preparations. Note that DDAVP produced a unimodal left/downward shift in the forward scatter and side scatter of the entire platelet population and the appearance of larger objects in R2 region (right upper corner).

This effect was dose dependent and was manifested by a marked increase in the percentage of counts in R1 (by 8%), R3 (by 9%) and R4 (by 3%) and a simultaneous drop (by 20%) in the percentage of events in R2. Of importance are the larger objects seen in the upper right corner of R2 which may reflect the presence of a subpopulation of swollen cells in the population of DDAVP-treated platelets. Analysis of the forward scatter *versus* side scatter dot plot of platelets preincubated with DDAVP reveals a unimodal decrease in both forward and side light scatters. Both the left-shifted and down-shifted new events on flow cytometry light scatter plots appear contiguous with the unchanged subfraction of the tested platelet population. We postulate that these new events (i.e., the events appearing in regions R1 and R3) are degranulated platelets with profound changes on their surface and/or fragmented cells.

Experiments shown in Fig. 2 were performed to determine whether inhibition of platelet Na^+/H^+ exchanger by EIPA affects the DDAVP-evoked changes in platelet morphology. As seen from Fig. 2, the light scatter profile returned to nearly normal when DDAVP was added together with EIPA.

We next studied how the incubation of platelets with DDAVP affects the PS expression on their surface. The rationale for this was the observation that DDAVP produces platelet procoagulant activity, known to be dependent on aminophospholipids that appear on their surface. As seen from Fig. 3, a one-hour incubation of platelets with 100 nM (panel B) and 200 nM DDAVP (panel C) produces a rise in the percentage of platelets with PS exposed on their surface by 16% and 23%, respectively.

Figure 4 shows that the DDAVP-evoked PS expression was abolished when the cells were incubated with EIPA.

The flow cytometry data analysis shown in Fig. 5 was performed to estimate the amount of surface expressed PS on each individual subset (small, normal, large events) of DDAVP-treated platelet population. To

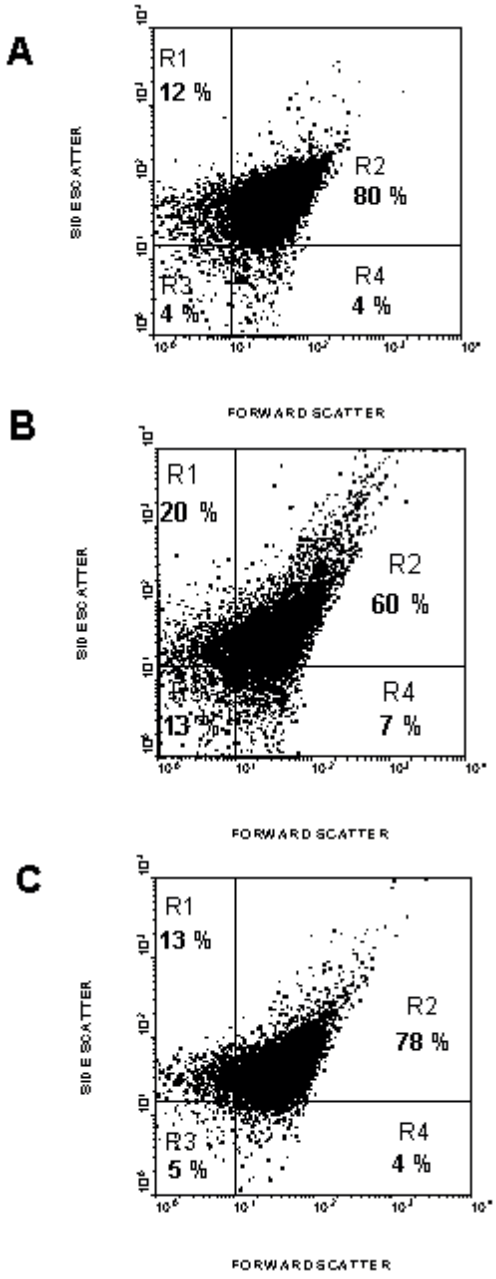

Figure 2. The effect of EIPA on the DDAVP-evoked changes in platelet morphology.

Representative dot plots of forward scatter *versus* side scatter of a platelet suspension (PRP) incubated for one hour at 37°C with 200 nM DDAVP or with 200 nM DDAVP and 100 μM EIPA. Panel A: control platelets treated with vehiculum (methanol control), B: platelets treated with DDAVP, C: platelets treated with DDAVP and EIPA. The percentage of events of the 10 000 total found in each region is shown. Dot plots are representative of 12 determinations performed on four different preparations. Note that the DDAVP-produced unimodal left/downward shift in the forward scatter and side scatter is reduced upon the inhibition of Na^+/H^+ exchanger by EIPA.

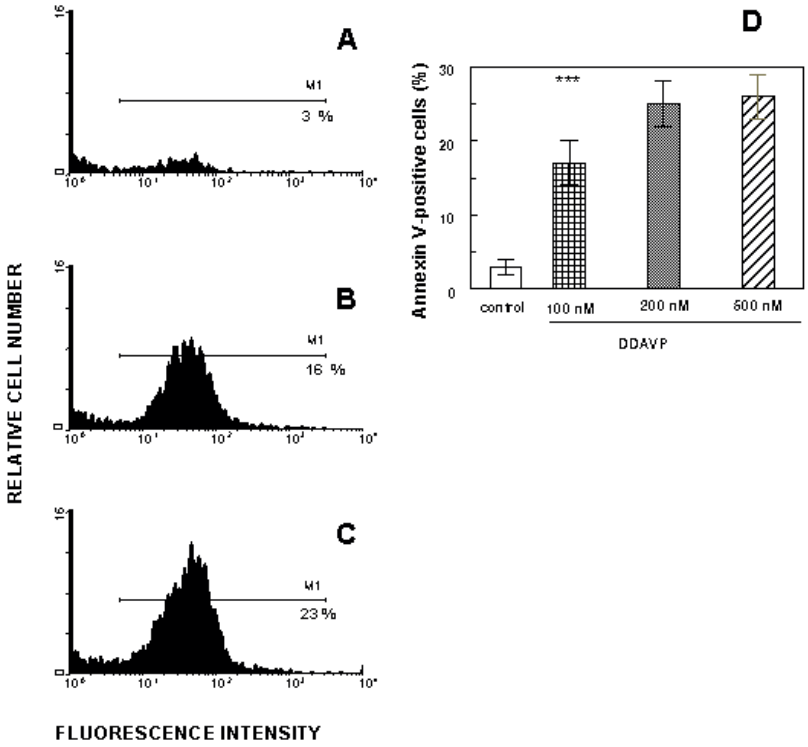

Figure 3. The effect of DDAVP on the appearance of PS on the platelet surface.

Platelets were preincubated for one hour at 37°C with no stimulator (A) or 100 nM (B) and 200 nM DDAVP (C). Panels A, B and C show representative histograms of annexin V-FITC binding. The data in panel D represent mean percentages of PS-positive platelets and standard deviations of four experiments each performed on separate platelet preparation (n = 12). Histograms of annexin V-FITC binding (fluorescence intensity) show a dose dependent DDAVP-induced conversion of platelets from PS-negative to PS-positive.

achieve this, we plotted forward scatter values with annexin-FITC fluorescence using the WinMDI program. As it is seen the PS-positive events are equally distributed in all subsets of the DDAVP-treated population of platelets.

Figures 6 and 7 show the results of experiments which were conducted to estimate how monensin and collagen affect platelet morphology and PS expression. As it is seen in Fig. 6 (panels A and B), a 10-min incubation of platelets with 10 μM monensin produces significant changes in platelet size and granularity. This was manifested by an increase in the events found in regions R1 (by 1%), R3 (by 43%) and R4 (by 24%) and a simultaneous decrease in their number in region R2 (by 68%). As is seen from Fig. 7 (panels A, B), similar changes in size and granularity are observed after 10 min of incubation of platelets with collagen (15 μg/ml). In this case we observed a rise of the number of events in regions R1, R3 and R4 by 1.5%, 41.5% and 23%, respectively, and a concomitant decrease of the events in region R2 by 66%. Analysis of forward scatter *versus* side scatter dot plots of platelets preincubated with monensin or collagen reveals a unimodal decrease in both forward and side light scatters. Both the left-shifted and down-shifted new events on the flow cytometry light scatter plots appeared contiguous with the unchanged subfraction of the tested platelet population (i.e., R2). We postulate that these new events (i.e., the events appearing in regions R1, R3 and R4) are degranulated platelets with profound changes on their surface and/or fragmented cells. As seen from Fig. 6 and 7 (panels C and D), a 10 min preincubation of platelets with monensin or collagen produces a rise in the

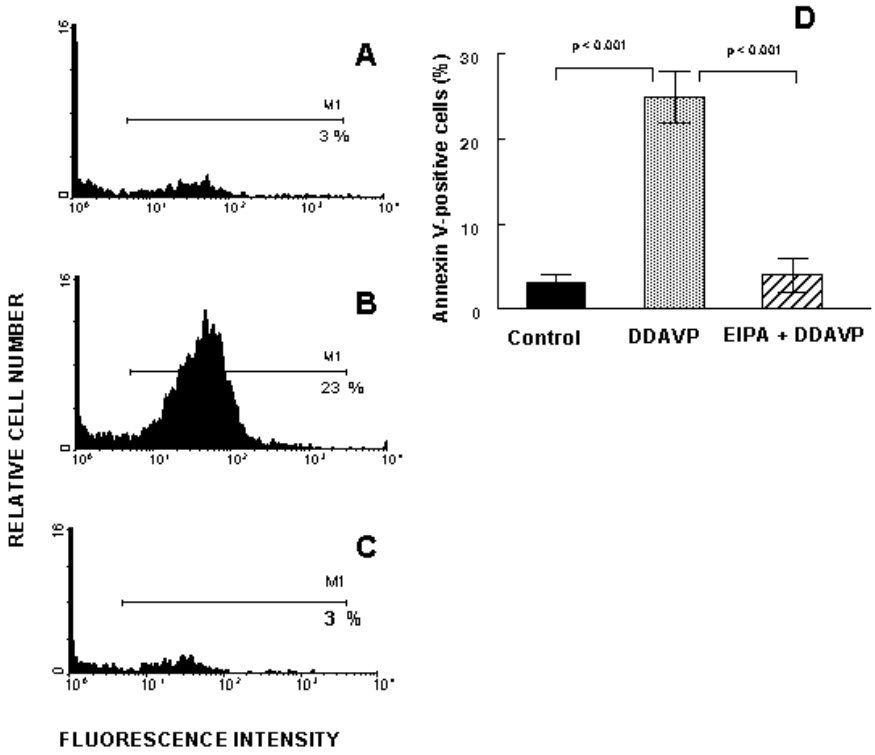

Figure 4. Inhibition by the Na^+/H^+ exchanger inhibitor (EIPA) of PS exposure in platelets treated with DDAVP.

Panel A: control platelets treated with vehiculum (methanol control). Panel B: platelets treated for one hour at 37°C with 200 nM DDAVP. Panel C: platelets treated for one hour at 37°C with 200 nM DDAVP and 100 μM EIPA. Panels A, B and C show representative histograms of annexin V-FITC binding. The data in panel D represent mean percentages of PS-positive platelets and standard deviations of four experiments, each performed on a separate platelet preparation (n = 12). Note that the DDAVP-produced annexin V-FITC binding is reduced by EIPA.

percentage of platelets with PS exposed on their surface to 41.5% and 78%, respectively.

Activation of Na^+/H^+ exchanger is expected to induce platelet swelling due to the rise in the intracellular sodium concentration. The results presented in Fig. 8 show the kinetics of platelet swelling following the addition of increasing (100–500 nM) concentrations of DDAVP. The effect of DDAVP was compared with that observed after artificially evoked (10 μM monensin) Na^+-H^+ exchange. As it is seen (panel A), DDAVP produces platelet swelling in a dose dependent manner. The DDAVP-evoked swelling was markedly slower than that produced by monensin (panel A) and was strongly reduced by EIPA (panel B).

The results presented in Table 2 show that a one-hour incubation of platelets with 200 nM DDAVP results in a small but marked drop in platelet count (by 25%) and crit (by 0.36 fl), and a rise (by 25%) in the mean platelet volume.

Figure 9 shows the results of experiments which were performed to estimate whether DDAVP is able to activate Na^+/H^+ exchanger in human platelets. It has been established that activation of Na^+/H^+ exchanger in platelets results in cytosol alkalinization (Siffert & Akkerman, 1988; Siffert *et al.*, 1989,). As it is seen (trace b), stimulation of platelets (loaded with the pH sensitive fluorophore (BCECF) with thrombin produces a rise in intracellular pH. The rise in pH_{cyt} was rapid and after 1 min it stabilized at the level of 7.1. Addition of DDAVP to BCECF-loaded platelets also initiated cytosol alkalinization (trace c) but it was very slow, and in contrast to that evoked

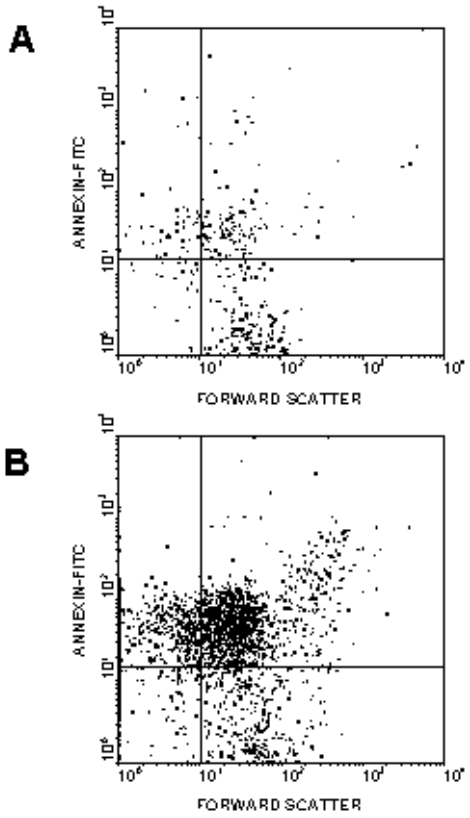

Figure 5. The effect of DDAVP on annexin V-FITC binding by separate subpopulations of platelets.

Dot plots of forward scatter *versus* annexin V-FITC fluorescence of a platelet suspension (PRP) untreated (panel A) and treated for one hour at 37°C with 200 nM DDAVP (panel B). Each dot plot is representative of eight determinations performed on four different preparations. Note the increased and uniform binding of annexin V to all subsets (small and degranulated platelets or platelet fragments, average volume platelets and swollen platelets) of DDAVP-treated platelet population.

by thrombin was long lasting. In platelets treated with DDAVP pH_{cyt} achieved the value of 7.1 after about 8 min.

DISCUSSION

The present results suggest that DDAVP behaves as a weak inducer of platelet procoagulant response. This is consistent with the observations of Horstman *et al.* (1995).

As it is reported here platelets treated *in vitro* with DDAVP express platelet factor 3 activity. This is manifested by the loss of plasma membrane asymmetry and appearance of PS on their surface. It is believed that the membrane area rich in aminophospholipds creates a thrombogenic surface that accelerates the blood coagulation process by providing catalytic sites for the assembly of the tenase and prothrombinase complexes (Zwaal & Schroit, 1997; Zwaal *et al.*, 1998). It is therefore apparent that the DDAVP-evoked platelet procoagulant response may results from the profound changes in the platelet plasma membrane.

How can DDAVP evoke the appearance of PS on the surface of platelets? It has been proposed that the main intracellular factor responsible for the loss of platelet plasma membrane asymmetry (and for the procoagulant response) is a large (micromolar) and long lasting (minutes) rise in the intracellular calcium concentration, $[Ca^{2+}]_{cyt}$ (Heemskerk *et al.*, 2002). This is, however, unlikely in the case of platelets treated with DDAVP since Horstman *et al.* (1995) failed to found a significant rise in $[Ca^{2+}]_{cyt}$ in Fluo-3-loaded platelets incubated with DDAVP. The rise in $[Ca^{2+}]_{cyt}$ seems not to be the only trigger of the loss of plasma membrane asymmetry and generation of procoagulant activity. One such factor may be an increase in the intracellular sodium concentration, $[Na^{+}]_{cyt}$ (Samson *et al.*, 2001; Stelmach *et al.*, 2002). The rise in $[Na^{+}]_{cyt}$ in activated platelets was suggested to be a consequence of the increase in the activity of the plasma membrane Na^{+}/H^{+} exchanger (Rosskopf, 1999).

Although it has never been specifically investigated, DDAVP is likely to be involved in the activation of the Na^{+}/H^{+} exchanger in platelets. This is based on the observation that DDAVP is able to displace vasopressin from its receptor (the V1 receptor) on the surface of platelets (Wun *et al.*, 1995). Vasopressin was reported to stimulate the platelet Na^{+}/H^{+} exchanger through the activation of

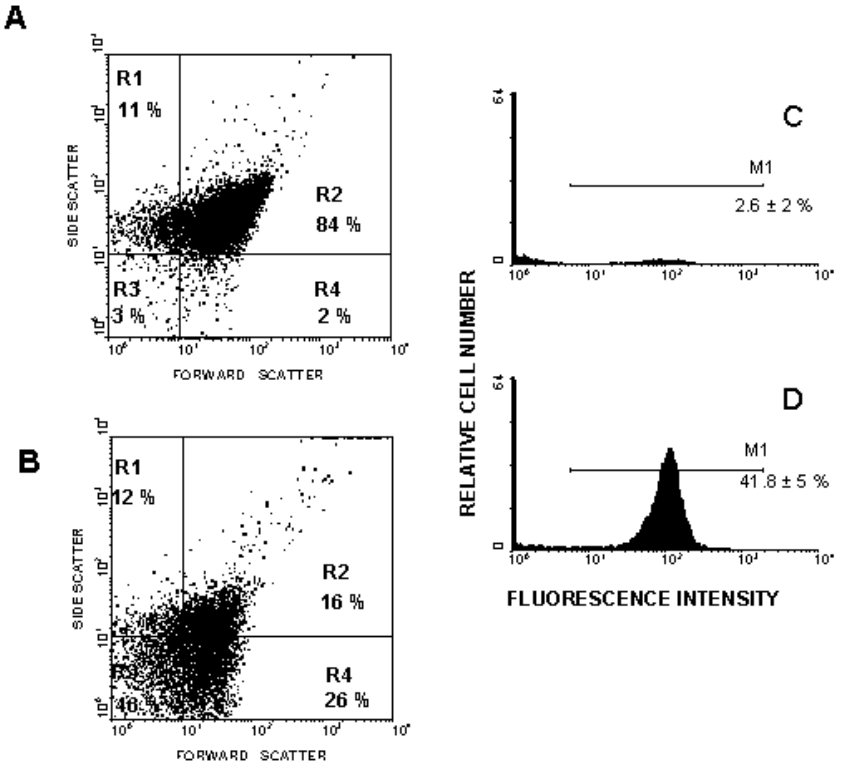

Figure 6. The effect of monensin on platelet morphology (A, B) and PS expression (C, D).

Platelets (PRP) were preincubated for 10 min at 37°C with ethanol vehiculum (panels A and C) or with 10 μM monensin (panels B and D). A, B, dot plots of forward scatter *versus* side scatter of platelets untreated (A) and treated with monensin (B). The percentage of events of the 10 000 total found in each region is shown. Panels C and D show histograms of annexin V-FITC binding. The dot plots and histograms are representative of 12 determinations performed on four different preparations. The data in panel C and D represent mean percentages of PS-positive platelets and standard deviations. Note that monensin produced a unimodal left/downward shift in the forward scatter and side scatter of the entire platelet population and the conversion of platelets from PS-negative to PS-positive.

V1 receptor (Aharonovitz & Granot, 1996). The present results show that DDAVP slowly alkalizes the cytosol in BCECF-loaded platelets, which also indicates the activation of the exchanger. The DDAVP-produced procoagulant response and PS expression were strongly reduced by blocking of the Na^+/H^+ exchanger by EIPA. EIPA is able to reduce also the collagen-evoked procoagulant response in porcine (Samson *et al.*, 2001; Stelmach *et al.*, 2002) and human platelets (Table 1). As revealed by flow cytometry, DDAVP produces a unimodal left shift in the forward scatter and side scatter of the entire platelet population, which is similar to that observed after artificially evoked Na^+-H^+ antiport and much less pronounced following blocking of the Na^+/H^+ exchanger by EIPA. It seems therefore possible that in platelets DDAVP may produce a loss of plasma membrane asymmetry, expression of PS and a rise in their procoagulant activity through the activation of the plasma membrane Na^+/H^+ exchanger.

How can the rise in the activity of Na^+/H^+ exchanger produce such profound changes in the platelet plasma membrane? Activation of Na^+/H^+ exchanger is accompanied by an acceleration in Na^+ influx (Borin & Siffert, 1990; Samson *et al.*, 2001). Since Na^+ ions transported to the cell are solvated by 6 tightly bound water molecules, sodium uptake is accompanied by the influx of water which is expected to be manifested as cell

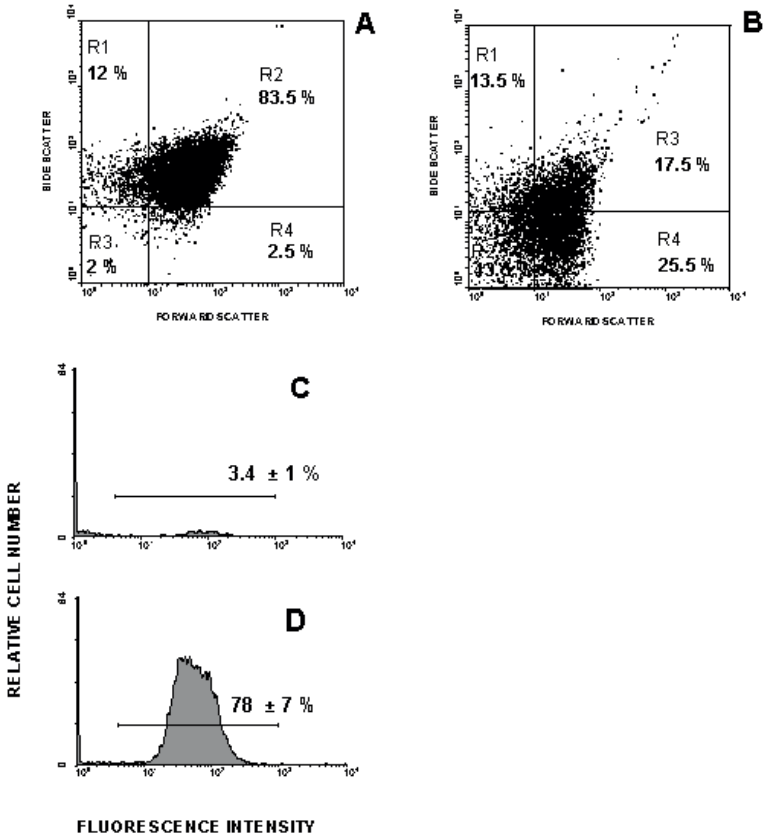

Figure 7. The effect of collagen on platelet morphology (A, B) and PS expression (C, D).

Platelets (PRP) were preincubated for 10 min at 37°C with saline (panels A and C) or with collagen added to the final concentration of 15 μg/ml (panels B and D). A and B — dot plots of forward scatter *versus* side scatter of platelets untreated (A) and treated (B) with collagen. The percentage of events of the 10 000 total found in each region is shown. Panels C and D show histograms of annexin V-FITC binding. The dot plots and histograms are representative of nine determinations performed on three different preparations. The data in panel E represent mean percentages of PS-positive platelets and standard deviations. Note that collagen produced a unimodal left/downward shift in the forward scatter and side scatter of the entire platelet population and the conversion of platelets from PS-negative to PS-positive.

swelling. As it is shown here this is really the case. Flow cytometry studies, the direct electronic cell sizing technique and optical swelling assay show that DDAVP is able to induce a rise in platelet volume. In addition, the optical swelling assay shows that the DDAVP-evoked swelling is relatively rapid, similar to that produced by artificially-evoked Na^+-H^+ antiport and strongly reduced after blocking of the Na^+/H^+ exchanger. In conclusion, the increase in platelet volume produced by the Na^+/H^+ exchanger-mediated sodium uptake may be the cause of the DDAVP- evoked platelet procoagulant response.

Little is known about the relationship between platelet swelling and their procoagulant activity. Flow cytometry studies show that DDAVP-evoked swelling is manifested by the appearance in the population of platelets not only of larger objects (exhibiting higher FS values) but also of smaller events (with low FS values). This picture is similar to that observed following artificially-evoked Na^+/H^+ exchange. Electronic cell sizing measurements show a rise in MPV and a reduction in platelet number and crit after *in vitro* DDAVP treatment. These observations indicate that following swelling a portion of the platelets

appeared to be quantitatively converted into fragments too small to be counted as platelets by clinical counters (Coulter type counters). This is consistent with the observation of Horstman *et al.* (1995), that DDAVP-treated platelets appear smaller in phase microscopy.

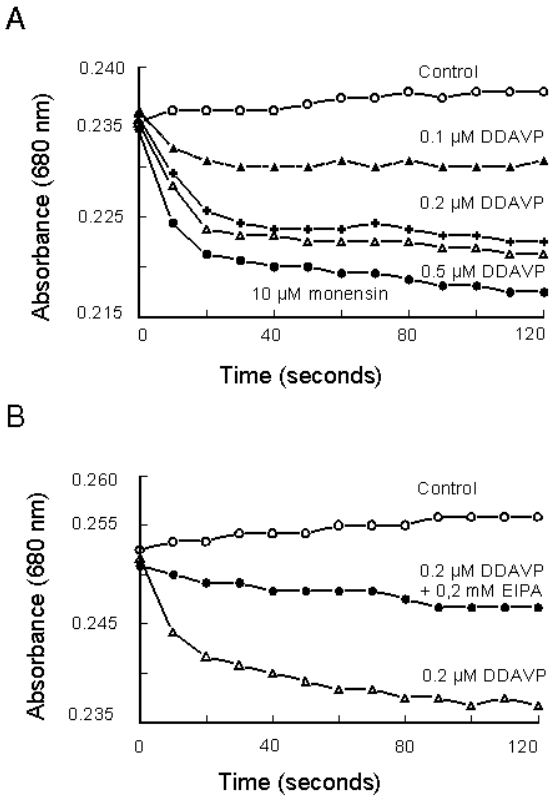

Figure 8. Kinetics of platelet swelling following addition of DDAVP or monensin.

Panel A. At time 0 suspensions of washed platelets were supplemented with DDAVP and monensin added to the final concentrations as indicated. In the control, vehiculum (ethanol) was added instead. Panel B. At time 0 suspensions of washed platelets were supplemented with DDAVP or DDAVP + EIPA added to the final concentrations as indicated. In the control, vehiculum (methanol) was added instead. Changes in platelet volume (swelling) were followed spectrophotometrically by recording absorbance as described in Materials and Methods. Measurements were performed at 37°C. Each trace is representative of at least 15 determinations performed on five different platelet preparations.

It is well known that ultrasonically disintegrated platelets also become procoagulant. In disintegrated platelets, clotting factors from the blood plasma have an easier access to the PS-rich inner leaflet of the platelet plasma membrane.

However, the disintegration of a fraction of DDAVP-treated platelet population cannot be the only explanation of the appearance of the procoagulant activity. Flow cytometry measurements show that in the population of DDAVP-treated platelets PS is expressed not only by the fraction of small events (possibly reflecting fragmented or disrupted cells) but it is rather equally distributed in all: small, normal and large objects. This may indicate that PS expression results not only from platelet disintegration but it is also a consequence of the changes occurring in the plasma membrane itself. Platelet swelling is expected to be accompanied by distending of the plasma membrane which may lead to a rise in the plasma membrane tension. Numerous experiments performed on model systems and cells other than platelets have shown that plasma membrane tension has a significant impact on exocytosis and endocytosis (Hamill & Martinac, 2001; Apodaca, 2002). In general, an increase in plasma membrane tension is followed by an increase in exocytosis. Activation of platelets by agonists evoking procoagulant response (e.g. collagen, thrombin) is always associated with exocytosis (Reed *et al.*, 2000). In exocytosis intracellular vesicles fuse with the plasma membrane supplying a vesicle membrane which becomes now the new fragment of the plasma membrane. This reduces plasma membrane tension but might have other important consequences. Assuming that intracellular vesicles (e.g. platelet granules) have an asymmetrical membrane (with PS in the inner leaflet) their fusion with the plasma membrane is expected to cause the appearance of PS on the surface of the platelet at the site of fusion (Sims & Wiedmer, 2001). Such a mechanism may explain the appearance of PS even at very early stages of platelet swelling. Further studies are needed to under-

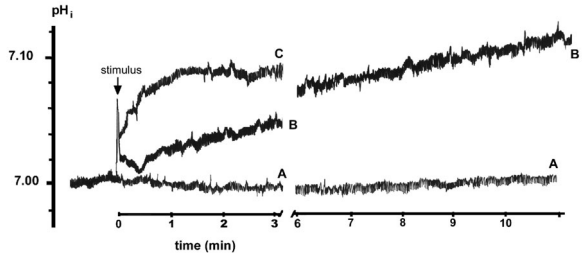

Figure 9. Effect of DDAVP and thrombin on platelet cytosolic pH.

BCECF-2-loaded platelets were untreated (control, trace A) and treated at 37°C with thrombin (trace B) or DDAVP (trace C) added (arrow) to the final concentrations of 0.1 U/ml and 200 nM, respectively. pH_{cyt} was calculated using the calibration procedure described in Materials and Methods. Each trace is representative of at least six determinations performed on three different platelet preparations.

stand how platelet swelling affects the appearance of PS on their surface.

Loss of plasma membrane asymmetry and PS externalization is an early event during apoptosis of a variety of cells (Martin *et al.*, 1995; van Engeland *et al.*, 1998). The appearance of PS in the outer leaflet of the plasma membrane of activated platelets may serve also as a catalytic surface required for the assembly of the coagulation factors and thrombus formation (Zwaal *et al.*,1997; 1998). Such a mechanism enable platelets to fulfill their major physiological function – to protect the integrity of the vascular wall. Following the contact of platelets with collagen (normally present in the subendothelium) platelets expose PS on their surface and shed PS-positive microparticles from their plasma membrane both of which contribute to the acceleration of thrombin formation (Zwaal *et al.*, 1997; 1998). This phenomenon is commonly defined as platelet procoagulant response (platelet factor 3) despite the fact that also the process of microparticle formation is morphologically similar to the membrane blebbing phase of nucleated cells undergoing apoptosis (Hacker, 2000). However, it should be stressed that in comparison to cells undergoing apoptosis the platelet procoagulant response is initiated by agonists, is very rapid (several minutes to one hour), is localized (to the site of vessel damage), and concerns a relatively large number of cells. As is shown in Fig. 2, in the case of platelets activated by collagen more than 70% of cells are PS-positive after 10 min.

In summary, a rapid expression of PS on a large fraction of platelets following addition of a stimulator is recognized as a criterium of procoagulant response.

Table 2. Platelet volume (MPV), count and crit following DDAVP treatment

	Platelet count (thousands)	Mean platelet volume (fl)	Platelet crit (%)
Control	218 ± 26	5.24 ± 0.18	0.21 ± 0.01
+ DDAVP	162 ± 19	5.6 ± 0.15	0.15 ± 0.01
p-value	1.2×10^{-3}	1.2×10^{-4}	1.5×10^{-3}

Aliquots of platelet-rich plasma were incubated for one hour at 37°C without (control) and with 200 nM DDAVP. Platelet count, mean platelet volume and crit were measured using electronic counting technique. The data represent mean values and standard deviations of four experiments, each performed on a separate platelet preparation (n = 16). Paired *t*-test was used to calculate *P*-values.

Although platelets are constitutively enucleated cells it was proposed that after 8–11 days they may undergo normal (typical) apoptosis (Li *et al.*, 2000; Bertino *et al.*, 2003). This can be observed in platelet concentrates prepared for transfusion and stored in blood

banking. In contrast to the procoagulant response this process is very slow (days) and is associated with the expression of PS on a limited subset of the whole platelet population. As reported by Shapira *et al.* (2000), in platelet concentrates stored for 1 day PS expression increased only from 2 to 5%. The results presented here indicate that DDAVP-treated platelets express PS on 23% of cells after one hour. In addition, flow cytometry studies show that the morphological changes observed in platelet population treated by DDAVP resemble those observed after addition of collagen (compare Figs. 1 and 7). Thus, DDAVP-treated platelets undergo programmed procoagulant response rather than programmed cell death.

How may the above observation be relevant to the *in vivo* situation? Platelet swelling produced by activated Na^+/H^+ exchanger can explain the transient thrombocytopenia observed in patients receiving DDAVP (Casteman *et al.*, 1993; Horstman *et al.*, 1995). The drop in platelet number may be produced by their disintegration (due to swelling). The appearance of PS at the platelet surface marks the cell as a pathologic (apoptotic) target for elimination by phagocytes (Sims & Wiedmer, 2001).

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