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Effects of divalent cations on lipid flip-flop in the human erythrocyte membrane

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Treatment of human erythrocytes with ionophore A23187 (10 μmol·l^{-1}) and Ca^{2+} (0.05–0.5 mmol·l^{-1}) or Sr^{2+} (0.2–1 mmol·l^{-1}) in results in a concentration-dependent acceleration of the transmembrane reorientation (flip) of the lipid probes lysophosphatidylcholine and palmitoylcarnitine to the inner membrane leaflet after their primary insertion into the outer leaflet. Mg^{2+}, Mn^{2+}, Zn^{2+} and La^{3+} do not accelerate flip. Ca^{2+}-induced flip acceleration depends also on the ionophore concentration. It is reversed by removal of Ca^{2+} with EDTA. A causal role of Ca^{2+}-induced membrane protein degradation and decrease of the polyphosphoinositide level in flip acceleration could be excluded. Likewise, calmodulin-dependent processes are probably not involved since the calmodulin antagonist calmidazolium (2–10 μmol·l^{-1}) does not suppress but even enhances the Ca^{2+}-induced flip acceleration. The same is true for the Ca^{2+} antagonist flunarizine. These drugs do not alter flip rate in the absence of Ca^{2+}. At high Ca^{2+} (1–5 mmol·l^{-1}) an initial flip acceleration is followed by flip normalization. High concentrations of Mn^{2+} and Mg^{2+} slow down flip rates. The selective acceleration of flip by Ca^{2+} and Sr^{2+} is discussed to be due to a local detachment of the membrane skeleton from the bilayer, whereas the unselective slow down of flip by divalent cations might be due to a stabilization of the membrane bilayer by the cations. After loading of cells with Ca^{2+} (but not with Mn^{2+}) the inner membrane leaflet phospholipid phosphatidylserine becomes rapidly exposed to the outer membrane surface, as detectable by its accessibility to phospholipase A_2 (5 min). This fast reorientation of phosphatidylserine is, however, not tightly coupled to the flip acceleration indicated by the lipid probes.

Introduction

Increase of the level of Ca^{2+} in human erythrocytes (about 0.02 μmol·l^{-1}, Ref. 1) to values above 10 μmol·l^{-1} results in the activation of the endogenous protease calpain I [2–4]. The first change in the membrane protein pattern upon calpain activation is the cleavage of ankyrin (band 2.1) followed by band 4.1 [2–4]. Both proteins connect the membrane skeletal proteins spectrin and actin to the intrinsic membrane domain [5]. Increased Ca^{2+} also activates the endogenous phospholipase C catalyzing cleavage of polyphosphoinositides [2,3], it stimulates the Ca^{2+} pump via calmodulin [6], modulates the activity of a variety of enzymes [7] and affects cytoskeletal proteins [8,9]. Moreover, an increased intracellular Ca^{2+} level activates a K^+ channel [10].

At intracellular Ca^{2+} levels in the millimolar range activation of transglutaminase produces cross-linking of spectrin [11]. Studies on model membrane lipid systems suggest that Ca^{2+} at millimolar concentrations increases the tendency of the phospholipids of the inner membrane leaflet to adopt non-bilayer organization even in the presence of cholesterol [12,13].

In platelets an increase of the intracellular Ca^{2+} level results in the proteolytic degradation of cytoskeletal proteins and concomitant stimulation of platelet procoagulant activity [14,15], presumed to be the result of exposure of inner leaflet phosphatidylserine to the outer membrane surface [16]. Phosphatidylserine exposure was therefore suggested to be due to breakdown of the cytoskeleton [14,15]. Since these Ca^{2+}-induced reactions

Abbreviations: TLCK, L-1-chloro-3-tosylamido-7-amino-2-heptanone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; DTE, dithioerythritol.

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occur within 10 s [14] the translocation of phosphatidylserine from the inner to the outer membrane leaflet should be very fast. A simultaneous inhibition by Ca^{2+} [17] of the fast ATP-dependent inward translocation of phosphatidylserine ($t_{1/2} = 7$ min, Ref. 18) may facilitate the fast exposure of phosphatidylserine to the outer membrane surface. In erythrocytes Ca^{2+} loading of cells by the ionophore also results in an increased exposure of phosphatidylserine to the outer membrane surface [19].

It was the aim of the present work to clarify whether reorientation of phosphatidylserine to the outer membrane surface following an increase of the intracellular Ca^{2+} level might reflect a general increase of the transversal dynamics of lipids in the erythrocyte membrane. To this end we studied the effect of Ca^{2+} loading of erythrocytes on the non-mediated flip of the lipid probes palmitoyllysophosphatidylcholine and palmitoylcarnitine, which have been shown to be useful for such purposes [20–22]. Ca^{2+} loading of cells is shown to accelerate flip, but accelerated flip is not tightly coupled to phosphatidylserine exposure.

Materials and Methods

Freshly taken human blood, anticoagulated with citrate, was stored at 4°C in a convential storage medium containing glucose and adenine and used within 5 days. Erythrocytes were isolated by centrifugation of blood (5 min at 6000 × *g*), plasma and buffy coat were removed and the cells were washed three times with isotonic saline at room temperature. Ionophore A23187, flunarizine, calmidazolium and TLCK were purchased from Sigma, fatty acid free bovine serum albumin and trypsin from Boehringer (Mannheim), L-[1-^{14}C]palmitoylcarnitine (spec. act. 2.04 GBq/mmol) from NEN and 1-[1-^{14}C]palmitoyllysophosphatidylcholine (spec. act. 1.67 GBq/mmol) from Amersham Buchler.

Incubation medium

One volume of washed erythrocytes was suspended in 10 vols of a medium containing (concentrations in mmol · l^{-1}) KCl 90, NaCl 45, Hepes 10 and sucrose 44 (= medium A; pH 7.4).

Inhibition of calpain activity

Erythrocytes were incubated in medium A containing 0.5 mmol · l^{-1} TLCK (15 min, 37°C, hematocrit 10%) and the cells subsequently washed 3 times with medium A.

ATP depletion

Erythrocytes were incubated in medium A containing 5 mmol · l^{-1} iodoacetate (15 min, 37°C, hematocrit 10%), washed three times with medium A, incubated for 60 min at 37°C in medium A containing 10 mmol · l^{-1} inosine and washed three times. This treatment decreases intracellular ATP to < 5% of the native level [22].

Entrapment of trypsin in resealed cells

Packed erythrocytes were lysed by mixing with the same volume of 4 mmol · l^{-1} $MgSO_4$ (0°C) containing trypsin (0.2 μg/ml cells). After 10 min incubation enough KCl was added to restore isotonicity and the suspension was incubated for 10 min at 0°C. The cells were then incubated (37°C, 15 min) and were washed three times with medium A.

Flip rates

Transbilayer movements of exogenously inserted probes were measured as described before [21,22]. Briefly, ^{14}C-labeled palmitoyllysophosphatidylcholine or palmitoylcarnitine was inserted into the outer membrane layer of erythrocytes (15 nmol/ml packed cells) by an incubation of a cell suspension in medium A (hematocrit 50%) for 5 min with the probe spread on the wall of an incubation tube by evaporation. Following centrifugation, the cells were washed once with medium A [4°C] and were resuspended in medium A (hematocrit 10%) containing Ca^{2+} or other divalent cations at the concentrations specified in the Results. Subsequently, variable amounts of ionophore A23187 (0.5–10 μmol · l^{-1}) were added to aliquots of the suspension. The reorientation of the lipid probe to the inner membrane layer at 37°C was quantified by following the time-dependent decrease of its extractability by albumin.

Reversal of divalent cation-induced flip acceleration

Erythrocytes were incubated in medium A containing either 0.1 mmol · l^{-1} Ca^{2+} or 0.5 mmol · l^{-1} Sr^{2+} in the presence of 10 μmol · l^{-1} ionophore A23187 (15 min, 37°C). To remove divalent cations cells were sedimented and were incubated in Medium A containing 10 mmol · l^{-1} EDTA (5 min, 22°C). The cells were sedimented and the EDTA treatment was repeated once. Subsequently, cells were washed twice with Medium A containing 1.5% albumin to remove the ionophore from the membrane followed by three washes with Medium A to remove albumin. The flip rate of the lipid probe was then measured in Medium A containing 5 mmol · l^{-1} EDTA.

Phospholipid asymmetry

To detect changes of phospholipid distribution between the two membrane leaflets of the erythrocyte the cleavability of phospholipids by phospholipase A_2 from bee venom was measured (20 IU/ml cells, 37°C, 5 min) in Medium A with 0.25 mmol · l^{-1} Ca^{2+} and Mg^{2+} [21]. Hemolysis was < 3%.

Membrane protein patterns

Erythrocytes were treated with divalent cations and ionophore in medium A (5–30 min, 37°C), EDTA (1–10 mmol · l^{-1} final concentration) was added and cells were washed three times in the presence of EDTA. After preparation of ghosts from the cells [23] membrane proteins were solubilized by SDS and separated by polyacrylamide gel electrophoresis [24].

Erythrocyte shape and microvesiculation

Changes of erythrocyte shape were assessed by interference light microscopy of a cell suspension between two plastic cover slips. The degree of echinocytosis was classified according to Bessis [25]. The extent of the release of microvesicules from erythrocytes loaded with divalent cations was quantified by the loss of phospholipid from the cells according to Ref. 26.

Results and Interpretation

Ca^{2+}- and Sr^{2+}-induced flip acceleration

The slow transmembrane reorientation (flip) of palmitoyllysophosphatidylcholine from the outer to the inner membrane leaflet ($t_{1/2}$ = 11 h; Ref. 27) of human erythrocytes becomes enhanced upon exposure of the cells to ionophore A 23187 (10 μmol · l^{-1}) and low concentrations of Ca^{2+} (Fig. 1). The acceleration of flip is clearly evident at a Ca^{2+} concentration of 0.05 mmol · l^{-1} and further increases upon an increase of the Ca^{2+} concentration to 0.5 mmol · l^{-1}. It is already detectable a few minutes after addition of the ionophore (Fig. 1A). The mere presence of ionophore, which results in Mg^{2+} depletion of the cells [28], does not affect flip (Fig. 1A, 0 mmol · l^{-1} Ca^{2+}). Under the above conditions the flip rate of palmitoylcarnitine, which is more than 5-fold higher than that of palmitoyllysophosphatidylcholine in unmodified cells [22], is also accelerated (data not shown).

The acceleration of the flip of the lipid probe by Ca^{2+} is not due to artifacts resulting from an interference of Ca^{2+} with the albumin extraction procedure, since on the one hand this extraction is done in the absence of Ca^{2+} (see Methods) and on the other hand flip kinetics in control cells in the absence of ionophore are not affected by Ca^{2+}.

The causal role of an increase of intracellular Ca^{2+} for the flip acceleration becomes evident from the dependence of the effect on the ionophore concentration. At high concentrations of the ionophore (10 μmol · l^{-1}), at which Ca^{2+} equilibration across the membrane will occur within a few minutes [29], flip acceleration in the presence of 0.1 mmol · l^{-1} Ca^{2+} is observed immediately. On the other hand, flip acceleration at lower ionophore concentrations only starts after a lag phase (Fig. 2). These lag phases last for about 30 and 90 min for ionophore concentrations of 1 and 0.5 μmol · l^{-1}. They can be explained by the slower attainment of the critical intracellular Ca^{2+} concentration required to accelerate flip, due to the slower rate of Ca^{2+} uptake at lower concentrations of ionophore [1]. In analogy an increase of the Ca^{2+} concentration from 0.1 to 1 mmol · l^{-1} at low ionophore concentration (1 μmol · l^{-1}) shortens the lag phase, too (Fig. 2).

At Ca^{2+} concentrations above 0.5 mmol · l^{-1} simple flip kinetics are no longer observed. At 1 and 2 mmol · l^{-1} Ca^{2+} a small fraction (8%) of the lipid probe reorients from the outer to the inner membrane leaflet within the first 5 min (Fig. 1B, hatched line). After this initial fast phase the flip slows down (Fig. 1B). At 5 and 10 mmol · l^{-1} Ca^{2+} the initial fast phase is less pronounced. A plot of the fraction of the lipid probe reoriented to the inner leaflet after 5 min against the

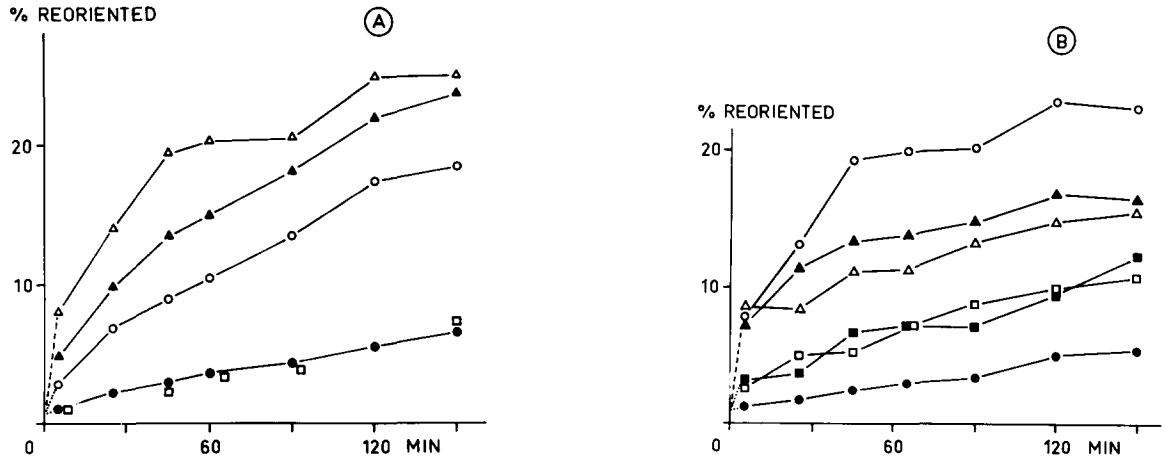

Fig. 1. Acceleration of lysophosphatidylcholine flip in erythrocytes by Ca^{2+}/ionophore A23187. After insertion of ^{14}C-lysophosphatidylcholine into the outer membrane leaflet of erythrocytes, cells were resuspended in Medium A at 37°C containing: (A) 0 (●), 0.05 (○), 0.1 (▲), or 0.5 (△) mmol · l^{-1} Ca^{2+}. Control cells (no Ca^{2+} and ionophore, □). (B) 0 (●), 0.5 (○), 1 (▲), 2 (△), 5 (■), or 10 (□) mmol · l^{-1} Ca^{2+}. At zero time ionophore (10 μmol · l^{-1}) was added and flip was measured. A typical result out of three experiments is shown.

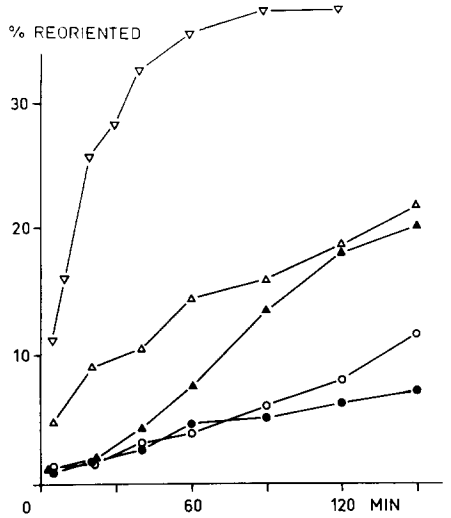

Fig. 2. Dependence of the Ca^{2+}-induced acceleration of lysophosphatidylcholine flip on ionophore concentration. ^{14}C-Lysophosphatidylcholine was inserted into the outer membrane leaflet of erythrocytes and the flip of the lipid probe to the inner leaflet was measured in the presence of 0.1 mmol·l^{-1} Ca^{2+} and 0 (●), 0.5 (○), 1 (▲), or 10 (△) μmol·l^{-1} ionophore A23187, added at zero time. Alternatively flip was measured at 1 mmol·l^{-1} Ca^{2+} and 1 μmol·l^{-1} ionophore (▽).

extracellular Ca^{2+} concentration (Fig. 3) demonstrates maximal flip acceleration in the range between 0.5 and 2 mmol · l^{-1} Ca^{2+}.

Sr^{2+} has a flip accelerating effect, too (Fig. 4). This is first observed at a somewhat higher concentration (0.2 mmol · l^{-1}) than with Ca^{2+} (Fig. 3). Above 2 mmol · l^{-1} Sr^{2+} the fraction of the lipid probe reoriented during the first 5 min decreases (Fig. 3).

In contrast to Ca^{2+} and Sr^{2+}, no accelerating effects of Mg^{2+}, Mn^{2+}, Zn^{2+} and La^{3+} are observed in the concentration range between 0.05 and 1 mmol · l^{-1} (data

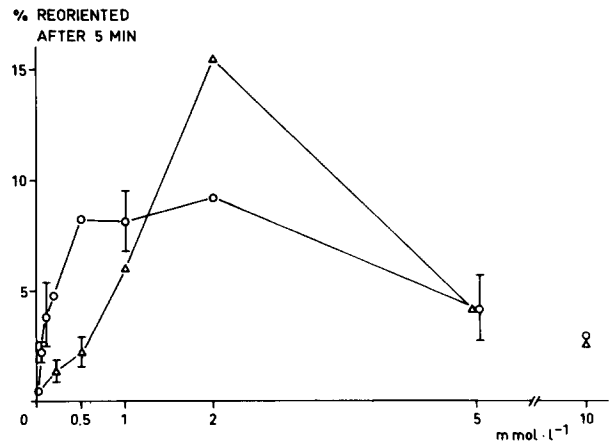

Fig. 3. Dependence of the initial phase of flip acceleration by Ca^{2+} and Sr^{2+} on their concentrations. ^{14}C-Lysophosphatidylcholine was inserted into the outer membrane leaflet and its translocation to the inner leaflet measured at varying concentrations of Ca^{2+} (○) or Sr^{2+} (△) in presence of ionophore (10 μmol·l^{-1}). The fractions translocated during the first 5 min are given and represent the mean values of 2–11 experiments (±S.D.).

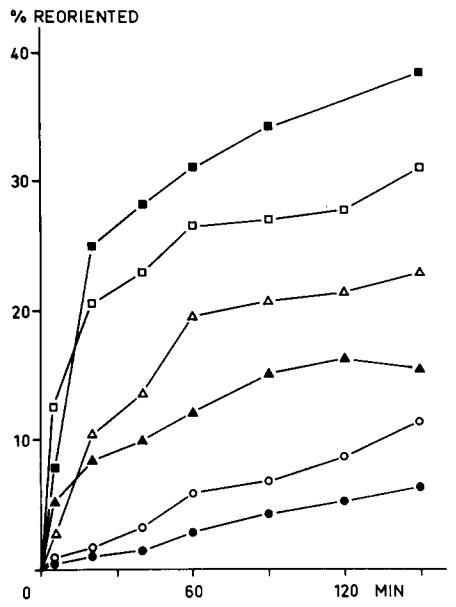

Fig. 4. Acceleration of lysophosphatidylcholine flip in erythrocytes by Sr^{2+}/ionophore A23187. After insertion of ^{14}C-lysophosphatidylcholine into the outer membrane leaflet cells were resuspended in Medium A at 37 °C containing 0 (●), 0.2 (○), 0.5 (△), 1 (■), 2 (□), or 5 (▲) mmol·l^{-1} Sr^{2+}, ionophore (10 μmol·l^{-1}) was added and flip of the lipid probe measured.

not shown), although these cations rapidly accumulate in the cells in the presence of the ionophore [30,31]. This result demonstrates the selectivity of flip acceleration and excludes a simple mechanism of flip acceleration by the membrane-bound ionophore/metal ion complex as previously observed for channel forming antibiotics [21,32].

Further evidence that the accumulation of Ca^{2+}

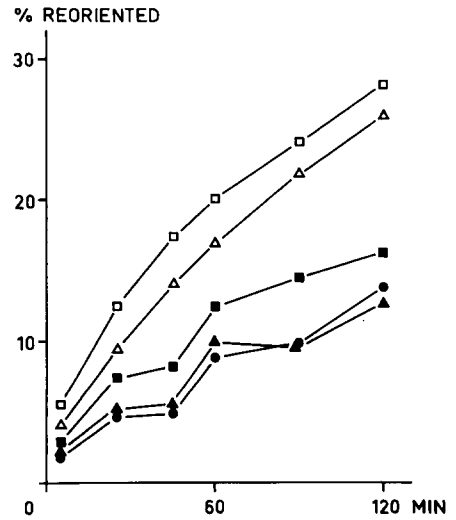

Fig. 5. Effect of ATP-depletion of erythrocytes on the flip acceleration of palmitoylcarnitine by Ca^{2+}/ionophore. ^{14}C-Palmitoylcarnitine was inserted into the outer membrane leaflet of control (filled symbols) and ATP-depleted (open symbols) erythrocytes and the flip of the lipid probe measured in the presence of 10 μmol·l^{-1} ionophore and 0 (●), 0.013 (▲, △), or 0.05 (■, □) mmol·l^{-1} Ca^{2+}.

causes acceleration of flip of palmitoylcarnitine and palmitoyllysophosphatidylcholine stems from the higher susceptibility of ATP-depleted cells towards Ca^{2+}/ionophore. In ATP-depleted cells acceleration of palmitoylcarnitine flip is already observed at 0.013 mmol $\cdot l^{-1}$ Ca^{2+} (Fig. 5), a concentration ineffective in control cells with a high ATP level. This difference is most probably due to the maintainance of a low intracellular Ca^{2+} level by the Ca^{2+} pump in ATP-rich cells. In line with this notion the difference in the Ca^{2+} induced flip rates between ATP-depleted and ATP-rich cells disappears at higher Ca^{2+} concentrations (0.1 mmol $\cdot l^{-1}$), at which the capacity of the Ca^{2+} pump (10–25 mmol/liter cells per h, Ref. 1) can no longer cope with the ionophore-mediated Ca^{2+} influx.

Activation of calpain I and flip acceleration by divalent cations

In order to investigate whether activation by Ca^{2+} and Sr^{2+} of the intracellular protease calpain I (human erythrocytes lack calpain II, Ref. 33) plays a causal role in flip acceleration, the membrane polypeptide patterns of cells loaded with the divalent cations were analyzed. The first activation of calpain I detectable by a degradation of ankyrin (band 2.1) to band 2.3 is observed after a 5 min exposure to 0.05 mmol $\cdot l^{-1}$ Ca^{2+} or 0.5 mmol $\cdot l^{-1}$ Sr^{2+} (Fig. 6, gel b, f). A more complete degradation of ankyrin to band 2.3 was found after 15 min exposure or at higher concentrations of the cations. Band 4.1 becomes degraded at Ca^{2+} > 1 mmol $\cdot l^{-1}$ but not with Sr^{2+} up to 5 mmol $\cdot l^{-1}$ (gel d, e and h, i). In the case of Ca^{2+} the concentrations required to obtain the first increase of band 2.3 in the gel and the first flip acceleration are very close, while in the case of Sr^{2+} they are not. Nevertheless, a causal role of calpain I in the flip acceleration should not be deduced from this finding,

TABLE I

Influence of calmidazolium and flunarizine on the Ca^{2+}-induced flip acceleration

Erythrocytes were loaded with ^{14}C-lysophosphatidylcholine and its flip to the inner membrane leaflet measured in Medium A containing Ca^{2+} and ionophore (10 μmol $\cdot l^{-1}$), as well as flunarizine or calmidazolium (10 μmol $\cdot l^{-1}$). The fractions (%) of the probe translocated represent the mean values of 2–5 experiments (±S.D.).

Ca^{2+} (mmol $\cdot l^{-1}$)	Antagonist	% translocated per 30 min
0	none	1.6
0	flunarizine	1.6
0	calmidazolium	1.6
0.1	none	6.3±1.3
0.1	flunarizine	11.6±1.7
0.1	calmidazolium	11.7±2.1
2	none	12.0
2	flunarizine	22.5
5	none	6.0
5	flunarizine	13.4

since a pretreatment of cells with the proteinase inhibitor TLCK, which suppresses degradation of band 2.1 (compare gel m and n), has no inhibitory effect on the Ca^{2+}-induced flip. In fact TLCK enhances the Ca^{2+}-induced flip (data not shown). This appears to be due to the superposition of a flip accelerating effect of TLCK on the flip acceleration by Ca^{2+}/ionophore. Further evidence against a causal role of proteolysis comes from experiments on resealed cells containing trapped trypsin. The flip rate of lysophosphatidylcholine in trypsin-free resealed cells as well as in resealed cells containing 0.2 μg trypsin/ml cells, which produced ankyrin cleavage to band 2.3, is quite similar to that in control cells (data not shown).

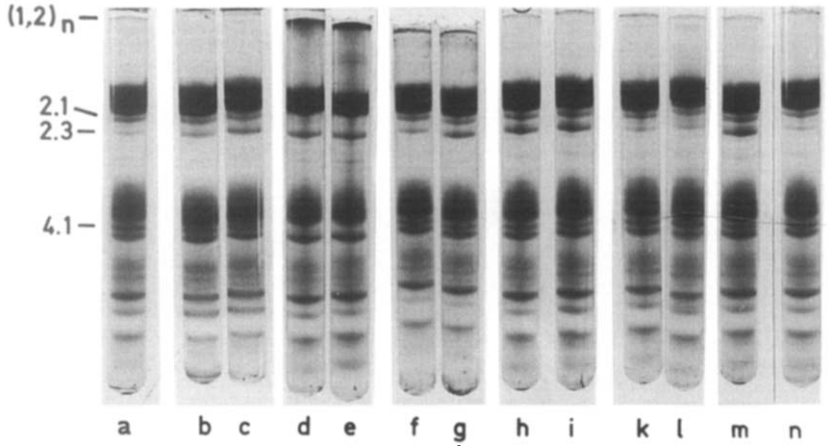

Fig. 6. Gel electrophoretic patterns of membrane polypeptides from erythrocytes treated with divalent cations and ionophore A23187 (10 μmol $\cdot l^{-1}$). Erythrocytes were incubated at 37°C in Medium A containing ionophore (10 μmol $\cdot l^{-1}$) and either Ca^{2+} (b–e), or Sr^{2+} (f–i) or Mn^{2+}1 (k, l). Details were as follows: Ca^{2+} 0.05 (b, c) or 5 (d, e) mmol $\cdot l^{-1}$ for 5 (b, d) or 15 min (c, e); Sr^{2+} 0.5 (f, g) or 5 (h, i) mmol $\cdot l^{-1}$ for 5 (f, h) or 30 min (g, i); Mn^{2+} 5 mmol $\cdot l^{-1}$ (k, l) for 5 (k) or 30 (1) min. Alternatively cells were incubated with 10 μmol $\cdot l^{-1}$ ionophore and 0.5 mmol $\cdot l^{-1}$ Ca^{2+} without (gel m) or with (gel n) TLCK pretreatment. Gel a represents the membrane polypeptide pattern of control cells.

Effects of calmodulin and Ca^{2+} antagonists on Ca^{2+}-induced flip acceleration

A possible involvement of calmodulin-dependent processes in the Ca^{2+}-induced flip acceleration seems unlikely since the calmodulin antagonist [7] calmidazolium does not suppress the flip acceleration. At 10 $\mu mol \cdot l^{-1}$ this drug has neither any effect on the flip rate of lysophosphatidylcholine in the absence of Ca^{2+} nor any inhibitory effect on the Ca^{2+}-induced flip acceleration (Table I). On the contrary, calmidazolium increases the susceptibility of the cells to Ca^{2+} in a concentration dependent way. The Ca^{2+} antagonist flunarizine [34] does not prevent the Ca^{2+}-induced flip either. As for calmidazolium, flunarizine enhances the Ca^{2+}-induced flip, but it does not have an effect on flip in the absence of Ca^{2+} (Table I).

Reversibility of divalent cation-induced flip acceleration

Removal of intracellular Sr^{2+} (or Ca^{2+}) by EDTA and of ionophore by albumin from cells originally loaded with Sr^{2+} (or Ca^{2+}) reverses the flip acceleration (Fig. 7A). This reversibility is also observed in ATP-depleted cells (data not shown). It does not only exclude a causal role of proteolytic degradation of membrane proteins, but also of a decrease of the polyphosphoinositide level in flip acceleration. In this latter case flip acceleration would be irreversible due to the impossibility to restore the polyphosphoinositide level in the absence of ATP.

Moreover, the Sr^{2+}-induced flip can be normalized by addition of 5 $mmol \cdot l^{-1}$ Mn^{2+} to the cells suspension during the measurement of flip kinetics (Fig. 7B).

Inhibition of flip by divalent cations

Mn^{2+} and Mg^{2+} ions which do not have a flip accelerating effect at low concentrations decrease the flip rates of lysophosphatidylcholine and palmitoylcarnitine to values below the rate in control cells when present at concentrations of 2–5 $mmol \cdot l^{-1}$ (Fig. 8). Mn^{2+} is more effective than Mg^{2+}. The Ca^{2+} antagonist flunarizine (10 $\mu mol \cdot l^{-1}$) does not reverse the suppression of flip rate (data not shown). Treatment of erythrocytes with Mn^{2+} does neither activate calpain I nor cellular transglutaminase. This latter enzyme catalyzes

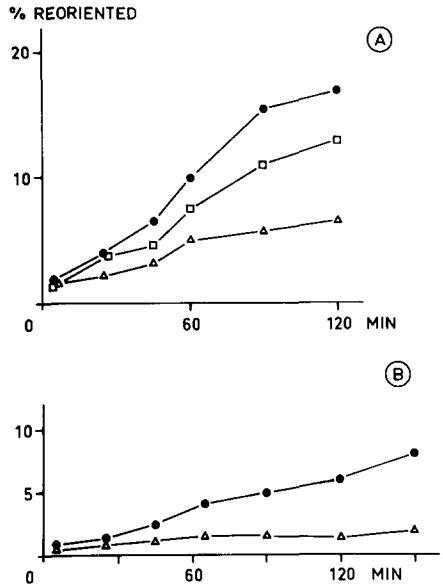

Fig. 8. Inhibition of flip of lipid probes by Mn^{2+} and Mg^{2+} (5 $mmol \cdot l^{-1}$). (A) Flip of ^{14}C-palmitoylcarnitine, measured in the presence of ionophore (10 $\mu mol \cdot l^{-1}$) and either no divalent cation (●) or Mn^{2+} (△) or Mg^{2+} (□). (B) Flip of ^{14}C-lysophosphatidylcholine was measured in the presence of ionophore (10 $\mu mol \cdot l^{-1}$) and either no divalent cation (●) or Mn^{2+} (△).

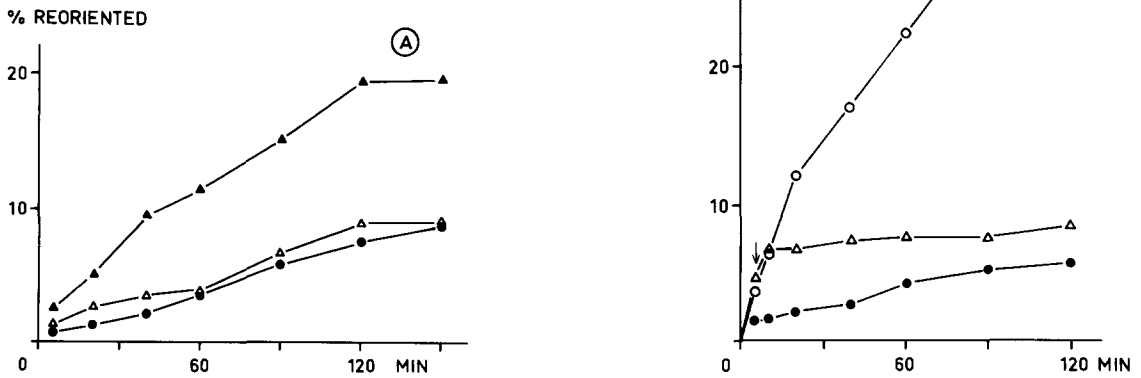

Fig. 7. Reversibility of the Sr^{2+}-induced flip acceleration. (A) Erythrocytes were incubated for 15 min at 37°C with 0.5 $mmol \cdot l^{-1}$ Sr^{2+} and ionophore (10 $\mu mol \cdot l^{-1}$). Flip of ^{14}C-palmitoylcarnitine was then measured directly (▲) or after treatment of cells with EDTA/albumin (△). Control (●). (B) Flip of ^{14}C-lysophosphatidylcholine to the inner membrane leaflet was measured in the absence (●) or presence (○) of 0.5 $mmol \cdot l^{-1}$ Sr^{2+} and 10 $\mu mol \cdot l^{-1}$ ionophore. After 5 min of flip Mn^{2+} (5 $mmol \cdot l^{-1}$) was added to an aliquot of the Sr^{2+}-containing suspension (△).

cross-linking of spectrin to oligomers $(1, 2)_n$ upon activation by high concentrations of Ca^{2+} (Fig. 6, gel d, e).

Exposure of phosphatidylserine to the outer membrane leaflet in the presence of divalent cations and ionophore

During a short treatment of erythrocytes (5 min at 37°C) with phospholipase A_2 (Table II, line A) in the presence of 0.25 mmol · l^{-1} Ca^{2+} and ionophore A23187, phosphatidylserine, normally restricted to the inner membrane leaflet, becomes accessible to cleavage by the lipase. This exposure to the outer membrane surface is also detectable when the cells are first treated with ionophore and Ca^{2+} and then washed with EDTA and albumin to remove ionophore and Ca^{2+} (Table II, line B1, B2) which normalizes the flip rate. This finding may be taken to indicate that an inward retranslocation of the exposed phosphatidylserine by the phospholipid flippase [35] does not occur. Most likely, the transporter is inhibited by the removal of cytoplasmic Mg^{2+} during the ionophore treatment [17]. On the other hand, Mg^{2+} depletion as such by a pretreatment of cells without Ca^{2+} or with EDTA in the presence of ionophore does not result in phosphatidylserine exposure (Table II, line B3, B4). Moreover, the extent of phosphatidylserine exposure by Ca^{2+} loading is not significantly enhanced by ATP depletion of cells which prevents [35] inward translocation of phosphatidylserine compare lines C1 and B1). ATP depletion of cells as such does not result in a significant exposure of phosphatidylserine to the outer surface, not even when the ATP depleted cells are incubated for 16 h at 37°C (compare lines C2 and D).

In line with the lack of flip accelerating effect Mn^{2+}-loading of cells does not result in phosphatidylserine exposure (line B5). Phosphatidylserine cleavability is observed in cells pretreated with low concentrations (0.2 mmol · l^{-1}) of Mn^{2+} followed by EDTA/albumin washes (data not shown). This is probably due to an incomplete removal of the ionophore from the membrane by albumin in the presence of Mn^{2+}, which leads to ionophore-mediated Ca^{2+} uptake and outward translocation of phosphatidylserine during the lipase treatment.

TABLE II

Influence of divalent cations on exposure of phosphatidylserine to the outer membrane leaflet of erythrocytes

(A) Freshly washed cells were exposed (5 min, 37°C) to phospholipase A_2 in the presence of Ca^{2+} and Mg^{2+} (0.25 mmol · l^{-1}) and ionophore A23187 (10 μmol · l^{-1}).
(B) In another set of experiments cells were pretreated with ionophore (10 μmol · l^{-1}, 10 min, 37°C) and either no additive, or EDTA, or Ca^{2+}, or Mn^{2+} (lines B1–B5). Subsequently, cells were washed sequentially with EDTA, albumin and Medium A.
(C) Alternatively, ATP depleted cells (see Methods) were treated with Ca^{2+}/ionophore (line C1). Subsequently, cells were washed sequentially with EDTA, albumin and Medium A. In another set of experiments cells were ATP depleted and then incubated for 16 h at 37°C (line C2).
(D) Freshly washed (line D1) and cells incubated for 16 h at 37°C with inosine (line D2) in the absence of Ca^{2+}/ionophore) served as controls.
The pretreated cells (lines B–D) were exposed to phospholipase A_2 (0.25 mmol · l^{-1} Ca^{2+} and Mg^{2+}, 5 min, 37°C, see Methods). PC, PE and PS are phosphatidyl-choline, -ethanolamine and -serine, respectively. Data represent mean values (± S.D.) of 2–4 experiments.

Experimental conditions		% degradation of		
		PC	PE	PS
A. No pretreatment, ionophore and Ca^{2+} (Mg^{2+}) present during lipase		77 ± 6	20 ± 6	22 ± 12
B. Pretreatment with ionophore in presence of	(mmol · l^{-1})			
1. Ca^{2+}	0.1	59 ± 5	23 ± 10	14 ± 4
2. Ca^{2+}	0.25	61 ± 9	25 ± 11	16 ± 2
3. Ca^{2+}	0	51	13	0
4. EDTA	5	38	0	0
5. Mn^{2+}	4	59	0	0
C. ATP depletion, then				
1. Ionophore and Ca^{2+}, 0.1 mmol · l^{-1}		61 ± 10	24 ± 10	20 ± 5
2. Incubation for 16 h, 37°C (no Ca^{2+}, no ionophore)		58 ± 5	16 ± 2	< 5
D. No Ca^{2+}, no ionophore				
1. No incubation		52 ± 3	0	0
2. Incubation for 16 h, 37°C		55 ± 7	7 ± 7	0

Effects of divalent cations on erythrocyte shape and microvesiculation

Loading of erythrocytes with Ca^{2+} or Sr^{2+} at concentrations which accelerate flip transforms biconcave erythrocytes into echinocytes. Incubation of cells for 15 min in media containing 0.1 mmol · l^{-1} Ca^{2+} or 0.5 mmol · l^{-1} Sr^{2+} in the presence of ionophore (10 μmol · l^{-1}) produces echinocytes type III, whereas incubation with 0.5 mmol · l^{-1} Ca^{2+} or 1 mmol · l^{-1} Sr^{2+} produces spheroechinocytes. Since spheroechinocytosis will ultimately lead to the shedding of microvesicles [25], we looked for this process by quantifying the appearance of phospholipid in the supernatant. After a 15 min exposure to 0.1 mmol · l^{-1} Ca^{2+} or 0.5 mmol · l^{-1} Sr^{2+} no significant release of vesicles could be detected. After 30 min at 0.5 mmol · l^{-1} Ca^{2+} or Sr^{2+} about 2% of the phospholipid are released as microvesicles. Incubation of cells with Mn^{2+} and ionophore does not produce speroechinocytes and microvesicle release.

No significant release of microvesicles (< 0.5%) is detectable after exposure (5 min) of Ca^{2+}-pretreated cells (shown in Table II, line B1, B2) to the lipase.

Discussion

Incubation of erythrocytes with Ca^{2+} or Sr^{2+} in the presence of ionophore A23187 accelerates the translocation of exogenously inserted lipid probes to the inner

membrane leaflet. At high extracellular levels of Ca^{2+} an initial acceleration of flip is followed by a suppression of flip in parallel to the time-dependent attainment of high intracellular Ca^{2+} levels. Flip acceleration is specific for Ca^{2+} and Sr^{2+}. Mg^{2+}, Mn^{2+}, Zn^{2+} and La^{3+} do not accelerate the flip, although these cations are transported into the cells by the ionophore [30,31]. Flip acceleration may be taken to result from an increase of the intracellular concentration of Ca^{2+} for the following reasons. One, ATP depletion of cells, which blocks active outward Ca^{2+} transport, increases the susceptibility of cells to Ca^{2+} Two, the time-point of the onset of flip acceleration after addition of the ionophore depends on its concentration. At low concentrations of ionophore and extracellular Ca^{2+} flip acceleration starts only after a lag phase, which is shortened by an increase of the Ca^{2+} or ionophore concentration.

An involvement in flip acceleration of a number of biochemical processes known to be triggered by an increase of intracellular Ca^{2+} can be excluded. After blockage of Ca^{2+} pump activity by ATP-depletion of the cells, flip acceleration became evident at an extracellular Ca^{2+} concentration of 13 $\mu mol \cdot l^{-1}$ (Fig. 5). Assuming a Donnan distribution, this corresponds to an intracellular Ca^{2+} concentration of about 30 $\mu mol \cdot l^{-1}$. This Ca^{2+} concentration is close to that required for the first activation of intracellular calpain I and phospholipase C [2,3]. However, a causal role of calpain-mediated degradation of membrane proteins or of a decrease of the polyphosphatidylinositide level by phospholipase C as well as of calmodulin-dependent processes in Ca^{2+}-induced flip acceleration could be excluded.

Alternatively, the Ca^{2+}-induced flip acceleration might be assigned to the interaction of Ca^{2+} with the negatively charged headgroups of the inner membrane layer lipid phosphatidylserine. Addition of divalent cations to monolayers and lipid bilayer membranes containing phosphatidylserine has been shown to result in an increase of the packing density of the phospholipid [36], lipid phase separation [13,37], decrease of the surface dielectric constant [38], the formation of non-bilayer structures [12,13], the acceleration of phospholipid flip [39] and the fusion of membranes [37,40]. A possible causal involvement of a perturbation of the inner phospholipid leaflet by Ca^{2+} or Sr^{2+} in flip acceleration would assume a selective interaction of these cations at submillimolar concentrations with the polar headgroups of the inner leaflet phospholipids. This seems to be unlikely since the strength of interaction of Mg^{2+}, Mn^{2+} and La^{3+} with phosphatidylserine are at least comparable to that for the smaller Ca^{2+} and Sr^{2+} ions [40–42]. On the other hand, a causal role of a stabilization of the inner membrane lipid leaflet by divalent cations in the unselective slow down of flip at millimolar levels of divalent cations (Fig. 8) seems possible.

Elevation of the intracellular Ca^{2+} level is known to transform biconcave erythrocytes into echinocytes [43,44] and, finally, spheroechinocytes from which microvesicles are released [2,3]. It could be argued that such a release process, which requires fusion of opposing membrane domains, produces at least transient membrane defects acting as flip sites for phospholipids. However, microvesicle release from cells (see Results) and cell fusion [45] requires Ca^{2+} or Sr^{2+} concentrations higher than those required to obtain the first flip acceleration. Microvesicle release in our studies is low, since studies were done at high extracellular K^+ levels which prevents cell shrinking due to activation of the Ca^{2+}-activated K^+ channel [10] and diminishes microvesiculation as compared to high Na^+ levels [2]. Interestingly, a Ca^{2+}-induced change of membrane structure preceding echinocytosis has been reported by two groups [43,44]. Shortly after addition of Ca^{2+} and ionophore to erythrocytes they develop cone-shaped extrusions (blebs) on their biconcave surface [43,44]. In the region of these blebs the submembranous space seen by transmission electron microscopy looks 'empty' [44]. It might therefore be postulated that flip acceleration by Ca^{2+} and Sr^{2+} is the result of membrane destabilisation as a consequence of a local detachment of skeletal proteins from the intrinsic membrane domain.

The flip acceleration by Ca^{2+} is accompanied by a very fast exposure of the inner membrane leaflet phospholipid phosphatidylserine to the outer membrane surface as detectable by its cleavability during a 5 min exposure to phospholipase A_2. The normal outward translocation of phosphatidylserine with a half time of 1 h at 37°C in the native human erythrocyte [46] is too slow to explain the fast accumulation of phosphatidylserine in the outer leaflet. At present we can only list a number of explanations not likely to account for Ca^{2+}-induced phosphatidylserine translocation. Since in our experiments vesicle release is low phosphatidylserine exposure can not be due to transient formation of flip sites during fusion which is required for microvesicle release. Phosphatidylserine exposure can not be due to enhanced translocation of this phospholipid during the lipase treatment, since it outlasts normalization of flip rate by removal of Ca^{2+} and ionophore. A mere inhibition of inward translocation due to removal by the ionophore of Mg^{2+} or ATP depletion by increased pumping rates following Ca^{2+} entry does not produce phosphatidylserine exposure (Table II).

The fast outward translocation of phosphatidylserine and accelerated flip rates of our probes are probably not tightly coupled. At Ca^{2+} concentrations of 0.1–0.5 $mmol \cdot l^{-1}$ (10 $\mu mol \cdot l^{-1}$ ionophore) the fraction of phosphatidylserine translocated to the outer leaflet does not significantly increase after a fast initial (< 5 min) translocation of a 15–20% fraction (Table II, Ref. 19), although the phase of flip acceleration lasts for 45–150

min (Fig. 1). In contrast to platelets [14] no equi-distribution of phosphatidylserine between the membrane leaflets of the erythrocyte occurs (Table II, Ref. 19). Assuming a suppression of flippase activity under our experimental conditions the incomplete loss of asymmetry may be due to an unfavourable ratio of the rate of the Ca^{2+}-induced outward translocation of phosphatidylserine and the time course of normalization of the tranversal lipid mobility, as detectable by the lipid probes (Fig. 1). As an alternative explanation the incomplete loss of asymmetry of phosphatidylserine in erythrocytes might be due to stabilizing forces of the membrane skeleton on this asymmetry. This stabilization is essentially maintained in Ca^{2+}-loaded erythrocytes, since in contrast to platelets [14] no proteolytic breakdown of skeletal proteins but only degradation of anchoring proteins occurs in the presence of internal Ca^{2+} (Fig. 6).

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