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THE *IN VITRO* EFFECT OF PROSCILLARIDIN ON PLATELET RESPONSES

KATARZYNA WINNICKA[a], HALINA STELMACH[b], TOMASZ RUSAK[b], and MARIAN TOMASIAK[b]

[a]Department of Drug Technology, [b]Department of Physical Chemistry, Medical University of Białystok, 1 Kilińskiego Str., 15-089 Białystok, Poland

Abstract: Proscillaridin is a potent inhibitor of Na^+/K^+-ATPase used to treat patients with atrial fibrillation. Elevated concentrations of endogenous proscillaridin are suggested to be present in the blood of subjects with essential hypertension. The rise in intracellular Na^+ and Ca^{2+} concentrations due to the inhibition of Na^+/K^+-ATPase are expected to affect platelet responses crucial for the generation of blood hypercoagulable state. The present study investigates the *in vitro* effect of proscillaridin on platelets. It was found that proscillaridin (100 μM) potentiated collagen-evoked platelet aggregation. Proscillaridin (20 – 100 μM) treated platelets expressed procoagulant activity which was dose and time related but weaker than that produced by collagen. Flow cytometry studies revealed that *in vitro* platelet treatment with proscillaridin produced left shift (decrease) in the forward and side scatter of the entire cell population. The shift was unimodal and less pronounced than that produced by collagen. Using flow cytometry and annexin V-FITC as a probe it was found that proscillaridin-treated platelets expressed phosphatidylserine (PS) which is crucial for the platelet-dependent thrombin generation. Proscillaridin-evoked PS expression was dose and time dependent but weaker than that produced by collagen. Proscillaridin *per se* was able to induce serotonin release from platelets. It is concluded that the therapeutic proscillaridin concentrations are unlikely to affect platelets in healthy subjects. However, the influence of endogenous proscillaridin on platelet responses critical for the generation of blood hypercoagulable state cannot be excluded.

Keywords: cardiac glycosides, proscillaridin, platelet responses, procoagulant activity

Proscillaridin is a potent and selective inhibitor of a plasma membrane Na^+/K^+-ATPase used to treat patients with atrial fibrillation and heart failure (1,2). Endogenously produced proscillaridin has also been suggested to circulate in increased concentrations in the blood of patients with low renin essential hypertension (3,4). Endogenous blockers of Na^+/K^+-ATPase constitute a group of so called endogenous cardiac glycosides which is now recognized as a new class of steroid hormones (5).

It has been reported that the concentration of endogenous cardiac glycosides is elevated in human blood upon increased sodium uptake, hypoxia and physical exercises (6). Enhanced plasma levels of endogenous cardiac steroids have also been observed in several forms of hypertension, diabetes mellitus and preeclampsia i.e. in clinical conditions associated with blood hypercoagulability (5,6,7). Since patients with all above mentioned clinical conditions as well as patients with atrial fibrillation are highly predisposed to thromboembolism, it is of importance to test whether cardiac glycosides are thrombogenic.

Activated platelets which have been proposed to provide a highly efficient catalytic surface that facilitates the reactions leading to thrombin formation (8), play a crucial role in the generation of blood hypercoagulable state. *In vitro* studies have shown that incubation of human platelets with ouabain results in a significant rise in intracellular Na^+ concentration (9) and in enhancement of intracellular Ca^{2+} concentration (10), which is expected to evoke a platelet procoagulant response (8,11). The effect of cardiac glycosides on platelet responses crucial for the generation of blood hypercoagulable state (e.g. procoagulant response and secretion) has never been studied before. It has been earlier reported that accumulation of Na^+ in platelets (produced by activated Na^+/H^+ exchanger) might be associated with the development of procoagulant response and shedding of platelet microparticles (microvesicles) from their plasma membrane (11,12). Preactivation of circulating platelets by cardiac glycosides is therefore likely to result in thrombin generation. The objective of the present study was to investigate the *in vitro* effect of proscillaridin on platelets with a special emphasis on their procoagulant response. Proscillaridin is one of the most common cardiotonic steroids currently in use. However, its effect on platelet function is largely unknown.

The obtained results indicate that the inhibition of platelet Na^+/K^+-ATPase by proscillaridin may result in the generation of procoagulant activity.

EXPERIMENTAL

Chemical reagents

N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes), ethylene glycol-bis-(aminoethyl-ether)-N,N,N',N'-tetraacetic acid (EGTA), proscillaridin, apyrase, prostaglandin E_1 (PgE_1), bovine serum albumin (BSA), Russell's viper venom (RVV), phosphatidylethanolamine were purchased from Sigma Chemical Co. (USA), as were most other chemicals used. Collagen (fibrillar, from equine tendon) was from Hormon Chemie (Germany), thrombin (human) was from La Roche (Switzerland). Fluorescein isothiocyanate (FITC)-labeled annexin V (annexin V- FITC), phycoerythrin (PE)-labeled anti GPIIb/IIIa MoAb (PE-CD41a), CD62P-FITC and PE-labeled isotypic mouse MoAb were from BD Biosciences-PharMingen. 5-hydroxy -[^{3}H] tryptamine creatinine sulphate ([^{3}H] 5-HT) with specific activity of 8.6 Ci/mmol was purchased from the Radiochemical Centre (England).

Blood collection

Venous blood was collected with minimum trauma and stasis via a 19-gauge needle 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 platelet-rich plasma (PRP), platelet-poor plasma (PPP) and washed platelets

Platelet-rich plasma was obtained by centrifugation of the blood at 200×g for 20 min at room temperature. Platelet-poor plasma was obtained by further centrifugation of PRP at 2700×g for 10 min. Washed platelets were prepared as described previously (13).

Measurement of procoagulant activity

The assay system was based on Russell's viper venom, which induces clotting by activation of factors V and X, and in the presence of Ca^{2+} ions is dependent on phospholipids (14). Assay of clotting time was done in plastic cuvettes with stirring bars using the optical coagulometer (Boehring) exactly as described previously (11). The clotting time in seconds (Russell's viper time) was converted to a procoagulant activity units (equivalent concentration of phospholipid expressed in µg/ml) using a standard curve that plotted log of clotting time versus log of phospholipid (cephalin) concentration essentially as described by Jy et al. (15).

Platelet activation and flow cytometry

Flow cytometry analysis was performed using Coulter EPICS XL (argon laser) flow cytometer. Measurements of phosphatidylserine exposure and morphology experiments were performed as described previously (16).

Assay of platelet aggregation

Platelet aggregation was followed turbidimetrically by recording the light transmission through a stirred platelet suspension in plastic cuvette of an aggregometer (Elvi, Logos, Milan) at 37°C (17). Samples of PRP (300 µl) were incubated with stirring for 2 min at 37°C. Then the threshold concentrations of the stimulus were added to induce the aggregation. For each platelet preparation, the threshold aggregating concentrations, defined as the minimum amount of the stimulus that induced at least 70% increase in light transmission within 3 min was selected. The tested substances were added to the platelet suspension 20 min prior to the addition of the stimulus. The extent of aggregation was expressed as a percentage of the maximum change of light transmission between platelet suspension and suspending medium. Quantification of aggregation tracings was performed by measuring the maximal increase in light transmission from the peak of shape change to the highest transmission level (the extent of platelet aggregation). All experiments were performed at least in quadruplicate using 4 – 5 different platelet preparations.

Measurement of platelet secretion

Secretion was determined by the release of ^{3}H--serotonin ([^{3}H] 5-HT) (18).

Data analysis

Data reported in this paper are the mean (± SD) 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.), accepting $p < 0.05$, as significant.

RESULTS

Experiments presented in Figure 1 were performed to establish whether proscillaridin may po-

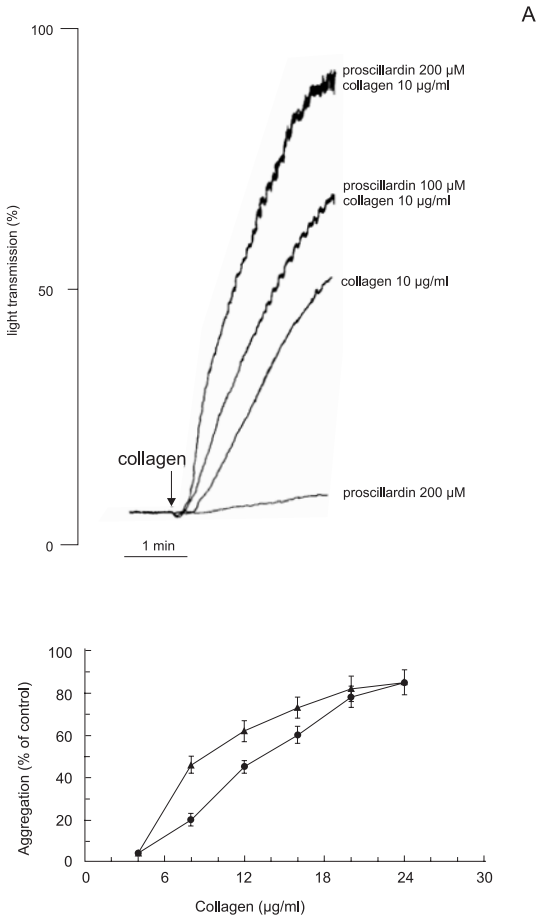

Figure 1. The effect of proscillaridin on collagen-induced platelet aggregation.
Panel A: Representative traces of aggregation of platelets, preincubated 20 min with 100 µM and 200 µM proscillaridin, evoked by subtreshold (10 µg/ml) collagen concentration.
Panel B: 0.6 ml of PRP was incubated without stirring for 20 min at 37˚C in the cuvette of aggregometer without (control – •) or with 100 µM proscillaridin (▲). Aggregation was initiated by the addition of collagen (4 – 24 µg/ml). The extent of platelet aggregation was measured 3 min after the addition of the agonist, and the maximum extent of aggregation was taken as 100%. Mean values ± SD from at least four assays made on five different platelet preparations are presented.

tentiate platelet aggregation induced by collagen. As it is seen from panel A, 20-minutes treatment of platelets with 100 and 200 µM proscillaridin augmented the aggregation response induced by subthreshold (10 µg/ml) concentration of collagen. Maximal potentiation of aggregation was recorded in the presence of 200 µM proscillaridin. Further increase in proscillaridin concentrations (not shown here) did not induce significant rise in potentialization of aggregation response. 50 µM proscillaridin (not shown here) failed to potentiate platelet aggregation. Proscillaridin *per se* up to the concentrations of 200 µM was not able to evoke platelet aggregation.

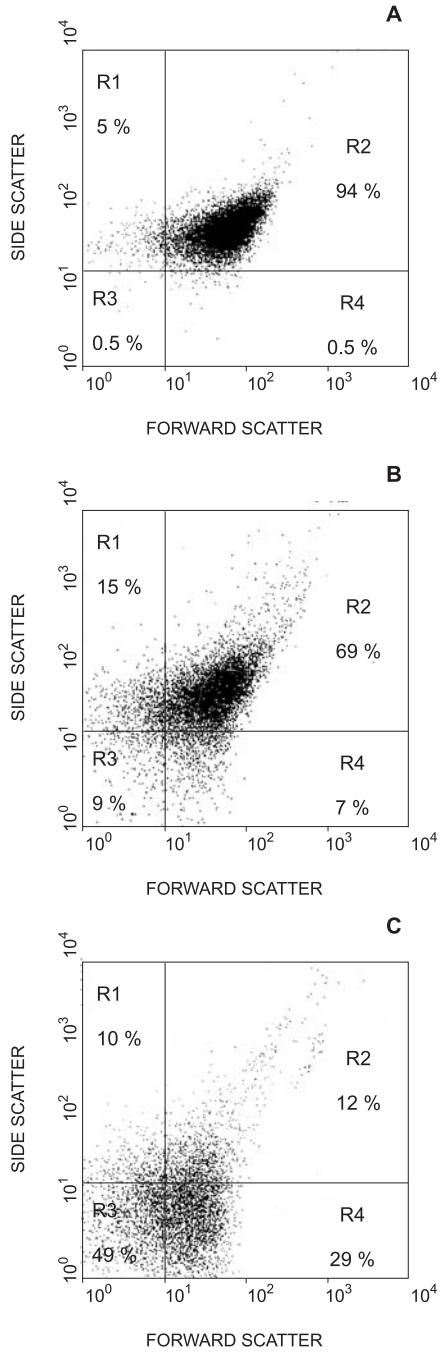

Figure 2. The effect of proscillaridin on the morphology (size and granularity) of platelets.
Dot plots of forward scatter versus side scatter of a platelet suspension (PRP) untreated (panel A) and treated for one hour at 37˚C with 100 µM proscillaridin (panel B) and for 10 min with 20 µg/ml collagen (panel C). The percentage of events of the 10 000 total found in each region is shown inset. Each dot plot is representative of 12 determinations performed on four different preparations. Note that proscillaridin and collagen 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).

Table 1. The effect of proscillaridin, collagen and thrombin on the serotonin release by blood platelets .

Experiment	Additions	Serotonin release (% of total)
A	None	1.5 ± 1.1
	Proscillaridin 20 µM, 60 min	1.7 ± 0.9
	Proscillaridin 50 µM, 60 min	5.6 ± 1.0**
	Proscillaridin 100 µM, 60 min	12.3 ± 1.2***
	Proscillaridin 200 µM, 60 min	12.9 ± 1.2***
	Thrombin 0.3 U/ml, 5 min	70.0 ± 6.4***
	Collagen 20 µg/ml, 5 min	50.0 ±4.9***
B	None	1.5 ± 1.1
	Proscillaridin 100 µM, 20 min	5.1 ± 0.6**
	Proscillaridin 100 µM, 40 min	7.6 ± 0.8***
	Proscillaridin 100 µM, 60 min	12.3 ± 1.3***

Aliquots of [^{3}H]5-HT-loaded platelets were incubated without stirring for 5 – 60 min at 37°C without (none) or with proscillaridin, thrombin or collagen added to the final concentrations as indicated. Results are expressed as a percent of [^{3}H]5-HT secreted to the total amount contained in unstimulated platelets and are presented as means ± SD. All experiments were performed at least in quadruplicate using five different platelet preparations. ** – $p < 0.01$, *** – $p < 0.001$

Table 2. Platelet procoagulant activity in the presence of proscillaridin.

Experiment	Additions	Procoagulant activity	
		RVV time (s)	PL conc. (µg/ml)
A	None	27.2 ± 1.2	1.56
	Proscillaridin 5 µM, 60 min	26.5 ± 1.8	2.22
	Proscillaridin 10 µM, 60 min	26.0 ± 1.1*	2.69
	Proscillaridin 20 µM, 60 min	25.1± 1.3**	3.55
	Proscillaridin 50 µM, 60 min	23.8 ± 1.3**	4.77
	Proscillaridin 100 µM, 60 min	21.4 ± 1.3***	7.04
	Proscillaridin 200 µM, 60 min	20.9 ± 1.0***	7.51
	Collagen 20 µg/ml, 10 min	15.1 ± 1.4***	12.99
B	None	26.1 ± 1.0	2.60
	Proscillaridin 100 µM, 20 min	24.5 ± 1.4*	4.11
	Proscillaridin 100 µM, 40 min	22.0 ± 1.3**	6.47
	Proscillaridin 100 µM, 60 min	20.1 ± 1.3***	8.27
C	Control (no platelets)	≥ 40.00	< 0.10

Procoagulant activity of washed platelets (3×10^8 cells/ml) after incubation at 37°C without stimulator (none) and with the tested substances added to the final concentrations as indicated. PL conc. (phospholipid concentration) – mean RVV time
converted to a procoagulant activity units. Mean values ± SD are reported (n = 16). * – $p < 0.05$, ** – $p < 0.01$, *** – $p < 0.001$

Panel B shows that proaggregatory effect of proscillaridin was significantly stronger at lower collagen concentrations. Thus, 100 µM proscillaridin augmented platelet aggregation evoked by collagen, added to the final concentration of 8, 12, 16 µg/ml by about 26, 17 and 7%, respectively.

Table 1 shows that proscillaridin, in a dose and time dependent manner, produced serotonin secretion from human platelets. Maximal secretion was observed following 60-minutes incubation with 100 µM proscillaridin. Further increase in proscillaridin concentration (up to 200 µM) did not induce significant rise in serotonin release. Proscillaridin behaves as a weak inducer of secretion since platelets treated for 5 min with collagen (20 µg/ml) or thrombin (0.3 U/ml) released 50 and 70% of serotonin, respectively.

As shown in Table 2, proscillaridin induced, in a dose and time dependent manner, a procoagulant response in platelets. Compared to control, a 60 min incubation of platelets with proscillaridin (10 – 200 µM) produced a 1.7 to almost 5-fold higher procoagulant activity. Maximal procoagulant response was ob-

Table 3. The effect of proscillaridin on the appearance of phosphatidylserine (PS) on the platelet surfaces.

Experiment	Additions	Annexin V – positive cells (%)
A	None	2.7 ± 1.2
	Proscillaridin 5 μM, 60 min	6.5 ± 1.2
	Proscillaridin 10 μM, 60 min	15.0 ± 1.3**
	Proscillaridin 20 μM, 60 min	17.1 ± 1.4***
	Proscillaridin 50 μM, 60 min	21.3 ± 1.5***
	Proscillaridin 100 μM, 60 min	22.5 ± 1.5***
	Proscillaridin 200 μM, 60 min	22.9 ± 1.6***
	Collagen 20 μg/ml, 10 min	78.0 ± 5***
B	None	2.5 ± 1.0
	Proscillaridin 100 μM, 20 min	14.4 ± 1.4**
	Proscillaridin 100 μM, 40 min	20.0 ± 1.3***
	Proscillaridin 100 μM, 60 min	26.1 ± 1.3***

Platelets (PRP) were incubated at 37°C without stirring with no stimulator (none), or with proscillaridin 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 ± SD of four experiments each performed on separate platelet preparation (n =12). ** – $p < 0.01$, *** – $p < 0.001$

served following the 1 h treatment of platelets with 100 μM proscillaridin. Further increase in proscillaridin concentrations (up to 200 μM) did not induce significant rise in procoagulant activity. Proscillaridin behaves as a weak inducer of procoagulant response since platelets treated for 10 min with collagen (20 μg/ml) expressed at least 8-fold rise in procoagulant activity. Collagen is the strongest physiological inducer of platelet procoagulant response (19).

Next it was studied how the incubation of platelets with increasing concentrations of proscillaridin affects the phosphatidylserine (PS) expression on their surface. The amount of PS expressed on a cell surface is a major determinant of the ability to support procoagulant complex assembly (8,20). Therefore, platelet PS expression (using fluorescein-conjugated annexin V), was directly examined. Annexin V binds to aminophospholipids in the presence of calcium with high affinity and strict specificity (21). The dose-dependent rise in the percentage of platelets with PS exposed on their surface following the 60 min treatment with 5 – 200 μM proscillaridin is depicted in Table 3 (panel A). As it is seen, one hour incubation of platelets with 10 – 200 μM proscillaridin produces a rise in the percentage of platelets with PS exposed on their surface by about 12.3 – 20.2%, respectively. The maximal amount of expressed PS was observed in the presence of 100 μM proscillaridin. Further increase in proscillaridin concentration up to 200 μM resulted in only insignificant rise in PS-positive events.

In comparison to collagen, proscillaridin is a relatively weak inducer of PS expression. A 10 min incubation of platelets with collagen (20 μg/ml) produced about 75% rise in the percentage of PS positive platelets. Panel B shows that proscillaridin effect is time dependent.

Figure 2 shows the results of flow cytometry performed to determine how proscillaridin affects platelet morphology (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). 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 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 in the population of normal (untreated) 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 (22).

A 1 h incubation of platelets with 100 μM proscillaridin (panel B) produces a much broader light scatter profile than in control (panel A). This effect was manifested by a marked increase in the percentage of counts in R1 (by 10%), R3 (by 8.5%) and R4 (by 6.5%) and a simultaneous drop (by 25%) 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 proscillaridin-treated platelets. In comparison to proscillaridin-treated cells, collagen-activated platelets (panel C) exhibited significantly stronger down/left shift in the side and forward scatters. It was manifested by a marked increase in the percentage of counts in R1 (by 5%), R3 (by 48.5%) and R4 (by 28.5%) and a simultaneous drop (by 82%) in the percentage of events in R2. Analysis of the forward scatter versus side scatter dot plot of platelets preincubated with proscillaridin and collagen 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. These new events (i.e. the events appearing in regions R1 and R3) seem to be degranulated platelets with profound changes on their surface and/or fragmented cells.

DISCUSSION

A rapid loss of plasma membrane asymmetry, manifested by the appearance of phosphatidylserine (PS) in the outer leaflet of the plasma membrane, and a process of secretion of clotting factors stored in platelet granules are important consequences of platelet activation. The membrane area rich in PS creates thrombogenic surface that accelerates blood coagulation process by providing catalytic sites for the assembly of the tenase and prothrombinase complexes (20). Prothrombinase complexes are able to produce large (up to 800 nM) quantities of thrombin which is responsible for the undesired and potentially lethal thrombotic events (23). The ability of stimulated platelets to catalyze the activation of factor X and conversion of prothrombin to thrombin is commonly called procoagulant activity, and is now recognized as a normal physiological response of these cells (19,24).

The present results suggest that *in vitro* proscillaridin behaves as a weak inducer of platelet procoagulant response. This is based on the observation that proscillaridin-treated platelets express PS on their surface, accelerate thrombin generation in the presence of factor Xa, factor Va, prothrombin and Ca^{2+}, and secrete the contents of platelet storage granules containing clotting factors. In addition, proscillaridin potentiates platelet aggregation evoked by subtreshold concentrations of collagen and initiates morphological changes similar to those produced by collagen.

To sum up, proscillaridin *in vitro* is able to preactivate platelets and make them procoagulant. However, it should be stressed that all the above mentioned effects are produced by micromolar concentrations of proscillaridin. Proscillaridin has been reported to affect purified ATP-ase preparations at nanomolar concentrations (25). Therapeutic concentrations of cardiac glycosides are about 1 ng/ml (26), whereas the concentrations of endogenously produced circulating cardiac glycosides are reported to be nanomolar (3,4). This may be interpreted to mean that therapeutic concentrations of proscillaridin are unlikely to produce platelet procoagulant response. However, the present experiments were performed on platelets obtained from healthy subjects. The platelets of patients with atrial fibrillation and heart failure may respond differently to proscillaridin therapy in the sense that they may be preactivated (27,28,29) and have an increased sensitivity to cardiac glycosides. It should be also emphasized that in our experiments, platelets were incubated with proscillaridin for maximum 60 min, whereas cardiac glycosides therapy usually continues for many years.

Assuming that *in vitro* proscillaridin affects platelets at micromolar concentrations, the question arises whether endogenously produced cardiac glycosides, which have been reported to be present in the blood stream within the nanomolar range (3,4), have a capacity to inhibit Na^+/K^+-ATPase in these cells. To answer this question, it should be taken into account that in mammals 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 (30). Both α and β isoforms of the sodium pump 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 has been reported to contain all of the sodium pump isoforms except α4 (31). The sodium pump of human erythrocytes is composed of the α1 and α3 subunits and of β1- β3 subunits (32).

Also little is known about the sensitivity of the diverse forms of the Na^+/K^+-ATPase to plant derived and endogenously produced cardiac glycosides. However, it has been reported that endogenously produced ouabain isolated and purified from bovine (or rat) blood inhibits the rat brain α1 Na^+/K^+-ATPase with 1000-fold higher potency than plant ouabain, while it has an IC_{50} similar to plant ouabain for the α3-isoform purified from rat brain (33). Both low and high ouabain affinity isoforms of Na^+/K^+-ATPase have also been reported to be present in arterial smooth muscle cells (30).

Platelets are likely to possess at least two isoforms of Na^+/K^+-ATPase since exogenously added

plant ouabain is not able to inhibit totally its activity measured by $^{86}Rb^+$ uptake (34). Platelets have been reported to express binding sites for ouabain which indicates that at least potentially their Na^+/K^+-ATPase may be controlled by physiological concentrations of endogenous cardiac glycosides (35). Therefore, at present it cannot be excluded that the platelet isoform (s) of Na^+/K^+-ATPase may be affected by physiological concentrations of endogenous cardiac steroids.

To sum up, the present data suggest that the occurence of proscillaridin in the blood may result in platelet preactivation which in turn may generate blood hypercoagulable state. It is concluded that the therapeutic proscillaridin concentrations are unlikely to affect platelets in healthy subjects. However, the influence of endogenous proscillaridin on platelet responses critical for the generation of blood hypercoagulable state cannot be excluded.

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