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The involvement of Na^+/K^+-ATPase in the development of platelet procoagulant response

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In circulation, platelets may come into contact with both exogenous (cardiac glycoside treatment) and endogenously produced inhibitors of Na^+/K^+-ATPase. We examined whether blocking of platelet Na^+/K^+-ATPase by ouabain results in generation of procoagulant activity. It was shown that an *in vitro* treatment of platelets with ouabain (20–200 μM for 20 to 60 min) is associated with an intracellular accumulation of sodium ($[Na^+]_i$), generation of a weak calcium signal, and expression of procoagulant activity. The ouabain-induced procoagulant response was dose- and time-related, less pronounced than that evoked by collagen and similar to that produced by gramicidin, not affected by EDTA or aspirin, and strongly reduced in the absence of extracellular Na^+ or by hyperosmolality. Flow cytometry studies revealed that ouabain treatment results in a unimodal left shift in the forward and side scatter of the entire platelet population indicating morphological changes of the plasma membrane. The shift was dose related, weaker than that evoked by collagen and similar to that produced by gramicidin. Ouabain-treated platelets express phosphatidylserine (PS). The ouabain-evoked PS expression was dose- and time-dependent, weaker than that produced by collagen and similar to that evoked by gramicidin. Electronic cell sizing measurements showed a dose-dependent increase in mean platelet volume upon treatment with ouabain. Hypoosmotically-evoked platelet swelling resulted in the appearance of procoagulant activity. Thromboelastography measurements indicate that, in whole blood, nanomolar (50–1000 nM, 15 min) concentrations of ouabain significantly accelerate the rate of clot formation initiated by contact and high extracellular concentration of calcium. We conclude that inefficiently operating platelet Na^+/K^+-ATPase results in a rise in $[Na^+]_i$. An increase in $[Na^+]_i$ and the swelling associated with it may produce PS exposure and a rise in membrane curvature leading to the generation of a procoagulant activity.

Keywords: ouabain, Na^+/K^+-ATPase, cardiac glycosides, platelets, procoagulant activity, atrial fibrillation

INTRODUCTION

Na^+/K^+-ATPase is a membrane enzyme which establishes and maintains the Na^+ and K^+ gradients across the plasma membrane of animal cells (Therien & Blostein, 2000). In resting platelets, low intracellular sodium concentration ($[Na^+]_i$) maintained by the Na^+/K^+-ATPase is critical for regulation of cell volume, cytoplasmic pH, and intracellular Ca^{2+} levels through the Na^+/H^+ and $3Na^+/Ca^{2+}$ exchangers, respectively (Borin & Siffert, 1991; Aviv, 1992; Marx *et al.*, 1992; Rosskopf, 1999).

Activation of platelets by specific physiological stimulators such as thrombin, collagen or ADP is accompanied by an increase in $[Na^+]_i$ (Borin & Siffert, 1990; 1991; Aviv, 1992). Elevated $[Na^+]_i$ in ac-

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Abbreviations: ATU, antithrombin units; FITC, fluorescein isothiocyanate; mAB, monoclonal antibody; PBS, phosphate-buffered saline; PE, phycoerythrin; PRP, Platelet-rich plasma; PS, phosphatidylserine; TEG, thromboelastography.

tivated platelets has been reported to be produced by activated Na^+/H^+ exchanger and/or following a reduction in Na^+/K^+-ATPase activity (Borin & Siffert, 1991; Rosskopf, 1999; Samson *et al*., 2001). In resting platelets in circulation, a rise in $[Na^+]_i$ is likely to occur following suppression of the Na^+/K^+-ATPase activity by endogenous blockers.

The endogenous blockers of Na^+/K^+-ATPase constitute a group of so-called endogenous cardiac glycosides, now recognized as a new class of steroid hormones (Kawamura *et al*., 1999; Dimitrieva & Doris, 2002; Schoner, 2002; el-Masri *et al*., 2002). Ouabain is believed to be the major endogenous cardiac glycoside, but several additional cardiotonic steroids have been isolated from the blood, adrenals, and hypothalamus (Hamlyn *et al*., 1991; Blaustein, 1993; Dimitrieva & Doris, 2002; Schoner, 2002). It has been reported that concentrations of endogenous cardiac glycosides are elevated in blood upon an increased sodium uptake, hypoxia, and physical exercise (Blaustein, 1993). Enhanced plasma levels of endogenous cardiotonic steroids have also been observed in several forms of hypertension, diabetes, and preeclampsia, i.e. in clinical conditions associated with blood hypercoagulability (Blaustein, 1993; Lopatin *et al*., 1997; Dimitrieva & Doris, 2002). Since patients with all of the above mentioned clinical conditions, as well as patients with atrial fibrillation undergoing prolonged cardiac glycoside treatment (Kamath *et al*., 2001), are highly predisposed to thromboembolism, it is of importance to test whether cardiac glycosides are thrombogenic.

Activated platelets play a crucial role in the generation of the hypercoagulable state of blood (Heemskerk *et al*., 2002). Platelets are a likely target of endogenous Na^+/K^+-ATPase inhibitors since specific ouabain binding sites have been reported to be present on their surface (Bork & Mrsny, 1993). The ouabain-induced rise in $[Na^+]_i$ is reported to produce water accumulation in platelets and their subsequent swelling. This has been proposed to lead to greater expression of fibrinogen receptors on platelets and to a rise in their sensitivity to stimulators (Marx *et al*., 1992). The effect of ouabain on the procoagulant activity of platelets has never been studied before.

We have reported that accumulation of Na^+ in platelets, produced by an activated Na^+/H^+ exchanger, may be associated with the development of a procoagulant response and the shedding of platelet microparticles demonstrating the procoagulant activity (Samson *et al*., 2001; Stelmach *et al*., 2002). Preactivation of circulating platelets by cardiac glycosides is therefore likely to result in the generation of thrombin.

The objective of the present study was to determine whether the rise in $[Na^+]_i$ evoked in blood platelets by inhibition of Na^+/K^+-ATPase may also produce a procoagulant response. Another aim was to determine how the sodium-induced platelet swelling is related to the generation of procoagulant activity.

The obtained results indicate that the rise in $[Na^+]_i$ in platelets produced by inefficiently operating Na^+/K^+-ATPase, and the swelling associated with it, may result in phosphatidylserine (PS) exposure and changes in membrane morphology leading to the generation of procoagulant activity. The data obtained suggest that the presence of ouabain-like substances in the blood may result in the generation of a hypercoagulable state of blood.

MATERIALS AND METHODS

Chemicals. Ouabain, gramicidin, *N*-methyl-D-glucamine, EGTA, Russel's viper venom, apyrase, hirudin and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Collagen (fibrillar, from equine tendon) was from Hormon Chemie, (Munich, Germany). Tirofiban was from Merck Scharp & Dohme B.V. (Holland). Fluorescein isothiocyanate-labelled annexin V (annexin-FITC), phycoerythrin (PE)-labelled anti GPIb/IIIa MoAb (PE-CD41a), and PE-labelled isotypic mouse MoAb were from BD Biosciences-PharMingen. [^{22}Na]Cl, specific activity 29.6 TBq/g, was from NEN Research Products. Stock solutions of ouabain were prepared in hot deionized water.

Blood collection and platelet preparation. Human venous blood (9 ml) 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. The study protocol was approved by the Ethics Committee at the Medical University of Bialystok. Platelet-rich plasma (PRP) was obtained from the fresh blood by centrifugation at 200 × *g* for 20 min at room temperature. After acidification to pH of 6.5 with 1 M citric acid, the PRP suspension was centrifuged at 1500 × *g* for 20 min to obtain a pellet which was resuspended in a Ca^{2+}-free Tyrode/Hepes buffer containing: 138 mM NaCl, 2.8 mM KCl, 8.9 mM $NaHCO_3$, 0.8 mM KH_2PO_4, 0.8 mM $MgCl_2$, 5.6 mM glucose, 10 μM EGTA, 10 mM Hepes pH 7.4, BSA (3.5 mg/ml) and apyrase (2 U/ml). The platelets were washed once and finally suspended in the same buffer without apyrase and EGTA. When indicated, platelets were suspended in a sodium-free medium which contained 152 mM *N*-methyl-D-glucamine, 0.8 mM $MgCl_2$, 5.6 mM glucose and 10 mM Hepes, pH 7.4. The osmolality of all media was adjusted to 300 mOsm/kg with the major salt.

Preparation of plasma. Plasma was obtained by centrifugation of the PRP at 1500 × *g* for 20 min

at room temperature. To remove smaller platelets and platelet fragments, the obtained supernatant was further centrifuged at 11000 × *g* for 5 min.

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 phosphatidylserine (PS). The preparation of defibrinated plasma, the 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 nanomoles of *p*-nitroaniline liberated during 1 min and was calculated for 1 ml of platelet suspension.

Platelet activation and flow cytometry. To measure PS exposure and for morphology experiments, 300 μl samples of PRP were placed in the polypropylene cuvette of an aggregometer and incubated at 37°C for two minutes without stirring. Ouabain, gramicidin, or collagen 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 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) and hirudin (2 ATU/ml). Thirty-microliter samples of the diluted suspension were combined with 20 μl of annexin-FITC (a marker of PS expression on the surface of platelets) and 10 μl of PE-CD41a (a marker of platelets) and incubated for 20 min in the dark. To stop the incubation, 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 made "particle-free" by filtration through a 0.2 μm filter (Millipore). Flow cytometry analysis was performed within 45 min of final dilution.

Flow cytometry analysis. Flow cytometry analysis was performed using a Coulter EPICS XL 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 color fluorescence 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 after triggering the PE fluorescence of the platelet marker CD41a at a preset threshold. The threshold was set above the background fluorescence with a PE-labelled isotypic mouse mAb. The CD41a-positive particle populations were separated by bitmaps (dot plots), where log forward scatter is the X-axis and log side scatter is the Y-axis.

Measurement of cytosolic free Ca^{2+}. Cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) was determined in Fura-2-loaded platelets. All procedures were conducted exactly as described previously (Samson *et al*., 2001).

Measurement of sodium uptake. Uptake of sodium ($^{22}Na^+$) by washed platelets (6×10^8 cells/ml) suspended in the Ca^{2+}-free Tyrode/Hepes buffer with 0.35% (w/v) BSA was measured using a filter technique employing Whatman GF/B filters to separate cells containing accumulated $^{22}Na^+$, as described previously (Samson *et al*., 2001).

Measurement of mean platelet volume. Changes in platelet volume were determined by electronic cell sizing using a hematological analyzer (Coulter Electronic GmbH). To prevent microaggregate formation, changes of platelet volume produced by ouabain were measured in the presence of tirofiban. Tirofiban is known to block the final step of platelet activation: the binding of fibrinogen to GPIIb/IIIa receptors and formation of platelet aggregates (Bednar *et al*., 1998).

Measurement of changes in platelet volume. Changes in platelet volume (swelling) were followed spectrophotometrically by recording optical density (OD) at 680 nm in diluted (to prevent aggregation) cell suspensions, essentially as described by Rosskopf *et al*. (1991). A drop in OD reflects a rise in cell volume. The changes in OD 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 vehicle) were added immediately. 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 to a personal computer.

Thromboelastographic coagulation analysis. The thromboelastogram test characterizes the kinetics of clot formation and its growth as well as the strength and stability of the formed clot (Wenker *et al*., 2000; Salooja & Perry, 2001). Thromboelastographic measurements are usually performed in fresh whole blood, but the analysis of coagulation in plasma is also possible. The principle of thromboelastography (TEG) is based on the measurement of the viscoelastic properties of blood as it is induced to clot under a low shear environment similar to that observed in veins (Wenker *et al*., 2000). TEG measurements were performed as described (Chandler, 1995; Wenker *et al*., 2000) using a thromboelastograph (ZIL, Moscow) connected to a personal computer *via* e-corder 401 (eDAQ Pty Ltd, Denis-

tone East, Australia). Clot formation was monitored at 37°C in an oscillating stainless steel cylindrical cuvette (0.34 ml) in which a stationary stainless steel piston was coaxially suspended in such a way that a 1-mm clearance between the surfaces of the cuvette and piston was kept. The cuvette oscillated through an angle of 5° in either direction every 9 s. The piston is suspended by a torsion wire that acts as a torque transducer. Following clot formation, the fiber strands which interact with activated platelets attach to the surface of the cuvette and the suspended piston. The clot thus formed transmits rotation of the cuvette onto the suspended piston. The torque experienced by the piston (relative to the oscillation of the cuvette) is plotted as a function of time. Initially, when no clot exists, the motion of the cuvette does not affect the piston and a straight-line trace is recorded (Fig. 1). As the blood in the cuvette clots, the motion of the rotating cuvette is transmitted to the piston and a TEG trace is generated. A weak clot stretches and therefore reduces the arc movement of the piston, which is graphically expressed as a narrow-amplitude thromboelastogram. A strong clot, by contrast, will move the piston simultaneously and proportionally to the cuvette movements, creating a wide-amplitude thromboelastogram. We measured (Fig. 1) three parameters of the TEG trace: the clotting time (CT), the alpha angle (α), and the maximum firmness of the clot (MCF). The CT is the latency between placing the blood in the cuvette and the formation of first fibrin strands. The α angle is the angle between the line in the middle of the TEG tracing and the line tangential to the developing "body" of the TEG tracing. The CT is a measure of the availability of factors necessary for clot formation. The α angle represents the acceleration of fibrin build-up and cross-linking. MCF (in millivolts) reflects the strength of the clot, and is dependent on the number and function of platelets.

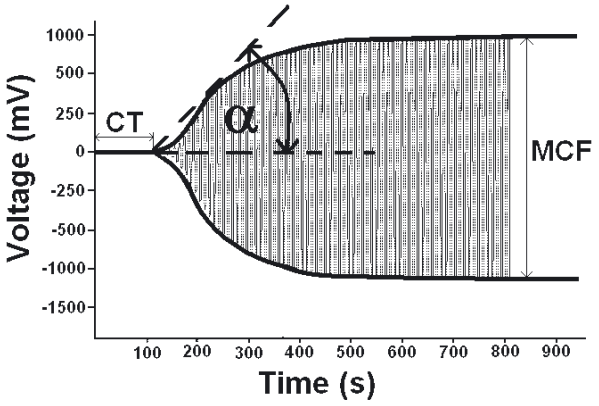

Figure 1. Typical thromboelastographic plot and parameters measured.
CT = clotting time; α = alpha angle, MCF = maximum clot firmness.

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 and elaboration of experimental data by the use of Slide Write plus (Advanced Graphics Software, Inc. Carlsbad, CA, USA).

RESULTS

To estimate whether an inefficiently operating Na^+/K^+-ATPase in platelets may result in a rise in the intracellular concentration of sodium, the effect of ouabain on the sodium influx was studied and compared with that exerted by gramicidin. The results of these experiments are shown in Fig. 2. Gramicidin is known to form Na^+-selective channels in the plasma membrane by the transmembrane association of two nonconducting monomers enabling rapid sodium influx (Koeppe & Andersen, 1996). As can be seen, a one-hour incubation of platelets with ouabain (10–200 μM) results in a dose-dependent uptake of sodium. A statistically significant sodium uptake was already observed in the presence of 20 μM ouabain. A substantial sodium uptake was produced following treatment of platelets with 100 μM ouabain. Since a further increase in the glycoside concentrations (up to 200 μM) resulted in an only small increase in sodium uptake, 100 μM oua-

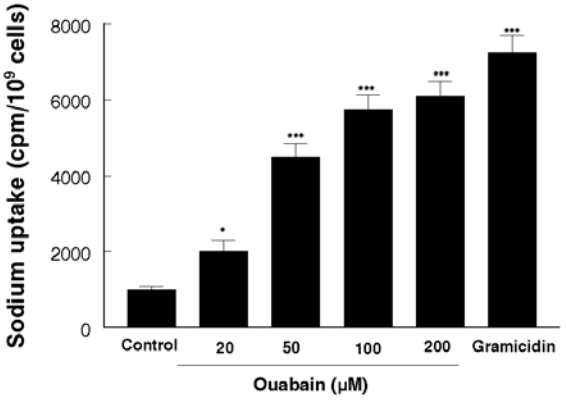

Figure 2. The effect of ouabain and gramicidin on sodium uptake by human platelets.
Aliquots (2 ml) of washed platelets (6×10^8 cells/ml) suspended in a Ca^{2+}-free Tyrode/Hepes buffer were supplemented with 5 μl (1 μCi) of [$^{22}Na^+$]Cl and incubated (in polypropylene tubes, without stirring) at 37°C for 10 min with gramicidin (10 μM final concentration), or for 60 min with ouabain added to the final concentration as indicated. In the control, PBS was added instead. After incubation samples of the incubation mixture were aspirated for filtration. The $^{22}Na^+$ that remained on the filters was measured. Data (c.p.m./10^9 cells) are the mean ±S.D. of four experiments, each performed on a separate platelet preparation (n=16). * $P<0.05$, *** $P<0.001$.

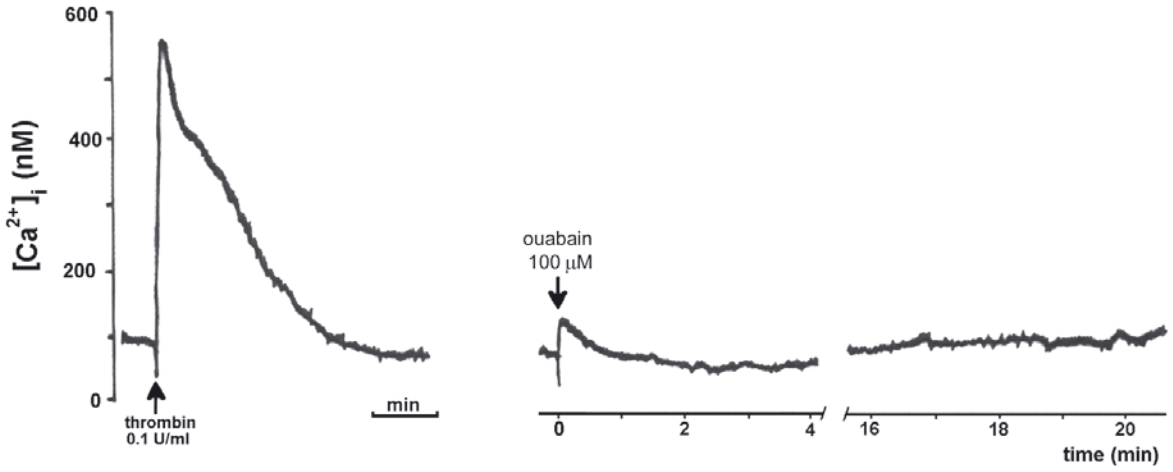

Figure 3. Effect of thrombin and ouabain on intracellular Ca^{2+} concentration in human platelets.
Fura-2-loaded platelets suspended in a Ca^{2+}-free Tyrode/Hepes buffer were treated (arrow) with thrombin or ouabain added to the final concentrations as indicated. Intracellular calcium concentration, $[Ca^{2+}]_i$, was calculated using the calibration procedure described in Materials and Methods. Each trace is representative of at least six determinations performed with three different platelet preparations.

bain was recognized to exert the maximal inhibitory effect on platelet Na^+/K^+-ATPase. Accumulation of sodium by platelets treated with 100 μM ouabain was lower than that observed following 10 min of incubation of these cells with 10 μM gramicidin. This data indicates that prolonged inhibition of platelet Na^+/K^+-ATPase by ouabain results in a rise in the intracellular sodium concentration.

We subsequently studied how ouabain treatment affects intracellular calcium concentrations ($[Ca^{2+}]_i$) in platelets. As seen in Fig. 3, in comparison to thrombin, a stronger physiological activator of platelets, ouabain (100 μM) produces a relatively small (below 50 nM) and transient (less than 1 min) rise in $[Ca^{2+}]_i$ in Fura-2-loaded platelets.

Table 1 summarizes experiments performed to determine whether prolonged inhibition of platelet Na^+/K^+-ATPase by ouabain may result in the generation of a procoagulant activity. As shown (Exp. A and B), ouabain induced a dose- and time-

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

Experiment	Additions	Thrombin generation (mU/ml)
A	None	4 ± 1
	Ouabain 20 μM, 60 min	7 ± 1*
	Ouabain 50 μM, 60 min	17 ± 1***
	Ouabain 100 μM, 60 min	37 ± 2***
	Ouabain 200 μM, 60 min	41 ± 3***
	Ouabain 100 μM + 1 mM EDTA, 60 min	34 ± 2***
	Ouabain 100 μM + 1 mM aspirin, 60 min	33 ± 2***
	Gramicidin 10 μM, 10 min	67 ± 3***
	Collagen 20 μg/ml, 10 min	97 ± 4***
B	None	4 ± 1
	Ouabain 100 μM, 20 min	9 ± 1*
	Ouabain 100 μM, 40 min	19 ± 1***
	Ouabain 100 μM, 60 min	37 ± 2***
	Ouabain 100 μM + 30 mM NaCl, 60 min	8 ± 1*
C	($-Na^+$)	4 ± 1
	($-Na^+$) Collagen 20 μg/ml, 10 min	80 ± 3***
	($-Na^+$) Ouabain 100 μM, 60 min	8 ± 1*
D	Control (no platelets)	< 0.5

Procoagulant activity of washed platelets (4×10^8 cells/ml) at 37°C without (None) and with the tested substances added to the final concentration as indicated. At the indicated time intervals samples of incubating mixture were taken for the measurement of phospholipid-dependent thrombin generation. Thrombin generation was evaluated as under Methods. The amidolytic activity of thrombin in international units (U) is reported. In the experiment designated as ($-Na^+$) collagen and ouabain were added to platelets suspended in sodium-free medium. Mean values ±S.D. are reported (n=16). * $P<0.05$, *** $P<0.001$.

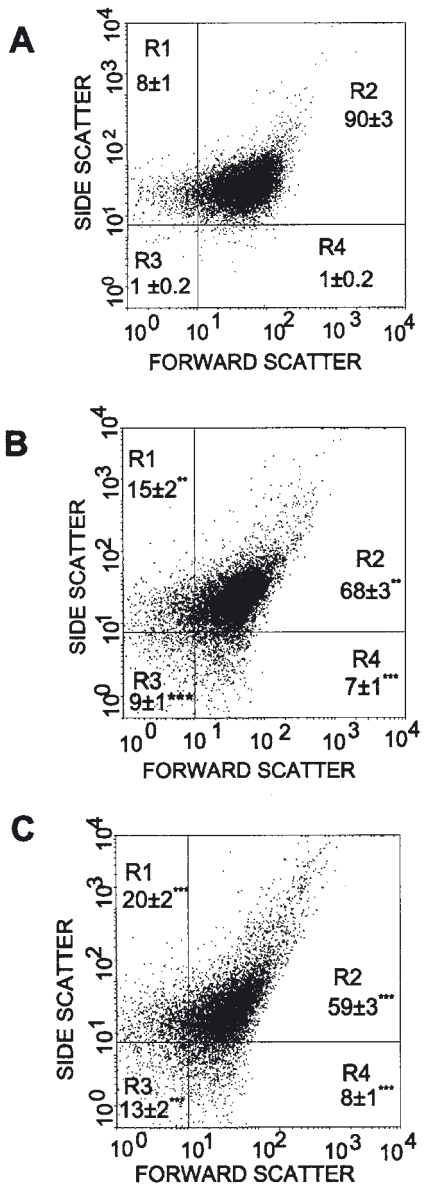

Figure 4. The effect of increasing concentration of ouabain on the morphology of platelets.
Dot plots of forward scatter *versus* side scatter of a platelet suspension (PRP) untreated (panel A) and treated for 1 h (without stirring) at 37°C with 50 μM ouabain (panel B) and 100 μM ouabain (panel C). The percentage of events detected in regions R1–R4 are indicated (mean ±S.D., n=12). $^{**}P<0.01$, $^{***}P<0.001$. Note that ouabain 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).

related procoagulant response. Compared to control, a sixty-minute incubation of platelets with ouabain (20–100 μM) produced an about 2- to 9-fold rise in the procoagulant activity. This is a relatively weak procoagulant response, since platelets treated for 10 minutes with collagen (20 μg/ml), the strongest physiological inducer of procoagulant response (Wolfs *et al*., 2005), demonstrate a 23-fold rise in procoagulant activity. The effect of ouabain was compared with that observed following gramicidin treatment. As can be seen (Exp. A), in comparison to control, a ten-minute incubation of platelets with 10 μM gramicidin produced a 16-fold increase in procoagulant activity. The ouabain-evoked procoagulant response was not affected by the presence of 1 mM EDTA in the medium (Exp. A) and was strongly reduced in the absence of extracellular Na^+ (Exp. C) or by the hyperosmolality produced by the addition of 30 mM NaCl (Exp. B). The ouabain-related procoagulant response cannot be explained by the activation of these cells by the surface of plastic cuvettes, since addition of aspirin, which is known to inhibit platelet activation (Roth & Calverley, 1994), failed to reduce the generation of the procoagulant activity. This data indicates that the ouabain-produced procoagulant activity appears to be related to the rise in intracellular sodium concentration.

Figures 4 and 5 show the results of flow cytometry studies performed to determine how ouabain affects platelet morphology (i.e. their size and granularity). To analyze the changes in platelet morphology, a bi-variate scatterplot (forward scatter *versus* side scatter dot plot) was arbitrarily split into four regions (R1–R4). 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 90%) of the aquisited events. The percentage of events of the 10000 total counted is shown in each region. Assuming that forward scatter and side scatter are the criteria of platelet size and granularity, respectively, in the population of normal platelets (Bode & Hickerson, 2000), R1 comprises the subpopulation of small platelets, R2 comprises 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. 4, sixty-minute incubation of platelets with 50 μM (panel B) or 100 μM ouabain (panel C) produces a much broader light scatter profile than in the control. This effect was dose-dependent and was manifested by a marked increase in the percentage of counts in R1 (by 7–12%), R3 (by 8–12%), R4 (by 6–7%) and a simultaneous drop (by 22–31%) 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 ouabain-treated platelets. As seen from Fig. 5, the effect of ouabain on platelet morphology is also time-related. This is because prolongation of the treatment of the platelets with ouabain (100 μM) correlated with a continuous in-

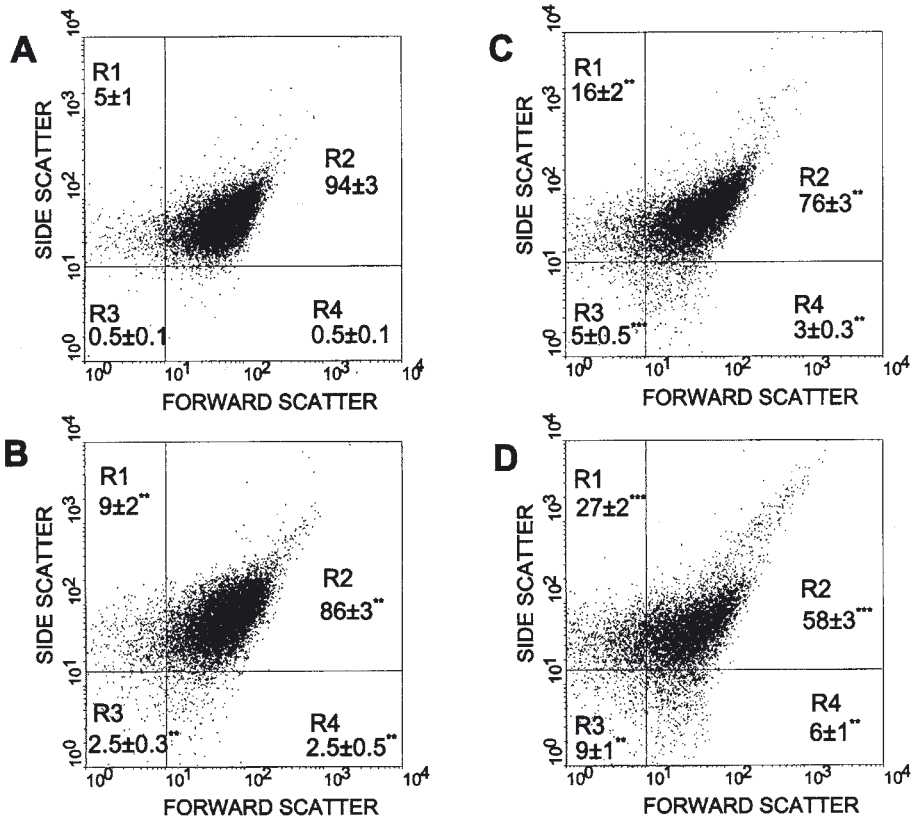

Figure 5. Time course of the morphological changes in platelets treated with ouabain.
Dot plots of forward scatter *versus* side scatter of a platelet suspension (PRP) untreated (panel A) and treated (without stirring) for 20 min (panel B), 40 min (panel C) and 60 min (panel D) with 100 μM ouabain at 37°C. The percentage of events detected in regions R1–R4 are indicated (mean ±S.D., n=12). $^{**}P<0.01$, $^{***}P<0.001$.

crease in the percentage of events in R1, R3, and R4 and simultaneous decrease in the percentage of counts in R2. Analysis of the forward scatter *versus* side scatter dot plot of platelets incubated with ouabain 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 appear contiguous with the unchanged subfraction of the tested platelet population. In agreement with the considerations of Bode and Hickerson (2000) we postulate that these new events (i.e. those appearing in regions R1 and R3) are degranulated platelets with profound changes on their surface.

Figure 6 shows the results of experiments which were conducted to estimate how gramicidin and collagen affected platelet morphology. As seen in panel B, a ten-minute incubation of platelets with 10 μM gramicidin produces significant changes in platelet size and granularity. This is manifested by an increase in the events found in regions R1 (by 15%), R3 (by 18%), and R4 (by 13%), and a simultaneous decrease in their number in region R2 (by 48%). As seen from panel C, similar changes in size and granularity can be 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 5.5%, 42.5%, and 24.5%, respectively, and a concomitant decrease of the events in region R2 by 72.5%. Analysis of forward scatter *versus* side scatter dot plots of platelets preincubated with gramicidin or collagen reveals a unimodal decrease in both forward and side light scattering.

The experiments shown in Table 2 were performed to determine whether prolonged treatment of platelets with ouabain affected PS expression. The amount of PS expressed on the cell surface is a major determinant of the ability of platelets to support procoagulant complex assembly and augment thrombin generation (Wolfs *et al.*, 2005). Therefore, platelet PS expression was directly examined using flow cytometry and fluorescein-conjugated annexin V (Tait *et al.*, 1989). Ouabain treatment increased the percentage of platelets with PS exposed on their surface. This effect was dose- and time-dependent. A sixty-minute incubation of platelets with 20–100 μM ouabain produced a rise in the percentage of PS-expressing cells from 3% in non-treated control to 8% in those treated with 20 μM ouabain and 37%

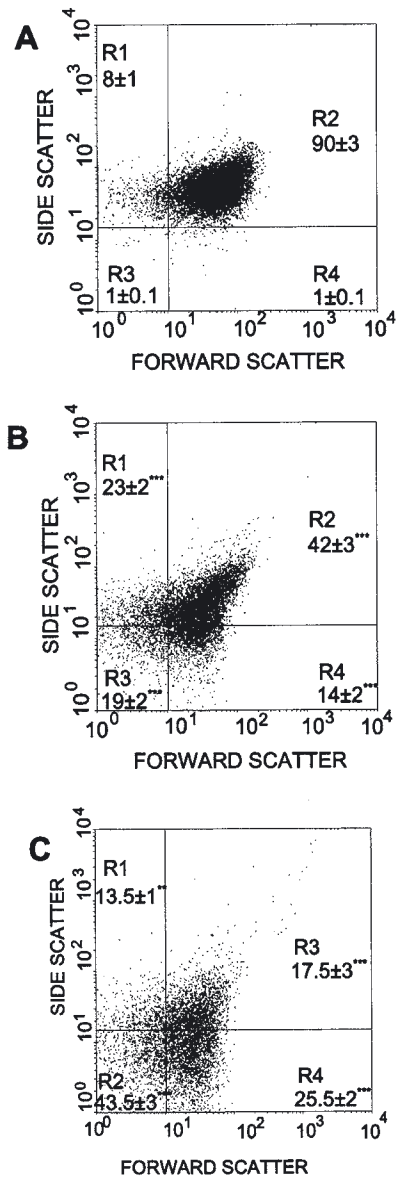

Figure 6. The effect of gramicidin and collagen on the morphology of platelets.
Dot plots of forward scatter *versus* side scatter of a platelet suspension (PRP) untreated (panel A) and treated for 10 min at 37°C with gramicidin (panel B) or collagen (panel C) added to the final concentrations of 10 μM and 15 μg/ml, respectively. The percentage of events detected in regions R1–R4 are indicated (mean ±S.D., n=12). $^{**}P<0.01$, $^{***}P<0.001$. Note that collagen and gramicidin produced a unimodal left/downward shift in the forward scatter and side scatter of the entire platelet population.

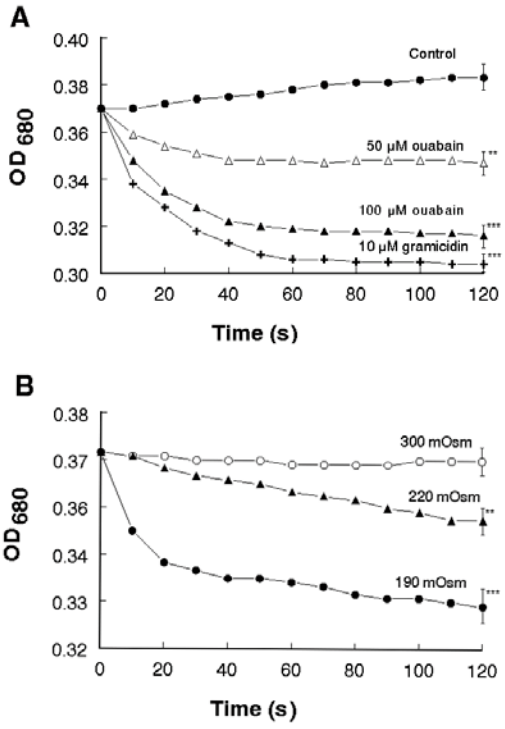

Figure 7. Kinetics of platelet swelling produced by ouabain, gramicidin or by reduction of medium osmolality.
Panel A. Treatment with ouabain or gramicidin. At time 0, suspensions of washed platelets (suspended in isotonic medium) were supplemented with ouabain or gramicidin added to the final concentrations as indicated. In the control, vehiculum (water) was added instead. Panel B. Reduction of medium osmolality. At time 0, samples of washed platelets were supplemented with water to achieve the desired final osmolality in the suspension medium. Changes in platelet volume (swelling) were followed spectrophotometrically by recording the 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. $^{**}P<0.01$, $^{***}P<0.001$

in cells treated with 100 μM ouabain. The ouabain-evoked PS exposure was less pronounced than that caused by a 10-minute incubation of platelets with collagen (76% of cells) or with 10 μM gramicidin (48%). The presence of aspirin did not affect the ouabain-evoked PS exposure.

Inhibition of Na^+/K^+-ATPase is expected to induce platelet swelling due to the rise in the concentration of intracellular sodium. Experiments shown in Figs. 7 and 8 were performed to assess whether the Na^+ influx evoked by ouabain or gramicidin produced a rise in platelet volume. An optical swelling assay (Fig. 7) and a Coulter-type counter (Fig. 8) were employed to measure changes in platelet volume. The optical method allows recording of the kinetics of cell swelling whereas the Coulter counter allows a direct measurement of mean platelet volume (MPV). The results presented in Fig. 7 (panel A) show the kinetics of (optical) platelet swelling following the addition of increasing (50–100 μM) concentrations of ouabain. The effect of ouabain was compared with that observed after an artificially evoked (10 μM gramicidin) Na^+ influx and with platelet swelling produced by the reduction of the medium osmolality (panel B). As seen, ouabain produced platelet (optical) swelling, in a dose-depend-

Table 2. The effect of ouabain, gramicidin and collagen on the appearance of phosphatidylserine on the platelet surface

Experiment	Additions	Annexin V-positive cells (%)
A	None	3 ± 1
	Ouabain 20 μM, 60 min	8 ± 1**
	Ouabain 50 μM, 60 min	34 ± 3***
	Ouabain 100 μM, 60 min	37 ± 3***
	Ouabain 100 μM + aspirin 1mM, 60min	35 ± 3***
	Gramicidin 10 μM, 10 min	48 ± 4***
	Collagen, 20 μg/ml	76 ± 6***
B	None	3 ± 1
	Ouabain 100 μM, 20 min	9 ± 1**
	Ouabain 100 μM, 40 min	24 ± 2***
	Ouabain 100 μM, 60 min	36 ± 3***

Platelets (PRP) were incubated at 37°C without stirring with no stimulator (none), or with ouabain, gramicidin or collagen added to the final concentrations as shown. At the indicated time intervals samples of incubating mixture were taken for the measurement of PS expression by means of flow cytometry. The data represent mean percentages of PS-positive platelets (expressed as percentage of annexin V-positive cells) and ±S.D. of four experiments, each performed on separate platelet preparation (n=12). $^{**}P<0.01$, $^{***}P<0.001$.

ent manner. The kinetics of platelet swelling evoked by 100 μM ouabain was similar to that observed in the presence of 10 μM gramicidin and to that recorded following the drop of medium osmolality to 190 mOsm. Figure 8 shows that the ouabain-evoked rise in $[Na^+]_i$ produced a dose-dependent increase in platelet volume. A sixty-minute treatment of platelets with 20-200 μM ouabain resulted in a rise in the mean platelet volume (MPV) by approximately 0.1 to 1 femtoliters. The rise in MPV evoked by 100 μM ouabain was slightly smaller (1 *versus* 1.2 femtoliters) than that observed following a 10-minute treatment of platelets with 10 μM gramicidin. The ouabain-evoked rise in MPV was not a result of microaggregate formation since measurements were performed in the presence of tirofiban (prevents microaggregate formation), and the changes in platelet volume were not associated with a drop in platelet count (not shown).

The experiments shown in Fig. 9 were performed to estimate whether platelet swelling produced following the suspending of cells in a medium with reduced osmolality (hypotonic shock) might result in the generation of a procoagulant activity. As can be seen, the reduction in osmolality from 300 to 190 mOsm resulted, after 10 min of incubation, in an about six-fold increase of procoagulant activity.

The experiments shown in Table 3 were designed to assess whether inhibition of Na^+/K^+-ATPase by ouabain affects the generation of thrombin in whole blood, i.e. in the presence of a high concentration of extracellular calcium. To follow the

Table 3. The effect of increasing concentrations of ouabain on the kinetics of clot formation in whole blood (A) and plasma (B)

Experiment	Additions	CT (s)	MCF (mV)	α (degree)
A	None	346 ± 41	1350 ± 95	40 ± 3
	Ouabain 20 nM	340 ± 40	1353 ± 90	40 ± 3
	Ouabain 50 nM	300 ± 37*	1390 ± 90*	41 ± 3
	Ouabain 100 nM	270 ± 32***	1405 ± 90***	42 ± 3
	Ouabain 1 μM	250 ± 36***	1425 ± 71***	42 ± 3
	Ouabain 5 μM	138 ± 16***	1614 ± 69***	51 ± 4***
B	None	488 ± 23	640 ± 86	12 ± 3
	Ouabain 5 μM	492 ± 34	623 ± 74	12 ± 3

Aliquots (0.5 ml) of fresh blood (A) or plasma (B) were supplemented with ouabain (5 μl) added to the final concentration as indicated, and incubated (in polypropylene tubes, without stirring) at 37°C for 15 min. After incubation the samples (0.34 ml) of the incubation mixture were transferred to a preheated TEG cuvette containing 20 μl of 0.2 M $CaCl_2$. The piston of the TEG was then raised and lowered into the cuvette 5 times to insure adequate mixing of the blood and additives. The TEG tracings were than begun and allowed to run until at least 20 min after achieving their MCF. Data (clotting time (CT), α angle and the maximum clot firmness (MCF)) are the mean ±S.D. of four experiments, each performed on a separate platelet preparation (n=16). $^{*}P<0.05$, $^{***}P<0.001$.

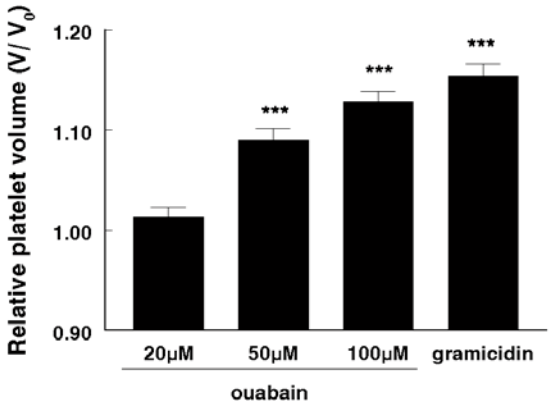

Figure 8. Platelet volume changes following ouabain or gramicidin treatment.
Aliquots of platelet-rich plasma were incubated (in polypropylene tubes, without stirring) at 37°C for 10 min with gramicidin (10 μM final concentration), or for 60 min with ouabain added to the final concentration as indicated. In control, PBS was added instead. To avoid platelet clumping tirofiban was added (prior to the addition of ouabain or gramicidin) to the final concentration of 1mg/ml. Mean platelet volume (MPV) was measured using electronic counting technique. No drop in platelet count was noted during incubation of platelets with 100 μM ouabain. Results (relative platelet volume) as the ratio of the MPV observed following platelet treatment (V) to the MPV of untreated cells (V_0) are expressed. MPV values in control varied from 7.3 to 8.3 femtoliters. Data are the mean ±S.D. of four experiments, each performed on a separate platelet preparation (n=16). Paired Student's *t*-test was used to calculate *P*-values. ***$P<0.001$.

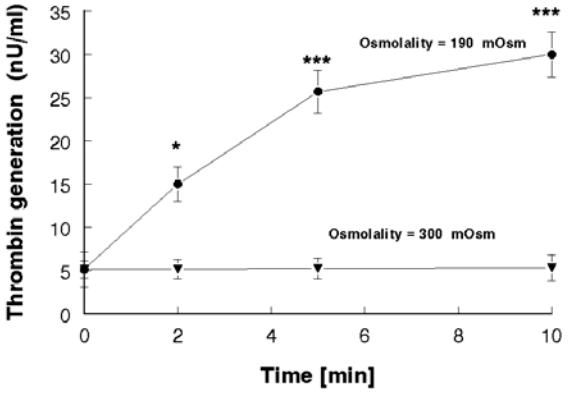

Figure 9. Time course of development of platelet procoagulant activity in medium with reduced osmolality.
Platelets (3×10^8 cells/ml) were incubated at 37°C in Tyrode/Hepes medium with osmolality reduced from 300 (control) to 190 mOsm. To achieve the desired osmolality, deionised water was added to the incubation mixture prior to the addition of platelets. In the control, PBS was added instead. At the indicated time intervals, aliquots of platelet suspension were removed and examined for procoagulant activity. Data are the mean ±S.D. of four experiments, each performed on a separate platelet preparation (n=16). *$P<0.05$, **$P<0.01$, ***$P<0.001$.

kinetics of thrombin formation in whole blood, we employed thromboelastography. In our experimental system, the initiation of clot formation (recorded by the instrument) starts after the generation of an appropriate (threshold) amount of thrombin. The thrombin formation results from the activation of the intrinsic pathway of coagulation initiated following activation (by contact) of factor XII (Davie, 2003). In these experimental conditions, the appearance of platelets with exposed PS will accelerate the synthesis of the threshold amount of thrombin, which should be manifested by a shortened clotting time (CT) and higher values of maximum clot firmness (MCF). As is seen in Table 3 (Exp. A), the treatment of whole blood with ouabain (50 nM – 5 μM) for 15 min appreciably reduced the clotting time and augmented the MCF values. Since ouabain did not affect the kinetics of clot formation in plasma (Exp. B), it is concluded that ouabain-treated platelets contributed to the generation of thrombin. The ouabain-related procoagulant effect measured in whole blood was dose-dependent and was observed at nanomolar concentrations of oubain, indicating that therapeutic doses of cardiac glycosides may produce a hypercoagulable state of blood.

DISCUSSION

The procoagulant activity is defined as the ability of stimulated platelets to catalyse the activation of factor X and to convert prothrombin to thrombin (Zwaal & Schroit, 1997; Heemskerk *et al*., 2002). Under normal physiological conditions, platelet-dependent thrombin generation is essential to the formation of a stable hemostatic plug (required to arrest hemorrhage), whereas in pathological states it contributes to the formation of stable thrombi that may occlude blood vessels (Coller, 1992; Monroe *et al*., 2002). It is well appreciated that the platelet procoagulant response is associated with remodelling of the plasma membrane leading to the exposure of PS in its outer leaflet (Sims & Wiedmar, 2001; Heemskerk *et al*., 2002; Wolfs *et al*., 2005). PS is recognized as a major component of the procoagulant platelet surface (Sims & Wiedmar, 2001; Wolfs *et al*., 2005). In resting platelets, the majority of PS is kept in the inner leaflet of the plasma membrane through the action of aminophospholipid translocase, which mediates a rapid inward-directed transport of PS from the outer to the inner leaflet of the plasma membrane (Daleke, 2003). Stimulation of platelets has been proposed to cause the aminophospholipid translocase to cease its activity, while simultaneously switching on the activity of phospholipid scramblase mediating a rapid bi-directional, transverse (flip-flop) movement of all classes of phospholipids within the plasma membrane. This was suggested to lead within min-

utes to the loss of the normal asymmetric distribution of phospholipids in the plasma membrane, resulting in surface exposure of PS (Sims & Wiedmar, 2001; Wolfs *et al*., 2005). It should be stressed, however, that despite several attempts, the identity of the membrane protein(s) involved in the scramblase activity remains unknown (Sims & Wiedmar, 2001; Zhou *et al*., 2002).

It has been proposed that a prerequisite and a key factor for the PS exposure in activated platelets is prolonged (minutes) and high (micromoles) elevation of intracellular calcium ($[Ca^{2+}]_i$) (Heemskerk *et al*., 2002; Keuren *et al*., 2005). This is based mainly on the observation that the maximal exposure of PS in platelets, as well as maximal procoagulant activity, is observed following the treatment of cells with calcium ionophores (A23187, ionomycin) in the presence of extracellular calcium (Keuren *et al*., 2005; Wolfs *et al*., 2005). Paradoxically, the activation of platelets by thrombin alone, which is considered to be the most potent physiological trigger of aggregation and secretion in these cells, known to produce in platelets a strong ($\Delta[Ca^{2+}]_{max}$ = 1000 nM) and persistent ($\Delta[Ca^{2+}]_{t=5\ min}$ = 360 nM) elevation in $[Ca^{2+}]_i$, is associated with only a very weak (two-fold increase in prothrombinase activity as compared with resting cells) procoagulant response (Keuren *et al*., 2005). By contrast collagen, which is recognized as the strongest physiological inducer of platelet procoagulant response (2.5-fold stronger than thrombin), causes a modest ($\Delta[Ca^{2+}]_{max}$ = 441 nM) and significantly less sustained calcium signal ($\Delta[Ca^{2+}]_{t=5\ min}$ = 198 nM) (Keuren *et al*., 2005). These findings can be interpreted to mean that in platelets an additional intracellular factor, besides Ca^{2+}, is likely to be involved in PS exposure and in the generation of procoagulant activity.

We have proposed that sodium uptake due to the activation of an Na^+/H^+ exchanger in platelets may result in PS exposure and in the development of a procoagulant response in a manner independent of the rise in $[Ca^{2+}]_i$ (Samson *et al*., 2001; Tomasiak *et al*., 2004; Tomasiak & Stelmach, 2005). These findings coincide with the results obtained by Courageot *et al*. (2004) who have demonstrated that in activated thymocytes a rise in $[Na^+]_i$, rather than the elevation of $[Ca^{2+}]_i$, is the major mediator of PS externalization.

Here, we have extended our previous work by demonstrating that in human platelets, in addition to elevated $[Ca^{2+}]_i$, the rise in $[Na^+]_i$ produced by inefficiently operating Na^+/K^+-ATPase may also result in PS exposure, coinciding with the development of a procoagulant response. This is based on the following observations: 1) Ouabain-treated platelets accumulate sodium, 2) The rise in ($[Na^+]_i$ evoked by ouabain and gramicidin is correlated with the appearance of a procoagulant activity, 3) Ouabain fails to produce a procoagulant response in platelets suspended in a medium depleted of sodium, 4) The blockage of Na^+/K^+-ATPase by ouabain is associated with only a relatively small elevation in $[Ca^{2+}]_i$ in both Fura-2- and aequorin-loaded platelets (Fig. 3, and Lees *et al*., 1989).

How can an elevation in $[Na^+]_i$ alone trigger a procoagulant response in platelets? A rise in $[Na^+]_i$ is likely to activate the plasma membrane $3Na^+/Ca^{2+}$ exchanger which, operating in the reverse mode, may elevate $[Ca^{2+}]_i$ (Blaustein & Lederer, 1999), eventually leading to PS exposure. Although such a mechanism is expected to function under physiological conditions (i.e. in the presence of high extracellular $[Ca^{2+}]$), it is unlikely to occur in our *in vitro* experimental system since an ouabain-evoked platelet procoagulant response was observed in the absence of extracellular Ca^{2+}.

Another possibility could be that the elevation of $[Na^+]_i$ may stimulate phospholipid scramblase. Courageot *et al*. (2004) have recently proposed that, at least in activated thymocytes, a rise in $[Na^+]_i$ can directly activate this enzyme. In sickle cells high $[Na^+]_i$ and low $[K^+]_i$ were reported to correlate with an increased scramblase activity (de Jong *et al*., 2001). However, a rise in $[Na^+]_i$ may also have some other consequences. Sodium uptake is necessarily accompanied by an influx of water which is expected to produce cell swelling (Lang *et al*., 1998). As shown here, this really is the case. Flow cytometry studies, direct electronic cell sizing, and optical swelling assay show that blocking of Na^+/K^+-ATPase by ouabain results in a rise in platelet volume. In addition, the optical swelling assay shows that the ouabain-evoked swelling is characterized by kinetics similar to those produced by the sodium ionophore gramicidin. The involvement of platelet swelling in the development of a procoagulant response is additionally confirmed by the observation that ouabain failed to make platelets procoagulant in a hypertonic medium, as well as by the fact that hypotonically-evoked platelet swelling may result in the generation of a procoagulant activity.

In conclusion, the rise in platelet volume resulting from the accumulation of Na^+ mediated by inefficiently operating Na^+/K^+-ATPase appears to be involved in the ouabain-evoked alterations in the plasma membrane leading to the development of a procoagulant response.

Little is known about the relationship between platelet volume and their procoagulant activity. We report here that hypoosmotically produced platelet swelling results in the generation of a procoagulant activity. Platelet swelling is accompanied by distending of the plasma membrane which may result in a rise in plasma membrane tension. Studies

performed on model systems and cells other than platelets have shown that a rise in plasma membrane tension may facilitate exocytosis (Straub *et al*., 2002). We have recently reported that in platelets activated by collagen, a rise in cell membrane tension (due to swelling) promotes the secretion of serotonin (Tomasiak *et al*., 2005). Activation of platelets by agonists evoking a procoagulant response (e.g. collagen) is always associated with exocytosis (Reed *et al*., 2000; Flaumenhaft, 2003). In exocytosis, secretory granules fuse with the plasma membrane delivering the granule membrane which then becomes the cell membrane (Fritz *et al*., 1994; Reed *et al*., 2000). Fusion of the secretory granules with the plasma membrane has been proposed to be associated with the appearance of PS on the platelet surface at the site of fusion (Zwaal & Schroit, 1997). Such a mechanism may explain the involvement of platelet swelling in the PS exposure. Further studies are necessary to understand how the rise in platelet volume affects PS exposure.

The flow cytometry studies presented here indicate that in ouabain- or gramicidin-treated platelets, PS exposure is associated with morphological changes on the platelet surface manifested as a unimodal left shift in the forward scatter of the entire platelet population (Figs. 4–6). The alterations in the morphology of platelet plasma membrane, visible in flow cytometry as a unimodal decrease in forward scatter, have been postulated to reflect filopod formation (Bode & Hickerson, 2000). Such morphological changes are likely to be associated with a dramatic increase in the membrane curvature, a phenomenon which was recently proposed to augment PS-related binding of activated factors V and VIII to the platelet surface and thus promote the assembly of tenase and prothrombinase complexes (Shi *et al*., 2004).

Altogether, this data indicates that prolonged inhibition of platelet Na^+/K^+-ATPase by ouabain may result in the development of a procoagulant response which can be mediated by a rise in $[Na^+]_i$. Elevation of $[Na^+]_i$ and the cell swelling associated with it may result in PS exposure and in changes in the membrane curvature which may ultimately promote the accelerated assembly of coagulation complexes.

How may the above observations be relevant to the *in vivo* situation? In our experimental system, i.e. in the absence of extracellular calcium, an appreciable rise in platelet procoagulant activity was observed following the treatment of cells with micromolar concentrations of ouabain for a relatively short (60 min) time. However, in the blood of patients treated with inhibitors of Na^+/K^+-ATPase (cardiac glycosides), platelets remain in contact with lower (nanomolar range) concentrations of the drug — but for a very long (several days) time. Taking into account that the ouabain-related platelet procoagulant activity is time-dependent (Table 1), prolonged treatment of cells with lower doses of the drug, which eventually only partly inhibit Na^+/K^+-ATPase, is also likely to result in an elevation of $[Na^+]_i$, leading to a rise in cell volume. Such a possibility is strongly supported by the observation that healthy volunteers receiving therapeutically relevant doses of digitoxin (a cardiac glycoside approved for treatment of atrial fibrillation) have demonstrated a marked (about 20%) drop in platelet count (Pettersen *et al*., 2002). Apparently part of the platelet population was disrupted, due to swelling, elicited by inefficiently operating Na^+/K^+-ATPase, then removed from circulation. In this context it is worth noting that in individuals with a history of cerebrovascular disease, an increase in platelet volume correlates with a rise in the hypercoagulable state manifested by an increased risk of stroke (Bath *et al*., 2004), and that artificially evoked platelet swelling may also result in the generation of procoagulant activity (Fig. 9).

One mechanism underlying the therapeutic effect of cardiac glycosides in patients suffering atrial fibrillation has been suggested to be associated with the activation of the $3Na^+/Ca^{2+}$ exchanger in the plasma membrane of cardiomyocytes, elicited by a rise in $[Na^+]_i$ (Reuter *et al*., 2002). Therefore, it cannot be excluded that a similar mechanism function also in platelets. Under physiological conditions, the platelets reside in a milieu containing high (millimolar range) concentrations of Ca^{2+}. Overloading of platelets with Na^+ in the presence of a high extracellular calcium concentration is therefore expected to result in the activation of the $3Na^+/Ca^{2+}$ exchanger which, operating in a reverse mode, can produce a persistent elevation in $[Ca^{2+}]_i$, eventually leading to the development of a procoagulant response or potentiation of the effect of other weak inducers of platelet procoagulant response. Recently published results indicate that a prolonged rise in $[Ca^{2+}]$, rather than its maximal concentration, is a major determinant of platelet procoagulant response (Keuren *et al*., 2005). The results presented here indicate such a scenario and show that, in the presence of high extracellular $[Ca^{2+}]$, ouabain added to whole blood distinctly accelerates the clot formation initiated by low concentrations of thrombin generated following activation (by contact) of the intrinsic pathway of blood coagulation (Table 3). Interestingly, this ouabain-related procoagulant effect, measured in whole blood, can be observed in the presence of therapeutic (nanomolar range) concentrations of ouabain (Table 3). Further studies are needed to confirm this observation and to define the exact role of the $3Na^+/Ca^{2+}$ exchanger in the development of platelet procoagulant response.

The results presented here indicate that ouabain behaves as a weak inducer of platelet procoagulant response. In fact, its procoagulant potency is about two-fold lower than that of collagen, which is considered to be the strongest individual inducer of the procoagulant response (Wolfs *et al*., 2005). This may be of minor importance in young healthy subjects with unimpaired endothelium able to produce sufficient amounts of antiplatelet agents (e.g. NO, prostacyclin) eventually reducing the excess of platelet-related hypercoagulability. However, the procoagulant effect of cardiac glycosides is expected to be an important thrombosis risk factor, especially in patients with atrial fibrillation who have been reported to have already preactivated platelets (due to fibrillation) with a significantly elevated volume (Kamath *et al*., 2001; Choudhury *et al*., 2007) as well as in older people with pathologically changed endothelium. This is even more likely if we realize that heart insufficiency treated with cardiac glycosides predominates in older people.

Assuming that *in vitro* ouabain affects platelets at nanomolar and micromolar concentrations, the question arises whether endogenously produced ouabaina-like factors reported to be present in the bloodstream at picomolar concentrations (Vakkuri *et al*., 2000) have the capacity to inhibit Na^+/K^+-ATPase in these cells. The fact that mammalian Na^+/K^+-ATPase is characterized by a complex molecular heterogeneity that results from the expression and differential association of multiple isoforms of both its α- and β-subunits (Blanco & Mercer, 1998) may help elucidate this problem. Both the α- and β-isoforms of the Na^+/K^+-ATPase exhibit a tissue-specific pattern of expression. It is not known yet which of the Na^+/K^+-ATPase subunits are present in platelets. Human bone marrow is reported to contain all of the Na^+ pump isoforms except $\alpha 4$ (Stengelin & Hoffman, 1997). The Na^+ pump of human erythrocytes is composed of the $\alpha 1$ and $\alpha 3$ subunits and of $\beta 1$–$\beta 3$ subunits (Hoffman *et al*., 2002). Also, little is known about the sensitivity of the diverse forms of Na^+/K^+-ATPase to plant-derived cardiac glycosides and endogenously produced ouabain-like factors. However, it has been reported that endogenously produced ouabain (probably a stereoisomer of plant ouabain) isolated and purified from bovine blood inhibits rat brain $\alpha 1$ Na^+/K^+-ATPase with a 1000-fold higher potency than does plant ouabain (Ferrandi *et al*., 1998). Platelets are likely to possess at least two isoforms of Na^+/K^+-ATPase, since exogenously added plant ouabain is not able to inhibit its activity totally (Marx *et al*., 1992). Activated platelets have been reported to express additional binding sites for ouabain, which may result in a rise in the sensitivity of their Na^+/K^+-ATPase to cardiac glycosides (Bork & Mrsny, 1993). Since patients with atrial fibrillation and heart failure are reported to have preactivated platelets (Kamath *et al*., 2001; Choudhury *et al*., 2007), it is likely that their Na^+/K^+-ATPase is also more sensitive to cardiac glycosides. To sum up, it cannot be excluded that the platelet isoform(s) of Na^+/K^+-ATPase, especially these from thrombosis-prone patients with heart failure and/or atrial fibrillation, may be affected by endogenously produced ouabain-like factors as well as by therapeutical concentrations of cardiac glycosides. Further studies are needed to assess the procoagulant effect of cardiotonic glycosides on platelet-related procoagulant activity in subjects suffering from atrial fibrillation.

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