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Research Article

Direct inhibition of phospholipid scrambling activity in erythrocytes by potassium ions

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Abstract. The exposure of phosphatidylserine (PS) at the cell surface plays a critical role in blood coagulation and serves as a macrophage recognition moiety for the engulfment of apoptotic cells. Previous observations have shown that a high extracellular [K^+] and selective K^+ channel blockers inhibit PS exposure in platelets and erythrocytes. Here we show that the rate of PS exposure in erythrocytes decreases by ~50% when the intracellular [K^+] increases from 0 to physiological concentrations. Using resealed erythrocyte membranes, we further show that lipid scrambling is inducible by raising the intracellular [Ca^{2+}] and that K^+ ions have a direct inhibitory effect on this process. Lipid scrambling in resealed ghosts occurs in the absence of cell shrinkage and microvesicle formation, processes that are generally attributed to Ca^{2+}-induced lipid scrambling in intact erythrocytes. Thus, opening of Ca^{2+}-sensitive K^+ channels causes loss of intracellular K^+ that results in reduced intrinsic inhibitory effect of these ions on scramblase activity.

Keywords. Phospholipid scramblase, potassium ions, erythrocytes, resealed ghosts, phosphatidylserine, morphology.

Introduction

Numerous studies have revealed the involvement of several lipid transporters in the mechanisms that control transbilayer lipid distribution in eukaryotic cells [1, 2]. The concerted action of an inward-directed aminophospholipid translocase and a less specific outward-directed floppase are thought to be responsible for maintaining the dynamic equilibrium distribution of phospholipids observed in the plasma membrane of quiescent cells. Activation of a putative protein, coined phospholipid 'scramblase', dissipates normal membrane lipid asymmetry by facilitating the bidirectional transbilayer movement of all phospholipid classes irrespective of the composition of the polar head group. The most pronounced effect of the perturbed lipid asymmetry is the appearance of the negatively charged phospholipid, phosphatidylserine (PS), in the external membrane bilayer leaflet. Once externalized, PS provides a platform for the assembly of enzyme complexes that regulate the propagation of coagulation [3] and serves as a 'death signal' for the recognition and ultimate phagocytosis of apoptotic

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cells [4]. In addition, a recently identified non-apoptotic form of PS exposure has been suggested to play a role in "inside-out" signaling in immune cells in response to pathogens [5].

Vessel wall injury causes platelets to interact with collagen and thrombin, which leads to scramblase activation resulting in surface exposure of PS. This enables the assembly of the tenase and prothrombinase complexes that ensures propagation of coagulation and rapid arrest of bleeding [3]. Compromised scramblase activity results in a bleeding disorder characteristic of Scott syndrome patients [6]. Although the activation pathways for scramblase activity may differ between cell types, a key downstream event for its activation seems to be sustained elevation in the intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, that occurs through the release of Ca^{2+} from intracellular stores and/or the influx of extracellular Ca^{2+}.

Despite considerable efforts, the identity of the scramblase has yet to be elucidated. Scramblase activity was demonstrated in proteoliposomes reconstituted from a membrane protein fraction isolated from platelets [7] and erythrocytes [8]. Although the protein from erythrocytes has been cloned [9], an increasing body of evidence suggests that the putative human phospholipid scramblase (hPLSCR) is not responsible for the rapid transbilayer migration of lipids in apoptotic cells or in activated blood cells [6, 10, 11].

We have recently obtained evidence that efflux of K^+ ions through Ca^{2+}-activated K^+ channels plays a regulatory role in PS exposure in platelets [12]. Selective K^+ channel blockers or increased levels of extracellular K^+, $[K^+]_e$, inhibit the procoagulant response of platelets to collagen and thrombin. Similarly, Ca^{2+}-induced PS exposure in erythrocytes is inhibited by K^+ channel blockers or elevated $[K^+]_e$ [13]. While it is possible that blocking K^+ efflux indirectly inhibits Ca^{2+} influx, we have not been able to clearly demonstrate a decreased Ca^{2+} response in platelets treated with K^+ channel blockers [12]. This raised the question as to whether scramblase activity is under direct control of the intracellular $[K^+]$, $[K^+]_i$.

To address this issue, we used erythrocytes and resealed erythrocyte ghosts as a model to study the relationship between Ca^{2+}-dependent scramblase activity and $[K^+]_i$. We show that K^+ specifically inhibits Ca^{2+}-dependent scramblase activity by a mechanism that is independent of changes in cell morphology and the formation of microvesicles.

Material and methods

Reagents. Ionomycin, charybdotoxin, clotrimazol and valinomycin were obtained from Sigma (St Louis, MO). The coagulation proteins prothrombin, factor Xa and factor Va were purified from bovine blood as described before [14]. Thrombin-specific chromogenic substrate, S2238, was obtained from Chromogenix (Mölndal, Sweden). Fluorescein isothiocyanate (FITC)-conjugated annexin A5, Fluo-3/AM, Rhod-2/AM and fluorescein-5 hydroxysuccinimide ester were from Invitrogen (Leiden, the Netherlands).

Isolation of erythrocytes. Human blood was obtained from healthy volunteers by venepuncture and collected in acid-citrate-dextrose in a ratio of 5 to 1 (v/v). Erythrocytes were washed at room temperature by centrifugation at 200 *g* for 10 min using buffer A (HEPES buffer: 10 mmol/l HEPES, 136 mmol/l NaCl, 2.7 mmol/l KCl, 2 mmol/l $MgCl_2$, 0.2 mmol/l EGTA, 5 mmol/l glucose, pH 7.4). Cells were resuspended in buffer A; cell count was adjusted at 10^8/ml using a Coulter counter (Beckman Coulter, Miami, FL).

Preparation of resealed ghosts. Washed erythrocytes were lysed by adding 19 vol ice-cold lysis buffer (1 mmol/l HEPES, 14 mmol/l NaCl, 0.3 mmol/l KCL, 2 mmol/l $MgCl_2$, 0.2 mmol/l EGTA, pH 7.4) to 1 vol packed cells (approx. 10^{10}/ml). After 30 min on ice, membranes were isolated (30 000 *g*, 15 min, 4°C) and washed once with 20 vol ice-cold lysis buffer. Isotonicity was restored by addition of 0.1 vol 'reseal' buffer (100 mmol/l HEPES, 1400 mmol/l KCl or NaCl, 2 mmol/l $MgCl_2$, 0.2 mmol/l EGTA, 50 mmol/l glucose, pH 7.4). After 30-min incubation at 37°C, resealed ghosts were collected by centrifugation at 2200 *g* for 10 min and subsequently washed three times in isotonic HEPES buffer with either Na^+ or K^+ or, when indicated, mixtures of both cations. The concentration of resealed ghosts was adjusted with buffer A to 10^8/ml using a Coulter counter (Beckman Coulter). Ghosts were kept on ice until use.

Prothrombinase assay and rate of lipid scrambling (scramblase activity). The rate of conversion of prothrombin to thrombin by the enzyme complex factor Xa-factor Va has been shown to be a convenient, rapid and sensitivity method to monitor in a semi-quantitative manner the extent of exposure of PS at the outer cell surface [15]. Briefly, samples taken from10^8/ml erythrocytes or resealed ghosts were diluted tenfold in buffer A and incubated at 37°C with factor Xa and factor Va at a final concentration of 0.2 and 2 nmol/l, respectively, for 2 min in the presence of 3 mmol/l $CaCl_2$. The reaction was started by addition of pro-

thrombin (final concentration 1 μmol/l); 2 min after addition of prothrombin, a sample from the incubation mixture was transferred to a cuvette containing 1 ml buffer composed of 50 mmol/l Tris, 120 mmol/l NaCl and 2 mmol/l EDTA pH 7.4. The amount of thrombin formed was determined by measuring the change in absorbance at 405 nm caused by hydrolysis of S2238 (0.2 mmol/l).

The time course of PS exposure (lipid scrambling) in erythrocytes or erythrocyte ghosts was determined by analyzing samples taken at different times and assaying for prothrombinase activity. As shown by Wolfs et al. [16], the scramblase activity in blood cells from different donors varies significantly. Therefore, the time courses presented in the figures are from single experiments that are representative of at least three identical experiments with erythrocytes obtained from different donors. Prothrombinase activity of erythrocytes from various healthy donors demonstrated that maximal activity required ~40–60 min of treatment with ionomycin. Therefore, activity measured after 15-min ionomycin treatment was chosen to reflect the rate of PS exposure, *i.e.*, the scramblase activity.

Mean cellular volume and intracellular K^+ content. Mean cellular volume (MCV) of erythrocytes and ghosts was determined using a Coulter ^{R} A^{C}.T diff ^{TM} Analyzer (Beckman Coulter). [K^+] of various buffers used in the experiments was determined using an IL 943 Flame photometer (Instrumentation Laboratory, Ramsey, MN). Osmolarity measurements were performed with a Osmomat 030 (Gonotek GmbH, Berlin, Germany). K^+ efflux of erythrocytes was determined by analyzing the [K^+] in the supernatant of 10^8 erythrocytes/ml.

Measurement of cytosolic Ca^{2+}. RBCs (1 % hematocrit, hct) were incubated for 1 h at 37°C with Rhod-2/am (5 μmol/l). The cells were then washed with buffer A and resuspended in the same buffer. RBC suspension (2 ml) was transferred to a fluorometer cuvette and incubated for the indicated time with ionophore (5 μmol/l). Briefly, Rhod-2 fluorescence (λ_{Ex}=552 nm, λ_{Em}=581 nm) was monitored following the addition of increasing [$CaCl_2$] from a 100 mmol/l stock solution. The increase in relative fluorescent intensity was recorded 5 min after each addition.

Assessment of ionophore/Ca^{2+}-induced vesiculation in intact RBC and resealed RBC ghosts. Intact RBCs were labeled for 2 h at 4°C with the *N*-hydroxysuccinimide ester of fluorescein-5 (250 μmol/l). The cells were then washed with 2 % BSA in buffer A to remove unlabeled reagent and resuspended in buffer A without BSA. To assess intact RBC, fluorescein-labeled RBC (2 % hct) were incubated for 1 h at 37°C with 1 mmol/l Ca^{2+} in the absence (control) or presence of ionophore. The cells were then centrifuged at 2500 *g* for 5 min and residual fluorescence in the supernatant was measured (λ_{Ex}=488 nm/λ_{Em}=525 nm). Data were expressed as the fraction of total fluorescence in the suspension before centrifugation. To assess the resealed RBC ghosts, fluorescein-labeled RBCs were lysed with hypotonic buffer (1:10 diluted buffer A), centrifuged at 30 000 *g* for 15 min at 4°C and washed twice with the same buffer. The ghosts were then resealed by bringing the suspension to iso-osmolarity by adding 0.1 vol of 'reseal' buffer followed by incubation for 30 min at 37°C. Resealed ghosts were collected by centrifugation at 2500 *g* for 5 min. The resealed ghosts were then incubated with Ca^{2+} in the absence or presence of ionophore, and the fluorescence in the supernatants assessed as described for intact RBC.

Morphology. For morphological analysis, cells or ghosts were diluted ten times in 1 % glutaraldehyde in buffer A. After fixation for 15 min at 4°C, the cells were washed in distilled water and subsequently taken through a graded series of ethanol and finally resuspended in absolute ethanol. Cells were deposited on plastic cover slips, air dried and sputter-coated with gold. Scanning electron micrographs were made using a Phillips CM-12 electron microscope at 15 kV (Phillips, Eindhoven, the Netherlands).

Results

Lipid scrambling in intact erythrocytes: Effect of $[K^+]_i$. Figure 1A shows that lipid scrambling can be induced in erythrocytes by treatment with the calcium ionophore, ionomycin, in the presence of extracellular Ca^{2+}. Scrambling is accompanied by an efflux of K^+ (Fig. 1B) and concomitant loss of Cl^- and water [13]. Charybdotoxin, isolated from the venom of the scorpion *Leiuris quinquestriatus*, blocks the Gardos channel, the intermediate conductance Ca^{2+}-activated K^+ channel [17–19]. This inhibitor reduced the efflux rate of K^+ ions (Fig. 1B, open circles) and the rate at which cells become PS positive as measured by the development of prothrombinase activity (Fig. 1A, open circles). Similar results were obtained with the antifungal agent, clotrimazol, another blocker of the Gardos channel [18, 19] (data not shown). Inhibition of scramblase activity achieved by blocking K^+ efflux was rescued by allowing K^+ efflux through the addition of valinomycin (Fig. 1, open squares). In the presence of valinomycin, the rate of PS exposure

induced by ionomycin is enhanced and, as expected, the efflux of K^+ ions is nearly instantaneous (Fig. 1A, B, closed squares). Importantly, treatment of erythrocytes with valinomycin in absence of ionomycin does not lead to the generation of PS-positive cells (open triangles), underscoring the essential requirement for Ca^{2+}. To confirm that varying levels of intracellular K^+ and/or valinomycin did not affect ionophore/Ca^{2+}-mediated increase in $[Ca^{2+}]_i$, thus causing changes in scramblase activity, Ca^{2+} levels were monitored with Rhod-2 in the presence and absence of valinomycin. Figure 2 shows that ionomycin-induced increases in $[Ca^{2+}]_i$ were identical in the presence or absence of valinomycin. Similar results were obtained in the presence of high $[K^+]_e$ or K^+ channel blockers (data not shown). These results indicate that scramblase activity is directly dependent on $[Ca^{2+}]_i$ and strongly suggest that an association exists between the rate of Ca^{2+}-dependent PS exposure and $[K^+]_i$. Support for this relationship is provided by the data shown in Figure 3, where the rate of scrambling was determined as a function of $[K^+]_i$. This was done by equilibrating erythrocytes for 15 min in buffers containing varying $[K^+]$ in the presence of valinomycin, prior to treatment with Ca^{2+}-ionophore. The results (Fig. 3A) indicate that scramblase activity, determined as the fraction prothrombinase activity developed after 15-min treatment with ionomycin, decreases with increasing $[K^+]$ up to ~0–30 mmol/l K^+. Similar conclusions were reached when the effect of high K^+ on the scramblase process was assessed with fluorescent-labeled annexin A5 instead of the prothrombinase assay (data not shown).

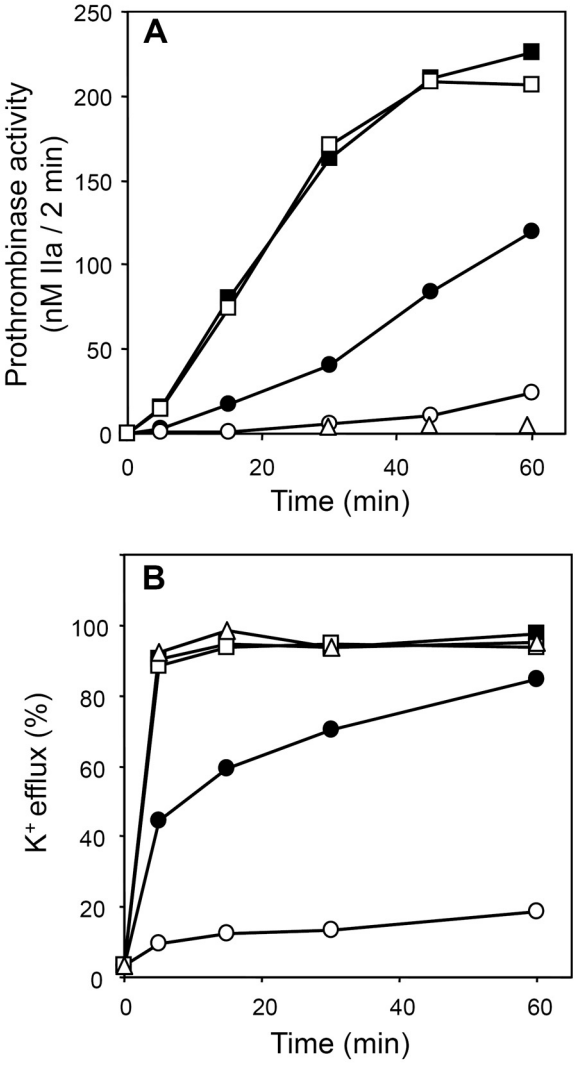

Figure 1. Ionomycin induced phosphatidylserine (PS) exposure and K^+ efflux in intact erythrocytes: inhibition by the K^+ channel blocker charybdotoxin and stimulation by the K^+ ionophore valinomycin. (*A*) Erythrocytes (10^8/ml) were preincubated with charybdotoxin (20 nmol/l) and/or valinomycin (3 μmol/l) for 15 min prior to treatment with ionomycin (5 μmol/l) in the presence of $CaCl_2$ (1 mmol/l) in buffer A ($[K^+]$ 2.7 mmol/l). At different times, samples were analyzed for prothrombinase activity to evaluate the extent of PS exposure. (*B*) Ionomycin-induced K^+ efflux in intact erythrocytes was measured by analyzing the K^+ content of the supernatant using a flame photometer. Under the experimental conditions used, $[K^+]$ in the supernatant appeared constant after 15 min pre-incubation with valinomycin (3 μmol/l); this value was set at 100 %. Symbols indicate: ● no additions; ○ charybdotoxin; ■ valinomycin; □ charybdotoxin + valinomycin; Δ valinomycin without ionomycin. Data presented are from a single representative experiment out of three identical experiments performed with cells obtained from different donors.

Lipid scrambling in intact erythrocytes: Effect of swelling and shrinkage. Depending on the $[K^+]_e$, loss or uptake of K^+ ions is likely accompanied by loss or uptake of Cl^- ions and subsequent water transport causing cell shrinkage or cell swelling, respectively. As shown in Figure 3A (inset), the MCV of the erythrocytes varies from 71 to 94 fl when the cells are incubated with valinomycin at $[K^+]_e$ from 0 to 140 mmol/l. In the absence of valinomycin, the MCV is 87 fl, irrespective the $[K^+]_e$. Considering that a change in cellular volume could affect the physical properties of the membrane and as a result the scrambling process, the rate of lipid scrambling was measured as a function of the MCV. This was done by preincubating cells in buffers of different osmolarity prior to inducing lipid scrambling with ionomycin and Ca^{2+}. Osmolarity was altered by changing only the [NaCl], keeping the concentration of the other buffer components constant. The results presented in Figure 3B illustrate that for cells in hypotonic medium (cell lysis was always <1 %), *i.e.*, in swollen cells, the rate of PS exposure is significantly enhanced compared to that of cells in isotonic medium; conversely, in hypertonic medium, scramblase activity is reduced.

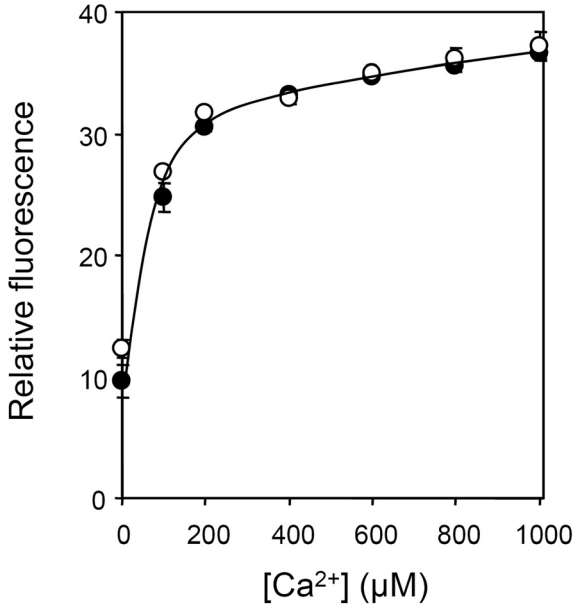

Figure 2. Effect of intracellular K^+ on ionophore-dependent Ca^{2+} influx. Rhod-2 labeled RBC in buffer A (low K^+) were incubated with ionomycin alone (●) or with ionomycin and valinomycin (○) as described in the experimental procedures section. The cells were then incubated with increasing amounts of Ca^{2+} and the change in fluorescence intensity was assessed after 5 min.

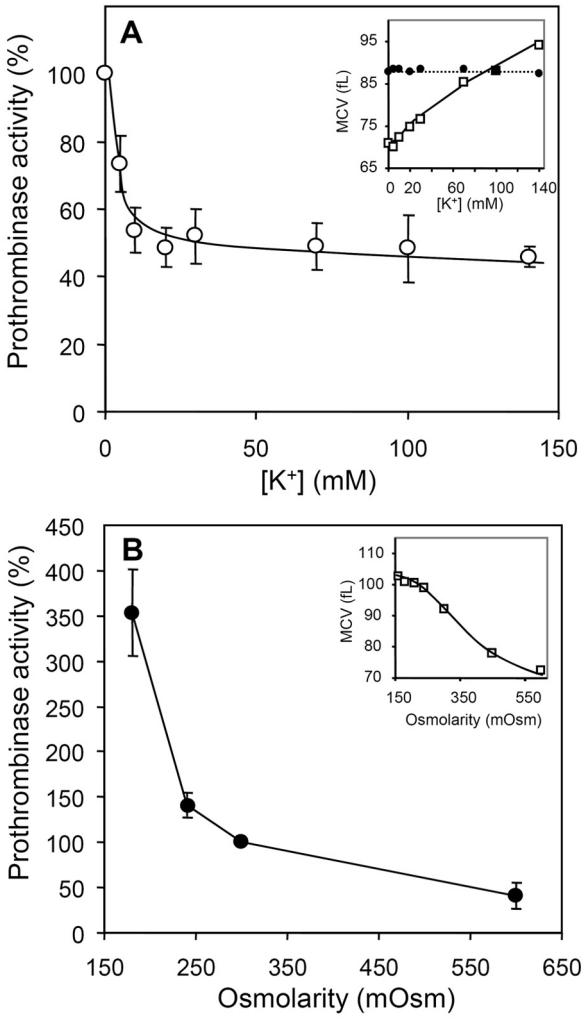

Figure 3. Ionomycin-induced PS exposure (scramblase activity) in intact erythrocytes as a function of the $[K^+]_i$ and the osmolarity. (*A*) $[K^+]_i$ was modulated by equilibrating cells (10^8/ml) in buffers containing graded $[K^+]$ in the presence of valinomycin (3 μmol/l) for 15 min prior to treatment with ionomycin (5 μmol/l) and $CaCl_2$ (1 mmol/l). Scramblase activity was assessed by measuring prothrombinase activity 15 min after the addition of ionomycin. The prothrombinase activity measured at zero K^+ was set at 100%. Inset: mean cellular volume (MCV) as a function of the $[K^+]$, determined before (●) and 15 min after equilibration with valinomycin (□). (*B*) Different osmolarities were obtained by changing [NaCl] in buffer A. The $[K^+]$ was maintained at 3 mmol/l. Scramblase activity was induced with ionomycin and $CaCl_2$ and measured as described for (*A*). The scramblase activity determined at 300 mOsm was set at 100%. Inset: MCV as a function of the osmolarity. For both insets, MCVs were determined before treatment with ionomycin/Ca^{2+}. Error bars represent SD (n=3).

This observation is particularly interesting since it suggests that cell size alone dramatically influences scrambling activity. Because of this, accurate determination of the relationship between PS exposure (prothrombinase activity at 15 min) and intracellular K^+ (as depicted in Fig. 3A) needs to be corrected for MCV. Thus, cells incubated with valinomycin at low $[K^+]_e$, will lose K^+ and, as a consequence, water, which will lead to cell shrinkage. Under these conditions, the rate of ionomycin-induced lipid scrambling is suppressed. On the other hand, at high $[K^+]_e$, uptake of K^+ and water will cause the cells to swell and lipid scrambling will be enhanced. Taking into consideration MCV, this suggests that the prothrombinase activity at zero K^+ is underestimated by ~50%, and the prothrombinase activity at 140 mM K^+ is overestimated by ~50%. Taken together, these data suggest a much stronger dependence of scrambling activity on $[K^+]_i$ than actually shown in Figure 3.

Lipid scrambling in resealed erythrocyte ghosts: Effect of K^+ and Na^+ ions. The K^+ content of intact erythrocytes affects the intracellular pH [20]. To explore the relationship between $[K^+]$ and scramblase activity in a pH-independent system, we built upon earlier observations with resealed erythrocyte ghosts. These studies showed that as long as the original membrane lipid asymmetry is maintained (by ensuring that the free $[Ca^{2+}]$ is kept low during the lysis and resealing procedures), Ca^{2+}-dependent scramblase activity can be demonstrated in resealed erythrocyte ghosts [21]. The rates of PS exposure induced by ionomycin and Ca^{2+} in ghosts resealed in high K^+ or in high Na^+ medium are shown in Figure 4A. The prothrombinase assay shows that the initial rate of

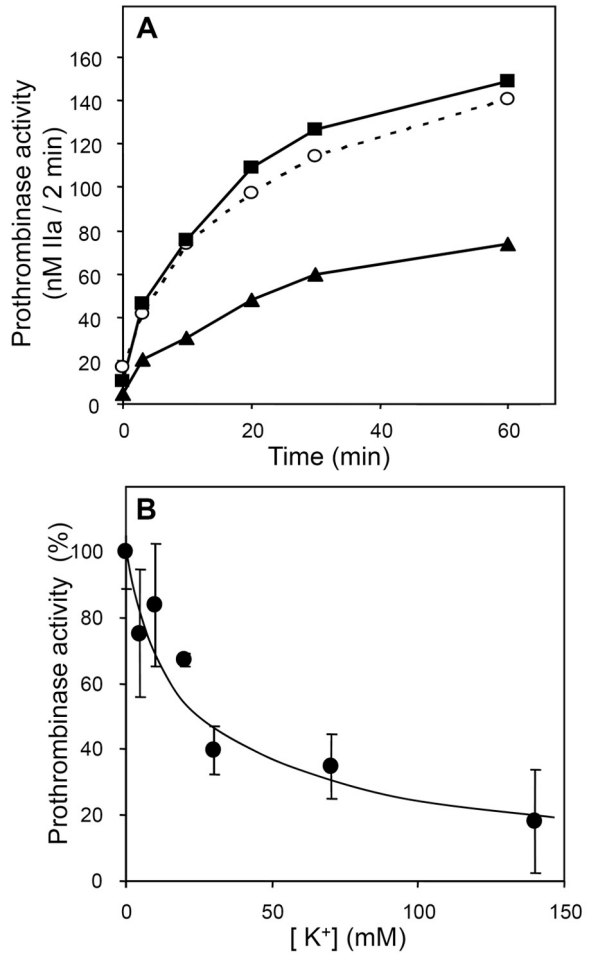

Figure 4. Ionomycin-induced prothrombinase activity in resealed erythrocyte ghosts: effect of monovalent cations. (*A*) Ghosts (10^8/ml), resealed in the presence of HEPES buffer containing 140 mmol/l Na^+-, K^+- or CholCl (choline chloride), were treated with ionomycin (3 μM) in the presence of $CaCl_2$ (1 mmol/and prothrombinase activity (*i.e.*, PS exposure) was monitored in time. ■, ghosts resealed in Na^+ buffer; ▲, resealed in K^+ buffer; ○, resealed in CholCl buffer. Data presented are from a single representative experiment out of three identical experiments performed with erythrocytes obtained from different donors. (*B*) Ghosts were resealed in HEPES buffers with varying $[K^+]$, in which osmolarity was kept constant at 300 mOsm by compensating with NaCl. Scramblase activity was assessed by measuring prothrombinase activity after 15-min treatment with ionomycin in the presence of 1 mmol/l $CaCl_2$. Error bars represent SD (n=3).

PS exposure for Na^+ ghosts is at least twofold higher than that for K^+ ghosts. Ghosts prepared in choline chloride display kinetics of PS exposure similar to the Na^+ ghosts (Fig. 4A, dashed line), indicating that K^+ ions inhibit scramblase as opposed to Na^+ ions stimulating scramblase. The effect of K^+ ions was further detailed by determining the rate of scramblase activity as a function of the $[K^+]$. Figure 4B shows the prothrombinase activity induced by 15-min ionomycin treatment of ghosts prepared in buffers containing graded concentrations of Na^+ and K^+. The inhibitory

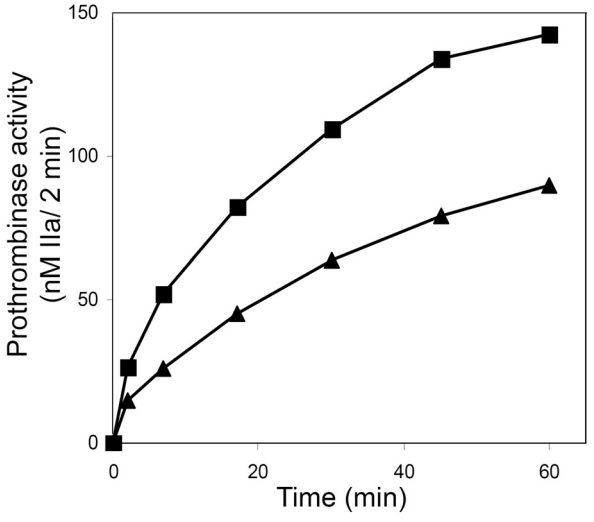

Figure 5. Time course of development of prothrombinase activity in ghosts resealed in the presence of Ca^{2+} and either K^+ or Na^+. Ghosts were resealed in HEPES buffer with 140 mmol/l K^+ (▲) or Na^+ (■), both in the presence of $CaCl_2$ (1 mmol/l) at 0°C. Scramblase activity was started by increasing the temperature to 37°C and monitored by prothrombinase activity. For details, see 'Materials and methods'. Data presented are from a single representative experiment out of three identical experiments performed with erythrocytes obtained from different donors.

effect is observed over the whole range of K^+ concentrations. It should be noted that unlike the results obtained for intact erythrocytes, the electrolyte concentrations inside and outside the ghosts are identical. The observed effects, therefore, are not related to cellular volume changes due to ion fluxes across the membrane.

To rule out the possibility that a high $[K^+]$ or $[Na^+]$ indirectly influenced the differences in scrambling activity by affecting the final $[Ca^{2+}]_i$ induced by ionomycin, an experiment was designed to avoid movement of Ca^{2+} ions across the membrane. To accomplish this, erythrocyte ghosts were resealed at 0°C in the presence of 1 mmol/l Ca^{2+} together with KCl or NaCl to restore osmolarity to 300 mOsm [although the reseal process is enhanced at 37°C, resealing also occurs upon prolonged incubation (120 min) at 0°C]. Figure 5 shows that membrane lipid asymmetry in both ghost preparations is preserved when kept at 0°C (time zero). Upon incubation at 37°C, however, prothrombinase activity increases with time indicating activation of scramblase. The results show that the rate of PS exposure in Ca^{2+}-containing ghosts resealed with K^+ is significantly lower (~50%) than in ghosts resealed with Na^+.

Morphology of erythrocyte ghosts during the process of lipid scrambling. Treatment of erythrocytes with Ca^{2+}-ionophore causes shedding of membrane vesi-

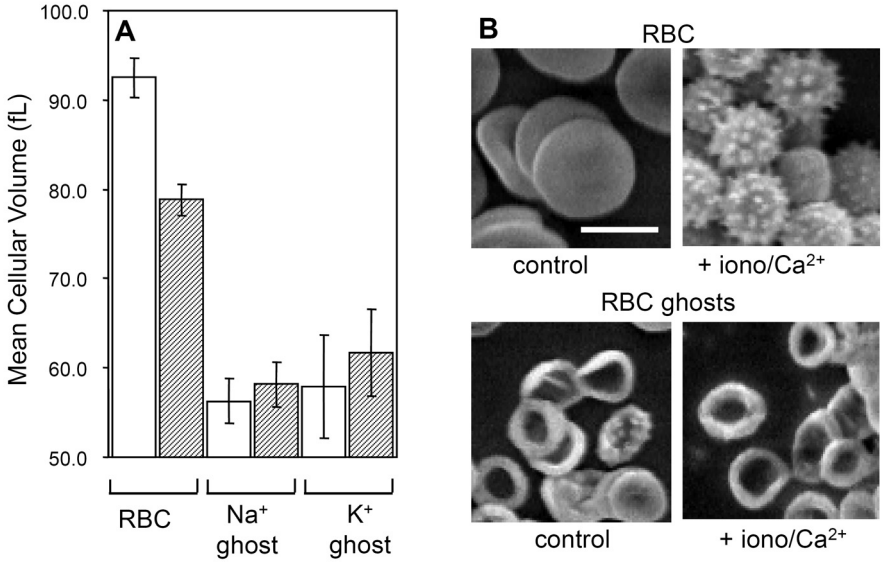

Figure 6. Morphological changes in erythrocytes and resealed ghosts upon treatment with Ca^{2+} ionomycin. (*A*) MCV of erythrocytes and erythrocyte ghosts before (white bars) and after (hatched bars) treatment with ionomycin (iono) in the presence of Ca^{2+} (1 mmol/l). Ghosts were resealed in HEPES buffer with 140 mmol/l Na^+ or 140 mM K^+, and subsequently washed and resuspended in the same high Na^+- and K^+-containing HEPES buffer, respectively, thus excluding monovalent cation fluxes resulting from ionomycin treatment. For details see 'Materials and methods'. Presented are mean values ± SD from three independent experiments. (*B*) Scanning micrographs of erythrocytes and erythrocyte ghosts before and after treatment with ionomycin. All micrographs are at the same magnification. Bar represents 5 μm.

cles and concomitant cell shrinkage that results in the formation of spherocytes. Increase of the $[Ca^{2+}]_i$ will cause opening of Ca^{2+}-dependent K^+ channels, which, depending on its concentration gradient, will result in efflux or influx of K^+ ions accompanied with efflux or influx of Cl^- ions and water. Indeed, we could confirm cell shrinkage by measuring the MCV of intact erythrocytes treated with Ca^{2+}-ionophore in the presence of 5 mmol/l extracellular K^+ (Fig. 6A). Alternatively, swelling occurs when cells are treated with Ca^{2+}-ionophore in the presence of 140 mmol/l extracellular K^+ (see Fig. 3A inset). Under conditions where ion fluxes across the membrane are excluded (as described in Fig. 5), ghosts show no significant change in MCV upon scramblase activation (Fig. 6A). These findings were confirmed by comparing the morphology of intact erythrocytes and resealed ghosts before and 60 min after treatment with Ca^{2+}-ionophore (Fig. 6B). Clearly, intact erythrocytes shrink and shed microvesicles that results in the formation of spherocytes with an approximate diameter of 5 μm. In contrast, for both K^+ and Na^+ ghosts, the morphology is virtually unaffected by treatment with Ca^{2+}-ionophore. The majority of the resealed ghosts retain the biconcave shape of the original erythrocyte, although they are smaller in size.

Microvesicle formation by erythrocyte ghosts during the process of lipid scrambling. Two independent experiments were carried out to determine whether Ca^{2+}-ionophore-treated erythrocytes and ghosts produce microvesicles. First, approximately 25 % of the prothrombinase activity was found in the supernatant of erythrocytes after 60-min incubation with ionomycin (Fig. 7A), in agreement with previous estimates of the amount of plasma membrane shed under these conditions [22]. In contrast, when ghosts were challenged with Ca^{2+}-ionophore, the activity remaining in the supernatant was less than 5 %. These results indicate that the supernatants did not contain procoagulant lipids, suggesting that microvesicles were not produced. To rule out the possibility that vesicles lacking PS were formed, ghosts labeled with *N*-hydroxysuccinimide-fluorescein were treated with Ca^{2+}-ionophore and residual fluorescence in the supernatants was determined. Figure 7B shows that, in contrast to erythrocytes that released a significant fraction of fluorescein-labeled membrane, fluorescent membrane was not detected in ionomycin-treated ghosts. Taken together, these results indicate that lipid scrambling occurs through a mechanism that is independent of cell morphology, cell size and microvesicle formation.

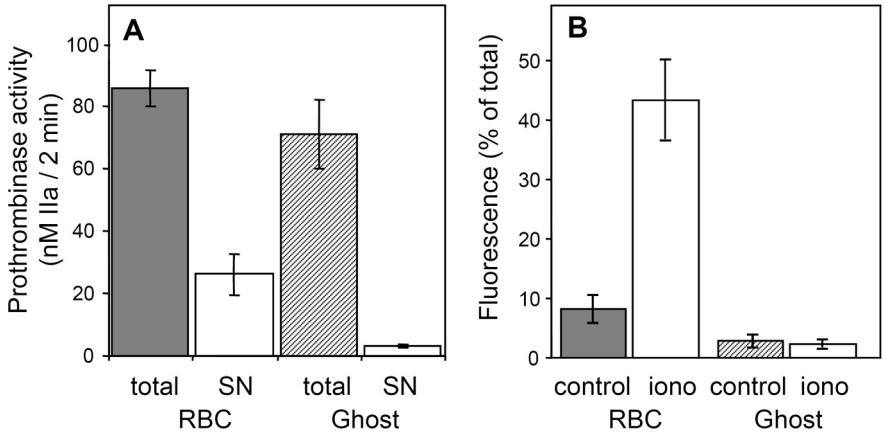

Figure 7. Microvesicle production in ionomycin-treated erythrocytes and erythrocyte ghosts. (*A*) Prothrombinase activity in erythrocytes (solid bar) and erythrocyte ghosts (hatched bar) after 60-min incubation with ionomycin in the presence of 1 mmol/l Ca^{2+}. Contribution of microvesicles was estimated by measuring the prothrombinase activity of the supernatant (white bars) obtained after centrifugation. Presented are mean values ± SD from three independent experiments. (*B*) RBC ghosts do not vesiculate following incubation with ionophore/Ca^{2+}. Fluorescein-labeled-RBC (solid bar) or resealed ghosts (hatched bar) were incubated with Ca^{2+} in the absence or presence of ionomycin (open bars) as described in the section experimental procedures. The RBC and ghosts were then centrifuged and the residual fluorescence in the supernatant measured. Data are expressed as the fraction of total fluorescence in the supernatant (SN).

Discussion

Increased intracellular Ca^{2+}, efflux of K^+ ions, cell shrinkage, shedding of membrane vesicles and collapse of lipid asymmetry are features typical of nucleated cells undergoing apoptosis. Similar phenomena also occur in anucleated cells such as platelets and red blood cells under conditions that result in a persistent elevation of $[Ca^{2+}]_i$ [3, 13]. Together with elevated Ca^{2+}, intracellular K^+ also plays an important role in apoptosis since preventing loss of K^+ by manipulating the $[K^+]_e$ or treatment with K^+ channel blockers blocks apoptosis [23–26]. We have previously observed inhibition of PS exposure in collagen/thrombin-activated platelets by high extracellular K^+ and blockers of the Ca^{2+}-activated K^+ channel (Gardos channel) [12]. Similarly, blockade of K^+ channels or high $[K^+]_e$ inhibits Ca^{2+}-ionophore induced PS exposure in red blood cells [13]. Valinomycin abolishes the inhibitory effect of the K^+ channel blockers. Using the red blood cell membrane as a model to study the relationship between intracellular K^+ and exposure of PS during lipid scrambling, the present study shows that the $[K^+]_i$ directly affects the rate at which membrane lipid asymmetry is lost when erythrocytes are challenged with high $[Ca^{2+}]_i$.

A possible explanation for the inhibitory effect of K^+ on scrambling could be the hyperpolarization following activation of Ca^{2+}-sensitive K^+ channels, which drives additional Ca^{2+} into the cell, augmenting cytosolic $[Ca^{2+}]$ [13]. The results presented in Figure 2, however, suggest that the $[K^+]_i$ does not influence ionophore-dependent $[Ca^{2+}]_i$. Conversely, elevation of cytosolic Ca^{2+} achieved with ionophore/Ca^{2+} in erythrocytes did not facilitate complete efflux of K^+ through opening of the Ca^{2+}-sensitive K^+ channels since the addition of valinomycin resulted in increased scramblase activity (Fig. 1A). Interestingly, Schneider et al. [27] recently showed that valinomycin-mediated loss of K^+ can induce PS exposure in erythrocytes in the absence of ionomycin. The inability of valinomycin alone to induce PS exposure in the present study is likely due to differences in the experimental conditions; the result by Schneider et al. required long incubation times (24 h) and is likely similar to the (unexplored) mechanism that is responsible for the exposure of PS upon *in vitro* aging that requires incubation for 4 days in the absence of Ca^{2+} [28].

Comparison of the effect of K^+ on intact erythrocytes and erythrocyte ghosts (Figs 3A, 4B) shows that the inhibitory effect of K^+ is significantly stronger in the ghosts. As explained in the results section, because of the osmolarity (size)-dependent effects on scramblase activity, the changes in the MCV of intact erythrocytes at different $[K^+]$ likely cause under- and overestimates of scramblase activity at low and high $[K^+]$, respectively. Moreover, it should be emphasized that $[K^+]_i$ in the intact erythrocytes treated with valinomycin is not necessarily identical to $[K^+]_e$. At electrochemical equilibrium in a dehydrating cell with a hyperpolarized membrane, $[K^+]_i$ is likely to exceed $[K^+]_e$. Thus, the K^+ titration in ghosts (Fig. 4B) more likely reflects the true inhibitory effect of K^+ on the rate of scrambling.

Efflux of K^+ is likely accompanied by acidification of the intracellular content. While we cannot exclude the

possibility that this will affect the scrambling process in intact cells, the experiments with resealed erythrocyte ghosts (Figs 4, 5) clearly demonstrate that lipid scrambling is directly modulated by $[K^+]_i$. Indeed, metal ions are known to alter the activity of various enzymes [29] and many enzymes are allosterically affected by Na^+ or K^+ ions, often in tandem with bivalent cations such as Mg^{2+} or Ca^{2+}. Inhibition of lipid scrambling by K^+ ions rather than a stimulation by Na^+ ions occurs since replacement of Na^+ with $choline^+$ had no effect on scrambling activity, whereas substitution with K^+ decreased the rate of lipid scrambling. The mechanism responsible for K^+ suppression of scramblase activity awaits identification of the scramblase protein(s). Considering that $[K^+]_i$ in human erythrocytes is normally high (100–140 mmol/l), scramblase activity in resting cells is likely suppressed. Thus, triggering scramblase activity by increasing intracellular Ca^{2+} will open Ca^{2+}-sensitive K^+ channels resulting in loss of intracellular K^+ that further enhances the Ca^{2+}-dependent scrambling activity.

A recent publication by Elliott et al. [30] raised the possibility that cell shrinkage-associated alterations in membrane lipid packing at the base and the apex of membrane blebs might be responsible for a scramblase-independent mechanism for PS externalization. This conclusion is based on the results of temporal labeling of lymphocytes with merocyanine 540 (a lipid packing-dependent dye) and annexin A5. Although PS exposure is known to be closely associated with membrane curvature [16], cell shrinkage [27], membrane blebbing and shedding of microvesicles [21, 31] in intact red blood cells, we show here that lipid scrambling in ghosts occurs in the absence of volume changes and microvesicle formation (Figs. 6, 7). Indeed, using spermine-containing ghosts, Bucki et al. [31] demonstrated that PS exposure and microvesicle formation are distinct and separable processes. Thus, while scrambling might be concurrent to cell shrinkage and microvesicle formation in intact cells, these phenomena are unrelated and mechanistically distinct.

Our observations on the inverse relationship between $[K^+]_i$ and Ca^{2+}-dependent lipid scrambling may provide some insight into the physiology and mechanism(s) involved in erythrocyte aging. Similar to the data reported here, RBC senescence is associated with reduced plasma membrane Ca^{2+}-ATPase activity that leads to the intracellular accumulation of the cation and concomitant loss of $[K^+]_i$ to less than 10 mM [32]. In addition, some pathological conditions, such as sickle cell anemia, are accompanied by an increased number of cells with substantial amounts of surface exposed PS, which may be partially related to changes in K^+ content of the cells (reviewed in Zwaal et al. [6]). Moreover, the inverse correlation between $[K^+]$ and PS asymmetry in erythrocytes is in agreement with recent data demonstrating inhibition of agonist (collagen and thrombin)-induced PS exposure at the platelet surface by K^+ channel blockers and their subsequent reversal with valinomycin [12]. While it is unclear whether loss of intracellular K^+ is critical for apoptosis regulated activation of scramblase activity in nucleated cells, there are reports that show an inhibitory role for intracellular K^+ on caspase and nuclease activity [33] and apoptosome activation [34, 35], suggesting a physiological role for intracellular K^+ as a negative regulator of several key events in apoptosis, including PS externalization.

In conclusion, we have shown that lipid scrambling can occur in isolated red blood cell membranes and that the activity of the scramblase is directly influenced by $[K^+]$. While cell shrinkage and changes in morphology affect the rates of lipid scrambling, they do not appear to be the driving force behind this process. Because of its significance in blood coagulation and in the removal of apoptotic cells, modulation of PS exposure by selective manipulation of the $[K^+]_i$ in Ca^{2+}-dependent lipid scrambling may offer new strategies to control this important physiological process.

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