Published in final edited form as:
Biochim Biophys Acta. 2006 February ; 1761(2): 195–204. doi:10.1016/j.bbalip.2005.12.008.

Involvement of the Na^+/H^+ exchanger in membrane phosphatidylserine exposure during human platelet activation

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Abstract

Platelet membrane phosphatidylserine (PS) exposure that regulates the production of thrombin represents an important link between platelet activation and the coagulation cascade. Here, we have evaluated the involvement of the Na^+/H^+ exchanger (NHE) in this process in human platelets. PS exposure induced in human platelets by thrombin, TRAP, collagen or TRAP+ collagen was abolished in a Na^+-free medium. Inhibition of the Na^+/H^+ exchanger (NHE) by 5-(N-Ethyl-N-Isopropyl) Amiloride (EIPA) reduced significantly PS exposure, whereas monensin or nigericin, which mimic or cause activation of NHE, respectively, reproduced the agonist effect. These data suggest a role for Na^+ influx through NHE activation in the mechanism of PS exposure. This newly identified pathway does not discount a role for Ca^{2+}, whose cytosolic concentration varies together with that of Na^+ after agonist stimulation. Ca^{2+} deprivation from the incubation medium only attenuated PS exposure induced by thrombin, measured from the uptake of FM1-43 (a marker of phospholipid scrambling independent of external Ca^{2+}). Surprisingly, removal of external Ca^{2+} partially reduced FM1-43 uptake induced by A23187, known as a Ca^{2+} ionophore. The residual effect can be attributed to an increase in $[Na^+]_i$ mediated by the ionophore due to a lack of its specificity. Finally, phosphatidylinositol 4,5-bisphosphate (PIP_2), previously reported as a target for Ca^{2+} in the induction of phospholipid scrambling, was involved in PS exposure through a regulation of NHE activity. All these results would indicate that the mechanism that results in PS exposure uses redundant pathways inextricably linked to the physio-pathological requirements of this process.

Keywords

Platelet; Thrombin; Phosphatidylserine; Na^+/H^+ exchanger (NHE) phosphatidylinositol 4,5-bisphosphate (PIP_2)

[1]Recipient of a grant from the Centre National de la Recherche Scientifique (CNRS).

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1. Introduction

In resting platelets, the constitutive plasma membrane aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) are almost exclusively restricted to the inner (cytoplasmic) leaflet, whereas sphingomyelin (SM) and phosphatidylcholine (PC) are more concentrated in the outer (exoplasmic) leaflet [1]. Randomization of the lipids over both membrane leaflets, i.e., lipid scrambling, is most clearly manifested by PS translocation to the exoplasmic leaflet in which PS becomes accessible to circulating blood-clotting factors [2]. Phosphatidylserine molecules bind to regulatory sites on factors Xa and Va and allosterically alter their proteolytic and cofactor activities, resulting in the enhancement of prothrombinase activity relative to intrinsic factor Xa activity in solution. Due to its important role in regulating the activity of critical enzymes, the exposure of PS on the surface of activated platelets is a key regulatory event in blood coagulation [3–6] suggesting an important role of PS in normal hemostasis and thrombotic disease.

A Ca^{2+}-dependent scramblase activation downstream of an increase in Ca^{2+} concentration was proposed initially as a necessary step for plasma membrane phospholipid scrambling; however, subsequent studies of pure recombinant scramblase activity reconstituted in phospholipid liposomes or large unilamellar vesicles (LUVs) show only slow and limited phospholipid redistribution after Ca^{2+} addition [7]. There is also evidence that scramblase activity is not the sole effector of phospholipid redistribution and that it may be regulated by factors other than Ca^{2+} [8,9]. Adult knockout phospholipid scramblase 1, PLSCR1 (−/−) mice, and mice overexpressing PLSCR1 show no obvious hematologic or hemostatic differences, and blood cells from PLSCR1(−/−) mice mobilize PS normally to the cell surface upon stimulation [10]. These data argue that the relationship between this protein and the scrambling activity observed in intact cells is uncertain. Other studies show that the increase in cytosolic Ca^{2+} is not sufficient to stimulate plasma membrane phospholipid scrambling [11] and the hypothesis that scrambling could result from the formation of microvesicles [12] induced by Ca^{2+} has been experimentally invalidated [13–15].

During platelet stimulation with agonists such as thrombin, a rapid rise in intracellular pH is observed. This alkalinization (found by some investigators to be preceded by brief acidification [16]) is mediated by an increase in the transport activity of the NHE1 isoform of the Na^+/H^+ exchanger. The regulation of NHE1 activity is complex, not completely understood, and includes several heterotrimeric G proteins, small G proteins (ras, cdc42, rhoA), mitogen activated protein kinase (MAP), protein kinase C (PKC) and the Ca^{2+}/calmodulin system [17,18].

Recent observations suggest that Na^+ influx through NHE is necessary for the creation of procoagulant activity of porcine platelets [19] and of human platelets treated by desmopressin [20], and an enhanced platelet NHE exchanger activity found in type 2 diabetic patients is associated with elevated phospholipid-dependent procoagulant activity and increased risk of vascular damage [21]. Additionally, Na^+ influx resulting from P2X7 receptor activation by extracellular ATP was recently reported to induce rapid PS externalization in thymocytes [22].

The relationship between Ca^{2+} mobilization and NHE1 function during platelet activation is not well established. There are data suggesting that inositol triphosphate (IP_3)-induced calcium mobilization is pH-sensitive [23] and inactivation of Na^+/H^+ exchange inhibits Ca^{2+} mobilization [17,24]. On the other hand, in human platelets stimulated with thrombin, Ca^{2+} mobilization was found to occur without a functional exchanger and in an acidified cytoplasm [25].

Phosphatidylinositol 4,5-bisphosphate (PIP_2) was reported previously to play a role in PS exposure as a direct Ca^{2+} target [9,26,27] by forming specific PIP_2 domains [9] and/or as a cofactor of an enzyme such as scramblase [26]. Since PIP_2 interacts with a large spectrum of proteins as well as peptides, cations and polycations, finding a PIP_2-regulated cofactor responsible for PS exposure is the long-term goal of our study. Based on a number of findings, we propose NHE activity, reported to be PIP_2-dependent [28] as a novel candidate for such an intermediate.

2. Materials and methods

2.1. Materials

Thrombin (T-6884), calcium ionophore A23187 (C-7522), BSA[1] (A6003), apyrase (A-6535), sepharose CL-2B (CL-2B-300), 5-(*N*-methyl-*N*-Isobutyl) Amiloride (EIPA) (A3085), *N*-methyl-D-glucamine (M-2004), FURA 2-AM (A-9210), quercetin (Q-0125), neomycin (N-6386), monensin (M-5273), gramicidin (G-5002), nigericin (N-7143), and lactate dehydrogenase (LDH) kit (500) were obtained from Sigma (St. Louis, USA). Thrombin receptor agonist fragment (SFLLRN) (TRAP); (H-2936) was from Bachem (King of Prussia, PA USA). Calf tendon collagen reagent (385) and luciferin-luciferase reagent (Chrono-Lume 395) were from Chrono-Log Corporation (Havertown, PA USA). Cell permeant sodium green Indicator (S-6901) and Pluronic F-127 (P-6867) were from Molecular Probes (Eugene, OR USA). Green synaptracer (FM1-43); (FPT2982A) was from Fluo Probes (Montluçon, France). IgG_1 PE-conjugated mouse monoclonal antibody to human CD41 (platelet GP II b) (12-0419) was from Bioscience (Franklin Lake, NJ USA), Annexin V-FITC (BMS306FI) and PE (BMS306PE) were from Bender MedSystems (Burlingame, CA, USA).

2.2. Preparation of human platelets

Human blood from several healthy donors was collected by venipuncture in a tube containing 0.1 vol of ACD (111 mM dextrose, 85 mM trisodium citrate, 71 mM citric acid) as an anticoagulant. Platelet-rich plasma (PRP) was prepared by centrifugation of the blood for 15 min at 150×*g* at room temperature (RT). After apyrase (0.5 U/ml) addition, contaminating erythrocytes were removed by centrifugation at 300×*g* for 5 min. The platelets were sedimented by centrifugation at 1100×*g* for 12 min and carefully resuspended in buffer A (145 mM NaCl, 2.8 mM KCl, 0.8 mM $MgCl_2$, 0.8 mM KH_2PO_4, 10 mM HEPES, 5.6 mM glucose, 0.3% albumin at pH 7.35) or in sodium-free buffer B in which Na^+ was replaced by *N*-methyl-D-glucamine ($NMDG^+$) [25,29] and filtered on a column of Sepharose 2B equilibrated with the respective buffers. Osmolarity of buffer A (309±10.5 mosM/kg) and B (315±5.7 mosM/kg) was determined using a vapor pressure osmometer (Vapor™, Vescor). Cell count was obtained using a Coulter Z2 particle count and size analyzer.

2.3. Thrombin activity

Enzymatic activity of thrombin in sodium buffer A and sodium-free buffer B was determined from optical density changes at 405 nm following cleavage of the synthetic peptide derivative tosyl-gly-pro-arg-4-nitroanilide (Chromozym TH, Roche, Indianapolis, IN, USA) based on the sequence at the N terminus of the alpha chain of human fibrinogen. The assay was done according to the manufacturer's instructions.

2.4. Platelet aggregation and secretion

Platelet suspensions (2×10^8/ml) in buffer A or B (supplemented with 2 mM $CaCl_2$) were added to a pre-warmed cuvette (37 °C) of a Chronolog Lumi-Aggregometer (Chrono-Log

Corp. Havertown, PA) and stirred at 1000 rpm before the addition of thrombin (0.1–1 U/ml) or TRAP (10–100 μM). The aggregometer was calibrated with a platelet suspension for zero light transmission and with a buffer for 100% transmission. Changes in light transmission were recorded for 7 min, using a PowerLab/200 instrument and MacLab Chart program version 3.2. ATP secretion was monitored in platelet suspensions (2×10^8/ml) supplemented with luciferin–luciferase reagent according to the manufacturer's instructions.

2.5. PS exposure

Samples of platelet suspension in buffer A or B were activated with different agonists (0.1–1 U/ml thrombin, TRAP 100 μM) and ionophores (A23187 2–5 μM, monensin 1–50 μM, gramicidin 1–50 μM, nigericin 10 μM) for 20 min at 37 °C in the presence of 2 mM $CaCl_2$. When indicated, platelets were treated for 1 min with 30 μM EIPA, one h with 100 μM quercetin or 5 mM neomycin before activation. Samples of activated platelet suspensions (0.5 μl) were mixed with 13 μl of filtered buffer A or B (supplemented with 2 mM $CaCl_2$), 1 μl of IgG_1 anti-CD41-PE, and 0.5 μl of annexin V-FITC or PE [14]. After 10-min incubation to ensure antibody and annexin binding, samples were diluted with 185 μl of the appropriate buffer and subjected to flow cytometry analysis. To determine the percentage of annexin V binding platelets, the population of platelets was first characterized by gating the platelets labeled by a PE-conjugated antibody to CD41 and this population was reanalyzed on the FITC fluorescence channel to determine the percentage of events CD41 positive (platelets) and FITC positive (platelets exposing PS). The gate containing FITC negative platelets was fixed with the non treated cells and in treated cells all the platelets with a higher fluorescence were considered as PS positive. In each sample run, at least 10,000 events were acquired. Platelet lysis occurring during this assay was determined by LDH activity in the supernatant of activated platelets. Depending on the experimental conditions, lysis amounted to 1–3% of total cells. To evaluate plasma membrane phospholipid redistribution in platelets suspended in calcium-free buffer, binding of Factor Va or uptake of styryl dye FM1-43 have been described. As activation of platelets induces secretion of endogenous Factor Va [30], it is not possible to use this technique (Data not shown). In contrast, FM1-43 uptake has been widely used to investigate PS externalization in various cells and the results have been justified by comparison with other methods [31–34]. After platelet activation for 20 min, 5-μl samples were mixed with 20 μl of 50 μM FM1-43 solution, followed by a 1-min incubation and a 100-fold dilution prior to flow cytometry analysis. In each sample run, at least 10,000 events were acquired.

In all experiments, cells were sorted by flow cytometry using a BD Biosciences FaxVantage cytometer. Fluorochrome excitation was performed by argon laser at 488 nm, and fluorescence emissions were analyzed with a 530 nm filter for FITC or FM1-43 fluorescences and with a 575 nm filter for R-phycoerythrin.

2.6. $[Ca^{2+}]_i$ measurement

Platelet suspensions in buffer A (4×10^8/ml) were incubated for 45 min at RT, in the dark, with 2 μM Fura 2-AM, centrifuged (10 min, 1000×*g*), and resuspended in buffer A or B containing 0.1 mM EGTA, to a density of 1.5×10^8/ml. After addition of platelets to a cuvette of a SLM-Aminco MC 200 fluorimeter, the transient Fura-2 fluorescence was recorded (I_{ex}=340 nm, I_{em}=510 nm) before and after addition of agonists. $[Ca^{2+}]_i$ was calculated from the general formula $[Ca^{2+}]_i = K_d(F - F_{min}/F_{max} - F)$ in which K_d is the dissociation constant of Fura-2 for Ca^{2+} binding (224 nM), and F the fluorescence intensity of the sample. F_{max} was determined after lysing the cells with 50 μM digitonin and F_{min} after adjusting the pH of the lysed cells to 8.5 with 20 mM TRIS base, followed by addition of 10 mM EGTA.

2.7. Cytosolic Na^+ changes

Platelets ($0.5–1 \times 10^8$/ml) suspended in buffer A (containing 0.1 mM EGTA) were incubated with 5 μM sodium green indicator (stock solution in 20% pluronic F-127/DMSO) for 40 min at RT. After this period, platelet suspensions were supplemented with 2 mM Ca^{2+} (when required), and 100 μl samples were treated for 1 min with 30 μM EIPA, one h with 100 μM quercetin or 5 mM neomycin and activated with various agonists for 10 min at 37 °C. 0.5 μl samples of activated platelet suspensions were mixed with 13.5 μl of filtered buffer A, 1 μl of IgG1 anti-CD41-PE, and after 10 min incubation (to ensure antibody binding), samples were diluted with 185 μl of buffer A for flow cytometry analysis. In each sample run, at least 10,000 events were acquired.

2.8. Data analysis

Data are means±S.D. of a minimum of three independent experiments. Differences between means were evaluated by paired *t* test and the *P* values are indicated in the tables and figures.

3. Results

3.1. Platelet activation through the PAR-1 pathway in Na^+-containing and Na^+-free buffer

Thrombin is a Na^+-activated protease [35,36]. Na^+ binding near the primary specificity pocket of thrombin promotes the procoagulant and signaling functions of the enzyme. The effect is mediated allosterically by communication between the Na^+ site and regions involved in substrate recognition [37,38]. Accordingly, the ability of thrombin to proteolyse the Chromozym substrate is drastically inhibited in a Na^+-free buffer (Fig. 1A). Thrombin-induced platelet secretion and aggregation were also inhibited in a buffer lacking Na^+ ions (Fig. 1 B and C), whereas the effect of TRAP peptide, a specific protease activated receptor 1 (PAR1) agonist, was not (Fig. 1B and D). These data confirm that in contrast to receptor activation through thrombin-mediated proteolysis, direct activation by TRAP is independent of external Na^+. To investigate the role of external Na^+ in the signaling cascades downstream of PAR1 activation, platelets were stimulated with TRAP.

3.2. Dependence of PS exposure on Na^+ influx through NHE activation

As previously described [39] and shown in Table 1, platelet PS exposure can be induced by activation of several receptors, including the thrombin receptor PAR1 activated with thrombin/TRAP [40] and procoagulant $\alpha_{II}\beta_3$ integrin and GpVI receptors activated with collagen/thrombin [41]. In a medium containing Ca^{2+} but no extracellular Na^+, PS exposure induced by TRAP, collagen, and a combination of TRAP and collagen was drastically inhibited (80–90% inhibition) (Table 1). Determination of PS externalization by annexin V-FITC binding was not affected in the medium containing Ca^{2+} but deprived of Na^+ as demonstrated by the increase of the percentage of annexin V-FITC positive cells when platelets were treated with A23187 (Table 1), and as in choline medium, PS externalization induced by ATP in thymocytes was always detectable [22]. Thrombin-induced PS exposure was also dependent on the presence of external Na^+ (Table 1). The simultaneous determination of PS exposure and $[Na^+]_i$ in platelets activated with thrombin provides evidence that $[Na^+]_i$ was increased in a significant percentage of the cell population that bind annexin V-PE as measured by FACS (Fig. 2B). Together, these data lead to the conclusion that Na^+ influx can be an effector of PS externalization in platelets. Na^+ influx can be mediated by NHE activation consecutive to a rapid and transient intracellular acidification [42]. We have investigated the implication of NHE in PS exposure by using an amiloride-derived specific inhibitor (EIPA) of the exchanger. Although less strongly that Na^+ deprivation, EIPA significantly inhibited (20–30% inhibition) PS exposure induced by

TRAP, collagen and TRAP+ collagen. The effects of thrombin and thrombin + collagen were also reduced, suggesting that thrombin-induced PS exposure was also dependent on Na^+ influx through NHE activation. The difference between the effect of Na^+ deprivation and EIPA on PS externalization could be linked to the inability of EIPA to reduce totally the increase in $[Na]_i$ even at 30 μM (the highest concentration that did not affect the resting platelet state) (Table 2). Assuming that EIPA is a full inhibitor of NHE1 [19,20], this finding indicates that Na^+ influx via other pathways than NHE could be involved in this process.

Nigericin, a K^+/H^+ ionophore known to induce a decrease in intracellular pH, was as effective as the other agonists at inducing PS exposure (Table 1). Na^+ deprivation and EIPA inhibited the effect of nigericin (Table 1). Nigericin also triggered an influx of Na^+ attributable to NHE activation, as did the other agonists (thrombin, TRAP, and TRAP+ collagen), and it was partially inhibited by EIPA (Table 2). NHE activation could be a consequence of nigericin-induced acidification that precedes alkalinization [16,17]. These data confirm the possible role of Na^+ influx through NHE activation but do not exclude the potential participation of pH variations in the PS exposure pathway.

PKC activation by PMA is known to activate NHE1 in platelets [17] by inducing its phosphorylation. Low concentrations of PMA (50–100 nM) were able to induce slow alkalinization in human platelets [16,17], but amplify recovery of pH after acidification induced by nigericin [16]. Higher concentrations (200 to 400 nM) were necessary to induce procoagulant activity [19] or serotonine secretion in porcine platelets [43]. Our data show that 50 nM PMA alone has no effect on PS externalization. However, the small increase of the effect of low or high concentrations of thrombin (Table 1) with PMA, argue in favor of a contribution of NHE in the regulation of PS exposure. Together, the data provide evidence that Na^+ influx subsequent to NHE activation participates in PS exposure induced through PAR1 activation in platelets.

3.3. Dependence of PS externalization on Na^+ influx, Ca^{2+} influx and intracellular pH

The involvement of Na^+ in PS exposure does not discount a role for Ca^{2+}. It is not easy to determine the respective contributions of these two cations as their concentrations vary concomitantly in response to the same agonists (Table 2 and Fig. 4A). Deprivation of either Na^+ or Ca^{2+} in the external medium has either a deleterious effect on cell viability or prevents the binding of fluorescent annexin V to PS (which requires the presence of about 2 mM Ca^{2+}). Moreover, the specificity of available cation ionophores is not absolute. The use of the Ca^{2+} ionophore A23187 has led to suggestions that Ca^{2+} is the major effector of phospholipid scrambling [44,45]. Here, A23187 induced PS externalization in 70% of platelets in a medium containing Na^+ and Ca^{2+}, while in the absence of Na^+ externalization was observed in 91% of the cells (Table 1). The increased effect of A23187 on the percentage of platelets exposing PS would be due to an increase in the influx of Ca^{2+} resulting from the suppression of the competition with Na^+ already described [46,47]. This hypothesis is supported by the fact that in a Na^+/Ca^{2+} medium, A23187 induced a rise in $[Na^+]_i$ similar to that of the other agonists, an effect enhanced in the absence of Ca^{2+} (Table 2) confirming the competition between Ca^{2+} and Na^+ [46,47]. To investigate the contribution of external Ca^{2+} on PS redistribution, we measured the uptake of FM1–43, which reflects phospholipid scrambling and PS externalization and does not require external Ca^{2+} [31–34]. A23187 induced FM1–43 uptake in the majority of platelets in the presence of Ca^{2+}, and a lesser uptake in its absence (Fig 3A and Table 3). The residual effect could be due to the release of Ca^{2+} from internal stores or to the Na^+ influx induced by A23187 in the absence of Ca^{2+} (see Table 2). In the same way, thrombin induced FM1–43 uptake in a significant population of platelets in the presence of Ca^{2+}, and this effect persisted but was attenuated without Ca^{2+} (Fig. 3B and Table 3). As for A23187, the residual effect of thrombin without external Ca^{2+} can be due either to Ca^{2+} released from internal stores or

Na^+ influx. However, since PS exposure mediated through PAR1 activation by TRAP was entirely abolished in solutions without Na^+ (Table 1), an increase in $[Ca^{2+}]_i$, due to release from internal stores or to an influx similar to that induced by TRAP or collagen (Fig. 4A) is unlikely to be the sole mechanism regulating PS exposure. Indeed as previously described in T cells, an increase in $[Ca^{2+}]_i$ was not sufficient to induce phospholipid redistribution [32], and the sole release from Ca^{2+} stores is likely insufficient as capacitive Ca^{2+} influx could be indispensable [48,49]. All together these data indicate that PS exposure induced not only by thrombin, TRAP, and collagen but also by A23187 was dependent, at least partially, on an influx of Na^+. An effect of Na^+ influx induced by A23187 was also suggested by the detection of an increase in $[Na^+]_i$ in a population of platelets exposing PS (annexin V-PE positive) (Fig. 2C).

Monensin, a Na^+/H^+ ionophore which mimics NHE function, has been shown previously to induce procoagulant activity in porcine platelets [19,20] and secretion in human platelets [43]. In human platelets, this ionophore raises the intracellular pH and Na^+ concentration within seconds [50]. In a medium containing Na^+ and Ca^{2+}, 50 μM monensin induced PS externalization (Table 1), an increase in $[Na^+]_i$ (Table 2), and no change in intracellular Ca^{2+} (Fig. 4B). Under these conditions, PS exposure appeared mostly dependent on Na^+ influx. FM1–43 uptake was not affected by Ca^{2+} removal (Fig. 3C and Table 3) and even in presence of Ca^{2+}, the $[Ca^{2+}]_i$ remained unchanged (Fig. 4B) confirming that Ca^{2+} influx did not affect PS exposure in a Na^+-containing medium. However, the effect of monensin on PS redistribution was not affected in a medium deprived of Na^+ (Table 1), consistent with the fact that, under these conditions, monensin induced a weak increase in $[Ca^{2+}]_i$ (Fig. 4B), which could favor PS exposure.

To investigate the effect of Na^+ influx independently of pH variation, we have used gramicidin, an ionophore that induces Na^+ influx without concomitant H^+ efflux. In a Na^+ medium with or without Ca^{2+}, gramicidin induced a marked increase in $[Na^+]_i$ (Table 2). Surprisingly, in a Ca^{2+} containing-medium, PS exposure occurred in a larger percentage of platelets (68.6%) in the absence of Na^+ than in its presence (9.6%) (Table 1). This latter effect was likely due to an increase in $[Ca^{2+}]_i$. Indeed, gramicidin has only relative specificity for Na^+ influx as it induced a drastic increase in $[Ca^{2+}]_i$ in medium devoid of Na^+ (Fig. 4B). The dependence of gramicidin-induced phospholipid redistribution on Ca^{2+} influx was confirmed by the abolition of FM1–43 uptake in a medium without Ca^{2+} (Fig. 3D and Table 3). In contrast, in medium containing Ca^{2+} and Na^+, gramicidin induced an increase of $[Na^+]_i$ comparable to that induced by agonists (thrombin, TRAP or collagen) (Table 2) but in contrast no increase in $[Ca^{2+}]_{i.}$ (Fig 4B) and no PS externalization (Table 1). These data suggest that the sole increase in $[Na^+]_i$ did not induce PS externalization and that an increase in $[Ca^{2+}]_i$. and/or variation in internal pH are necessary factors, which are induced in activated platelets.

These data indicate that the effect of monensin on PS exposure can be mediated through an increase in $[Na^+]_i$ without any increase in $[Ca^{2+}]_i$. In contrast, the effect of gramicidin would require an increase in $[Ca^{2+}]_i$ which is inhibited in the presence of external Na^+, presumably as a result of a competition between Ca^{2+} and Na^+ influx. In a normal medium (Na^+ and Ca^{2+}), both ionophores induced a significant increase in $[Na^+]_i$. Remarkably, only monensin induced PS externalization. In contrast to gramicidin, only monensin induced a change in cytosolic pH [50], thus mimicking NHE activation, leading to the hypothesis that variations in cytosolic pH linked to an increase in $[Na^+]_i$ are necessary to induce PS externalization.

3.4. Implication of PIP_2 in NHE-regulated PS exposure

The activity of NHE is optimized by PIP_2 [28]. To investigate the role of PIP_2 on PS exposure and Na^+ influx through the NHE regulation, we have tested the effects of

quercetin, a modulator of PIP_2 concentration in platelets that inhibits phosphoinositide kinases and reduces phosphoinositide synthesis (^{32}P-phosphate incorporation) by 50–60% or agonist-induced increase of PIP_2 resynthesis by 70% [51]. We have also used neomycin, known for its high affinity interaction with PIP_2 [52,53] preventing PIP_2 interaction with proteins and resulting in NHE inhibition [28]. Preincubation of platelets with 100 μM quercetin or 5 mM neomycin significantly inhibited PS exposure induced by thrombin, TRAP, collagen or collagen + TRAP, (Table 1). The inhibitory effect of quercetin and neomycin on thrombin-induced PS exposure was partially reversed by a direct activation of NHE with PMA (Table 1). Quercetin and neomycin not only decreased PS externalization, but also abolished the increase in $[Na^+]_i$ induced by thrombin and TRAP (Tables 1 and 2). This inhibition was larger than that promoted by EIPA, indicating that the blocking effect of quercetin and neomycin would affect NHE and other mediator(s) of Na^+ influx. These data agree with the implication of NHE in PS externalization and suggest that PIP_2 could play a regulatory role on Na^+ influx and NHE activity. Quercetin and neomycin had no effect on PS exposure induced by monensin (Table 1), showing that this ionophore mimics NHE activation without involving PIP_2.

4. Discussion

PS exposure on the surface of platelets is essential for their ability to activate the coagulation pathway. The intracellular signals responsible for triggering PS exposure are incompletely understood, with most studies focusing on the effects of a rise in intracellular Ca^{2+} on this process. Previous studies comparing the kinetics of Ca^{2+} influx and Na^+ influx – Ca^{2+} influx being transient although Na^+ influx is prolonged – have proposed a major role for Na^+ through NHE activation, in procoagulant activity induced by collagen in porcine platelets [19] and by desmopressin in human platelets [20]. In the present study, when human platelets were activated by several physiologic agonists (thrombin, TRAP, collagen or combinations of collagen with thrombin or TRAP) or drugs that mimic agonist action, PS exposure was abolished in the absence of external Na^+ and significantly diminished by EIPA. Accordingly, even for thrombin treatment, whose proteolytic action is dependent on external Na^+ (see Results), Na^+ influx through NHE activation plays a role in PS externalization.

Classically, Ca^{2+} influx has been implicated in PS exposure in activated platelets [14,54] and cell apoptosis [55,56]. In erythrocytes, Ca^{2+} channels have been shown to be regulated by PKC and phosphatases [57] and osmotic shock, oxidative stress or glucose depletion open Ca^{2+}-permeable cation channels that induce apoptosis (cell-shrinkage and PS exposure) only partly dependent on Ca^{2+} entry [58]. Other effectors than Ca^{2+} have been implicated in phospholipids scrambling. Acidification induced phospholipid analog redistribution in inside-out vesicles from membrane erythrocytes [8,9]. Production of ceramides promotes PS externalization in erythrocytes exposed to osmotic shock [59], and oxidation of PS itself could play a role [60]. By measuring FM1–43 uptake, which reflects membrane remodeling and PS exposure independently of Ca^{2+} [31], we show that phospholipid redistribution generated by thrombin was not fully abolished by the suppression of external Ca^{2+}, suggesting that Ca^{2+} influx is not the sole effector. Surprisingly, the effect of A23187 was also partially maintained without external Ca^{2+}, an effect that can be attributed to an increase in $[Na^+]_i$ mediated by the ionophore, due to a lack of competition with Ca^{2+}. However, the deprivation of external Na^+ increased the externalization of PS induced by A23187, an effect mediated this time by a larger increase in $[Ca^{2+}]_i$, in the absence of Na^+, again resulting from the lack of competition between the two cations (Table 2) and previous reports [19,46,47]. In a similar way, in thymocytes, activation by external ATP of the purinergic P2X7 receptor, a non-specific cationic channel, induced a simultaneous Na^+ and Ca^{2+} influx, and the very early PS externalization was

predominantly generated through an effect of Na^+ [22]. Our data provide various lines of evidence for the function of NHE in Na^+-mediated PS exposure in activated platelets: (1) monensin, which mimics NHE activation by inducing an increase in Na^+ influx and $[pH]_i$ promoted PS exposure as did the physiologic agonists; (2) nigericin, which activates NHE through primary acidification, induced Na^+ influx and PS exposure; (3) EIPA, a specific inhibitor of NHE, significantly inhibited Na^+ influx and PS exposure induced by the agonists and nigericin; (4) PMA, which activates NHE through a PKC-dependent phosphorylation [61], had no effect on PS exposure by itself, but enhanced the effect of thrombin.

Activation of NHE suggests that in addition to Na^+ influx, a change in intracellular pH could be involved in the regulation of platelet PS exposure. In a previous report, the involvement of pH in procoagulant activation of porcine platelets was discarded [19]. However, only the effect of a cytosolic alkalinization was tested independently of agonist activation and consecutive variations of $[Na]_i$ and or $[Ca^{2+}]_i$. In our study, gramicidin, which triggered an increase of $[Na^+]_i$ without changing the pH, had no effect on PS externalization. In contrast, the effect of A23187, which could be partly due to Na^+ influx (see above), is potentially linked to intracellular alkalinization, as this carboxylic ionophore functions as a Ca^{2+}/H^+ exchanger [46]. The influence of pH on the effect of A23187 would deserve to be investigated. Changes in pH can modulate PS exposure by several mechanisms: (1) a direct effect of an increase in the concentration of protons as suggested from the induction of the redistribution of phospholipid analogs in IOVs by acidification [8,9]; (2) an inhibition of the aminophospholipid translocase mediated by acidic intracellular pH [62] could contribute to PS externalization, as previously shown in apoptotic cells [63]; (3) pH variations could modulate the affinity of intracellular messengers such as Ca^{2+} or Na^+ for their targets, which include scramblase or phosphoinositides [15,26,64,65]. Since NHE activation leads to a prolonged Na^+ influx coupled with alkalinization in response to platelet activation, only the latter hypothesis has to be considered in our conditions.

Polyphosphoinositide (PPI) turnover is a key signaling mechanism in platelets. During initiation of activation, PIP_2 hydrolysis leads to Ca^{2+} signaling. Next, PIP_2 resynthesis creates a new pool, which could be essential for the regulation of various processes by acting as a ligand or a cofactor in a variety of proteins. We have previously proposed that PIP_2 could serve as a target for Ca^{2+} and other effectors, or as a cofactor of scramblase to induce phospholipid redistribution [9,15,26,64,65]. NHE1, the isoform of the Na^+/H^+ exchanger presumably implicated in activated platelets [19–21], possesses two potential PIP_2-binding domains required for optimal activation [28]. Furthermore, intracellular regulation of pH has been shown to depend on PIP_2 concentration or accessibility in plasma membranes [28,66]. Pretreatment of platelets with quercetin induces a decrease in PIP_2 concentration [51], whereas neomycin, which interacts with PIP_2, affects its accessibility [52,53]. A first consequence of quercetin and neomycin is an inhibition of the agonist-induced increase in $[Ca^{2+}]_i$ [51,67] which can partly affect PS externalization. Here, quercetin and neomycin significantly inhibited Na^+ influx and PS externalization when induced by agonists, suggesting that PS redistribution could depend on PIP_2 metabolism through its regulatory role on Na^+ influx mediated by NHE activation. Interestingly, PS externalization induced by monensin which mimics NHE activation was not prevented by quercetin or neomycin. In the same way, direct activation of NHE with PMA through PKC activation, overcame the inhibitory effect of the quercetin or neomycin.

In this work, we confirm that an increase of cytosolic Na^+ concentration triggers PS exposure in activated platelets, similarly to PS exposure induced by P2X7 receptor in thymocytes [22]. Furthermore, the role of PIP_2 in PS exposure may involve modulation of Na^+ influx by regulating NHE activity. Together, these data suggest that NHE would be a

checkpoint in the regulation of PS externalization. The reason for the existence of redundant pathways and/or effectors to induce PS exposure is inextricably linked to the physio-pathological requirements of this process. The identification of these pathways represents an opportunity for future investigations to provide new targets for anti-thrombotic therapies.

Abbreviations

BSA	bovine serum albumin
EIPA	5-(N-Ethyl-N-Isopropyl) amiloride
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PS	phosphatidylserine
LUVs	large unilamellar vesicles
IP_3	inositol triphosphate
PIP_2	phosphatidylinositol 4, 5-bisphosphate
NHE	Na^+/H^+ exchanger
TRAP	thrombin receptor agonist fragment
PLSCR1	phospholipid scramblase 1

Acknowledgments

We gratefully acknowledge Dr. M. Hours from the Service de Cytometrie, Institut Federatif de Recherches 46, bat 440, Universite Paris XI for help with sample analysis using FACS. This study was supported by NIH grant AR38910.

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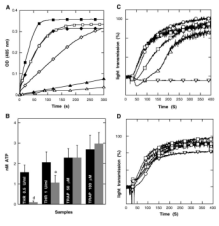

Fig. 1.

Na^+-dependence of thrombin proteolytic activity (panel A). Filled and empty symbols represent thrombin activity in Na^+-containing and Na^+-free buffer, respectively, with equal concentration of chromozym substrate and after thrombin addition at a concentration 0.1 U/ml (triangles) 0.5 U/ml (diamonds) and 1 U/ml (squares). Data shown are representative of three independent experiments. Na^+-dependence of platelet secretion after addition of thrombin (THR) or TRAP was evaluated by monitoring ATP release (panel B). Black and gray columns represent Na^+-containing and Na^+-free buffers, respectively. Data are means ±S.D. of 3–4 experiments ($^aP<0.05$; $^dP<0.001$ vs. the respective value in Na^+-containing buffer). Na^+-dependence of platelet aggregation expressed as the percentage of light transmission in suspensions activated with thrombin (panel C) or TRAP (panel D). Platelet aggregation was induced with 0.1 U/ml thrombin or 10 μM TRAP (triangles up—with extracellular Na^+, triangles down without extracellular Na^+), 0.5 U/ml thrombin or 50 μM TRAP (squares—with extracellular Na^+, pointed squares—without extracellular Na^+), 1 U/ml thrombin or 100 μM TRAP (circles—with extracellular Na^+, pointed circles—without extracellular Na^+). Data shown are representative of three independent experiments.

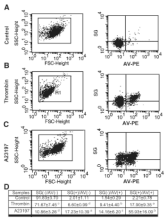

Fig. 2.

Simultaneous analysis of PS exposure and Na^+ influx in sodium green (SG) loaded platelets (suspended in buffer A supplemented with 2 mM $CaCl_2$) incubated for 10 min with annexin V-PE (AV-PE), after activation with thrombin (1 U/ml) (row B) or calcium ionophore (A23187; 5 μM) (row C). Data shown are representative of 4–6 independent experiments. Percentage of positive cells in each quadrant (row D). SG (+) Na^+-positive, AV (+)-annexin V-PE positive ($^aP<0.05$; $^bP<0.02$; $^cP<0.01$; $^dP<0.001$ vs. the respective value in control).

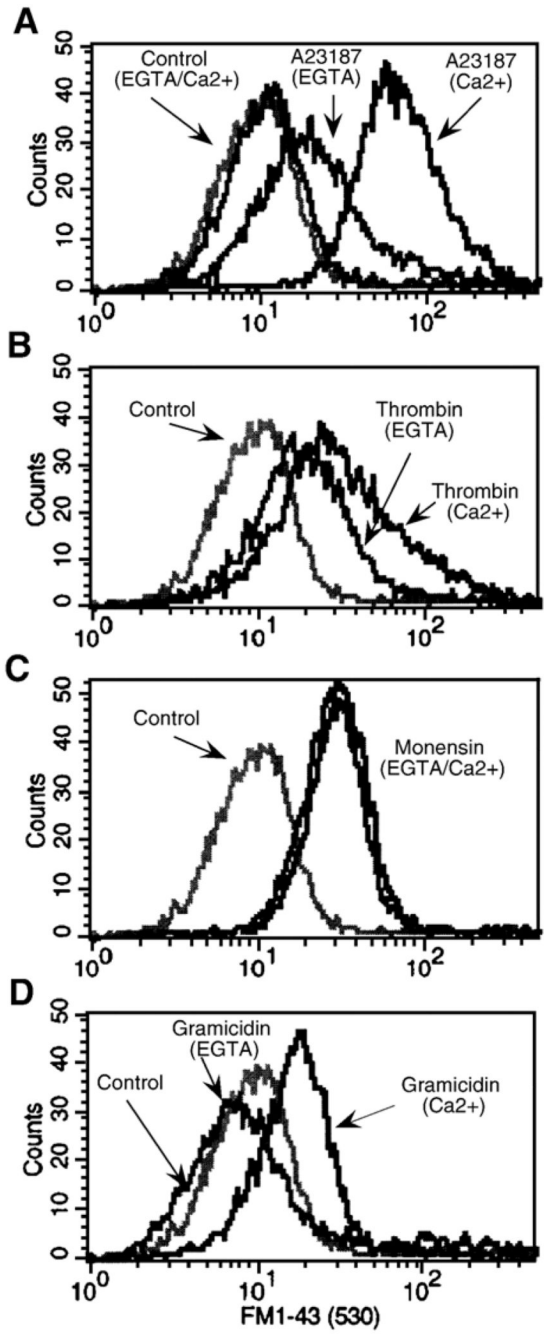

Fig. 3.

Evaluation of PS exposure in calcium-containing (2 mM) and calcium-free buffers (0.1 mM EGTA), 20 min after platelet treatment with 2 μM A23187 (panel A),1 U/ml thrombin (panel B), 50 μM monensin (panel C) and 50 μM gramicidin (panel D). Remodeling of plasma membrane was assessed from FM1–43 staining. Data shown are representative of four independent experiments.

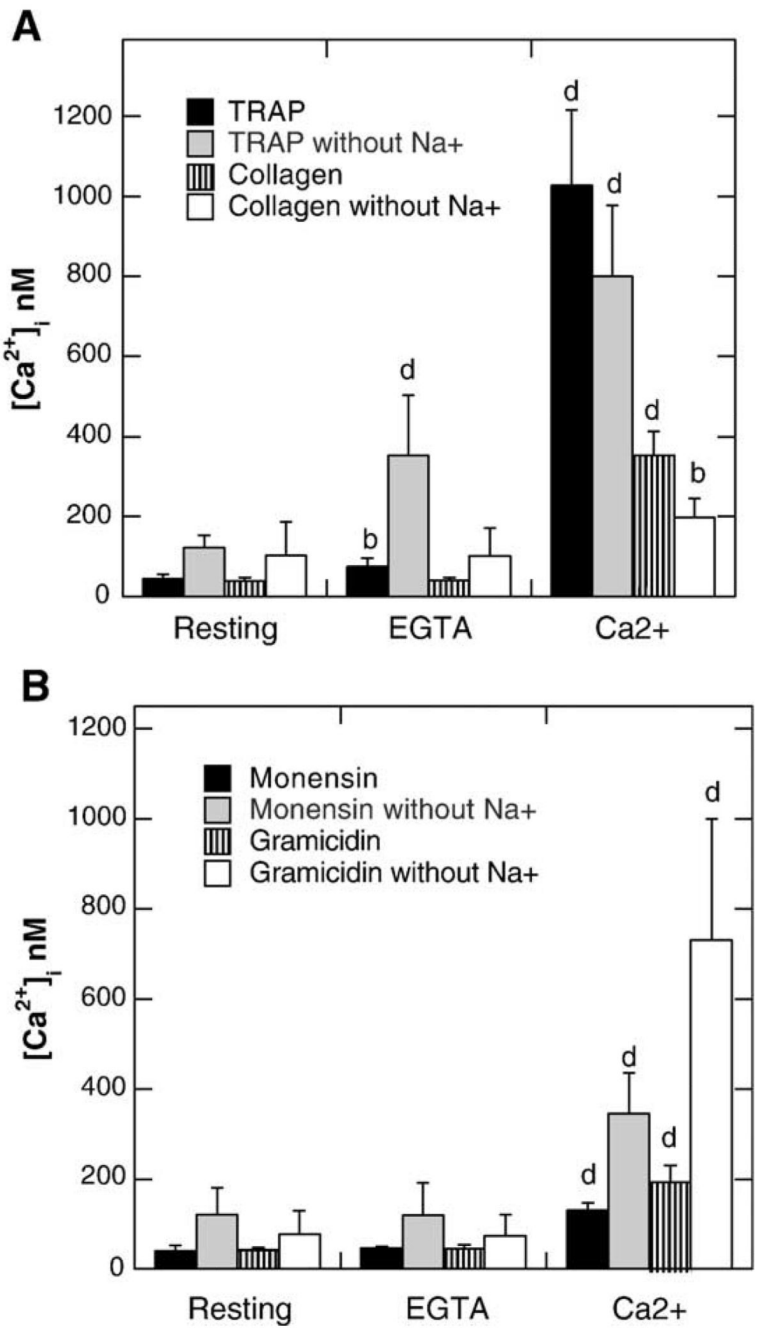

Fig. 4.

Evaluation of intracellular calcium concentration in platelets loaded with Fura-2 and suspended in buffer A supplemented with 2 mM [Ca^{2+}] (right and left columns) or 0.1 mM EGTA (center columns). Changes in $[Ca^{2+}]_i$ after activation with TRAP, collagen (panel A), 50 μM gramicidin or 50 μM monensin (panel B). Data are means±S.D. of 4–5 experiments ($^{b}P<0.02$; $^{d}P<0.001$ vs. the respective value in control).

Table 1

Annexin V-FITC positive platelets (% of CD41-PE positive), suspended in media with (buffer A) or without Na (buffer B) after treatment with different agonists for 20 min at 37 °C

Agonist	Buffer A (Control)	Buffer B	Buffer A+EIPA 30 μM	Buffer A+Quercetin 100 μM	Buffer A+Neomycin 5 mM
Control	1.2±0.4	1.7±0.3	1.7±1.3	1.7±1.0	1.2±0.3
Thrombin 0.1 U/ml	7.6±3.1	2.7±0.7^{b}	ND	ND	ND
Thrombin 1U/ml	30.1±7.9	2.8±0.8^{d}	11.7±6.1^{b}	11.0±2.7^{c}	3.0±1.5^{c}
TRAP 100 μM	11.6±4.6	2.9±0.8^{b}	5.5±3.4^{a}	5.7±3.6^{a}	4.4±3.5^{a}
Collagen 20 μg/ml	15.8±4.9	2.6±0.6^{d}	8.1±1.2 ^{b}	9.2±2.3^{a}	7.1±2.9^{b}
TRAP+Collagen	36.0±4.9	6.7±2.1^{d}	13.4±4.9^{c}	16.1±1.7^{d}	12.5±2.0^{d}
Thrombin 1U/ml+Collagen	39.7±7.8	ND	29.9±4.3^{a}	ND	ND
PMA 50 nM	3.9±1.6	1.9±0.9^{a}	ND	3.1±2.3	3.3±1.1
Thrombin 0.1 U/ml+PMA	14.7±4.5	4.8±2.3^{a}	ND	ND	ND
Thrombin 1U/ml+PMA	35.4±3.5	6.4±3.7^{d}	ND	21.2±6.4^{b}	11.6±2.7^{d}
Nigericin 10 μM	28.3±9.1	5.8±3.0^{c}	8.4±4.2^{a}	ND	ND
Monensin 50 μM	50.2±11.4	45.2±5.1	ND	50.4±21.9	44.9±9.4
Gramicidin 50 μM	9.6±9.1	68.6±10.1^{d}	ND	ND	ND
A 23187 2 μM	70.2±11.3	91.2±7.8	ND	ND	ND

Data are means±S.D. of 4–8 experiments ($^{a}P<0.05$; $^{b}P<0.02$; $^{c}P<0.01$; $^{d}P<0.001$ vs. the respective value in control) ND: not determined.

Table 2

Mean fluorescence intensities of sodium green indicator in platelets (CD41-PE positive) activated by different agonists for 20 min at 37 °C

Agonist	Buffer A/Ca^{2+}	Buffer A/EGTA	Buffer A/Ca+30 μM EIPA	Buffer A/Ca+100 μM Quercetin	Buffer A/Ca+5 mM Neomycin
Control	18.9±1.7	19.4±1.4	18.7±0.6	16.6±2.6	19.5±2.2
Thrombin 1 U/ml	34.9±2.5	32.8±1.8	28.3±4.4	23.3±1.4^{d}	23.4±3.1^{c}
TRAP 100 μM	32.8±3.1	27.9±1.3	22.8±1.6^{c}	20.6±2.3^{c}	24.5±4.0^{a}
Collagen 20 μg/ml	24.1±0.9	26.1±0.3	20.9±0.6^{c}	16.6±0.3^{d}	(22.1)^{e}
TRAP+Collagen	36.7±5.4	29.6±4.5	24.3±1.1^{b}	20.7±1.3^{c}	(27.8)^{e}
Nigericin 10 μM	34.9±2.7	ND	21.8±3.0^{c}	ND	ND
Monensin 50 μM	38.7±3.9	32.1±2.0^{a}	34.4±2.2	28.2±9.5	34.5±2.0
Gramicidin 50 μM	28.9±3.0	28.4±2.4	30.9±2.0	24.3±7.4	29.5±2.3
A23187 2 μM	38.1±9.9	51.8±1.5	ND	ND	ND

Data are means±S.D. of 4 experiments ($^{a}P<0.05$; $^{b}P<0.02$; $^{c}P<0.01$; $^{d}P<0.001$ vs. the respective value in buffer A/Ca^{2+}). ND: not determined, ()^{e}—means of two experiments.

Table 3

Mean fluorescence values (Geo mean) for data from Fig. 3

Samples	EGTA	Ca^{2+}
Control	2.70±0.24	2.78±0.42
A23187	9.90±2.92[c]	29.55±6.27[d]
Thrombin	6.81±0.91[d]	14.8±7.05[a]
Monensin	12.04±3.78[d]	10.38±5.14[a]
Gramicidin	2.86±0.54	6.18±1.28[c]

Data are means±S.D. of 4 experiments ([a] $P<0.05$; [c] $P<0.01$; [d] $P<0.001$ vs. the respective value in control).