Micropipette Aspiration of Human Erythrocytes Induces Echinocytes via Membrane Phospholipid Translocation

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ABSTRACT When a discocytic erythrocyte (RBC) was partially aspirated into a 1.5-μm glass pipette with a high negative aspiration pressure ($\Delta P = -3.9$ kPa), held in the pipette for 30 s (holding time, t_h), and then released, it underwent a discocyte-echinocyte shape transformation. The degree of shape transformation increased with an increase in t_h. The echinocytes recovered spontaneously to discocytes in ~10 min, and there was no significant difference in recovery time at 20.9°C, 29.5°C, and 37.4°C, respectively. At 11°C the recovery time was significantly elevated to 40.1 ± 6.7 min. At 20.9°C the shape recovery time varied directly with the isotropic RBC tension induced by the pipetting. Sodium orthovanadate (vanadate, 200 μM), which inhibits the phospholipid translocase, blocks the shape recovery. Chlorpromazine (CP, 25 μM) reversed the pipette-induced echinocytic shape to discocytic in <2 min, and the RBC became a spherostomatocyte-II after another 30 min. It was hypothesized that the increase in cytosolic pressure during the pipette aspiration induced an isotropic tension in the RBC membrane followed by a net inside-to-outside membrane lipid translocation. After a sudden release of the aspiration pressure the cytosolic pressure and the membrane tension normalized immediately, but the translocated phospholipids remained temporarily "trapped" in the outer layer, causing an area excess and hence the echinocytic shape. The phospholipid translocase activity, when not inhibited by vanadate, caused a gradual return of the translocated phospholipids to the inner layer, and the RBC shape recovered with time.

INTRODUCTION

When micropipette aspiration is used to measure RBC membrane shear modulus and RBC relaxation times (Chien et al., 1978; Hochmuth et al., 1979; Evans, 1983; Mohandas and Chasis, 1993, Artmann, 1995), small aspiration pressures are applied and only a small portion of the membrane is aspirated into the pipette. Such aspirations do not cause shape change of the discocytic RBC after its release from the pipette. However, when a discocytic RBC is aspirated into a pipette with a diameter of ~1.5 μm by using high negative aspiration pressures (e.g., −3.9 kPa), it becomes echinocytic after release. Such discocytic-echinocytic shape transformation following aspiration and release has been observed previously, but it was either not explained or considered to be an artifact (Rand, 1964; Jay, 1973; Nash and Meiselman, 1985).

It is generally accepted that the discocytic shape of the normal RBC and the inducible shape alterations are primarily influenced by the lipid distribution in the membrane bilayer (Zachowski and Devaux, 1990; Mohandas and Chasis, 1993). The bilayer couple mechanism accounts for the echinocytic or the stomatocytic shape changes observed in response to a variety of chemical agents (Deuticke, 1968; Bessis et al., 1973; Artmann et al., 1996). According to the bilayer couple hypothesis (Evans, 1974; Sheetz and Singer, 1974; Sheetz et al., 1976; Sackmann et al., 1986; Farge and Devaux, 1992), echinocytic shape results when the surface area of the outer layer increases relative to the inner layer, whereas a stomatocytic shape results when the surface area of the inner layer increases relative to the outer layer (Kuypers et al., 1984; Fischer, 1993). The lipid bilayer of the RBC membrane is asymmetric in its organization and composition (Daleke and Huestis, 1989; Sugihara et al., 1992; Devaux and Zachowski, 1994). This asymmetry is maintained by the ATP-dependent phospholipid translocase (Seigneuret and Devaux, 1984; Daleke and Huestis, 1985, Zachowski et al., 1986) which can be inhibited by sodium orthovanadate (Lin et al., 1994). If the translocase has been inhibited, the transmembrane diffusion of lipids between the two leaflets would disturb this asymmetry (Van Meer et al., 1980; Devaux and Zachowski, 1994). Several membrane-active substances can change the lipid distribution (Lin et al., 1994; Devaux and Zachowski, 1994; Schrier et al., 1992a) and usually lead to RBC morphology changes (Deuticke, 1968; Bessis et al., 1973; Daleke and Huestis, 1989; Lin et al., 1994).

We propose the hypothesis that the discocytic-echinocytic shape transformation is due to a pipette-induced transmembrane phospholipid translocation and the ensuing area excess of the outer lipid layer. To test this hypothesis, we studied the echinocytic shape change after the release of RBCs that had been aspirated into a small micropipette (with a typical inner diameter of 1.5 μm) under a high negative pressure.

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METHODS

Materials

EDTA, chlorpromazine hydrochloride (CP) ($C_{17}H_{20}Cl_2N_2S$, molecular weight 355.3), and bovine serum albumin (BSA, 96–99% albumin) were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium orthovanadate (vanadate, Na_3VO_4, 99%, molecular weight 183.91) was purchased from Janssen Chimica, Geel, Belgium. A phosphate-buffered saline (PBS: 300 ± 5 mOsmol/l, pH 7.4 ± 0.1, containing 1 mM EDTA) was used as the standard suspension solution.

RBC suspension preparation

Approximately 120 μl of blood was obtained from the finger tip of healthy adult donors and added to 10 ml PBS without BSA; the RBCs were immediately washed twice with PBS at 3500 rpm for 10 min. The final RBC suspension was prepared by re-suspending 20 μl of packed RBCs in 50 μl PBS.

Micropipette aspiration

A small rectangular micropipette chamber was glued on top of a coverslip (0.17 mm thickness) and mounted on the heating stage of a temperature controller (TS-4 Controller, Sensortec Inc., Clifton, NJ). The suspension temperature was monitored continuously using a Fluke 52 thermometer (John Fluke Mfg. Co., Everett, WA). The micropipettes were pulled with the use of a Flaming brown micropipette puller (type P80/PC, Sutter Instruments Co., San Rafael, CA). With appropriate settings of the puller, the pipettes were made with the inner radius ranging from 0.5 to 1 μm. The wall thickness was ~0.2 μm and the opening angle was ~9°. The negative aspiration pressure, which was induced hydrostatically and kept constant during the aspiration time, was between −2.9 and −3.9 kPa.

Observations of the micropipette chamber system were made with an inverted microscope (Nikon, Japan), using an immersion oil lens objective (100 × NA 1.25) and a 20× eye piece. The video image was monitored and recorded with the use of a CCD-camera type NC-67 M (Dage-MTI Incorp., Michigan City, IN) and a video recorder BV-1000 (Mitsubishi, Japan). The time base was provided by a video timer model VTG-33 connected in series with a digital time base corrector model FA-200 (both FOR-A Co., Japan). The photographs were printed from the video tapes using a video printer type UP 5000 (Sony, Japan).

Induction of echinocytes with the use of micropipettes

Five μl of the RBC suspension was added to the chamber, which had been pre-filled with 250 μl PBS without BSA. The RBCs were allowed to settle for 10 min to attach to the chamber bottom. To reverse the "glass effect" (Trotter, 1956; Erikson, 1990), the PBS without BSA was gently replaced by PBS with 0.5 g % BSA, while the RBCs remained attached to the glass. The micropipette tip was positioned at the edge of a RBC. The membrane was aspirated into the pipette by using a step negative pressure. This step negative pressure was maintained for a predetermined period of holding time (t_h, usually 30 s) and then removed as a step function. This led to a time-dependent recession of the aspirated segment in the micropipette, ending in the total exit of the cell. During and after the aspiration and release processes, the cell remained attached to the chamber bottom and was continuously observed for shape recovery studies.

Vanadate and chlorpromazine experiments

After the attachment of RBCs and the reversal of the glass effect, vanadate was added to the chamber at a final concentration of 200 μM. The pipette aspiration experiment was carried out as described above in the presence of vanadate.

The experiments with chlorpromazine hydrochloride (CP) were carried out with a new RBC preparation. To obtain the photograph shown in Fig. 8, the pipetting was carried out (see Fig. 8, *a–c*) in PBS containing 0.5 g % BSA. After the RBC was released from the pipette, this solution was gently replaced by PBS containing 0.5 g % BSA and 25 μM CP, while the RBCs remained attached to the glass.

Studies on shape recovery time

The RBC shape was monitored as a function of time. A sufficient number of RBCs were measured for statistical analysis of shape recovery from the echinocytosis. After the study of each new RBC, all RBCs pipetted before in the experiment were again brought into the view of the video camera to check their actual shape. After the completion of the pipette experiments, video photographs were printed from video tapes. The shape recovery of each single cell could be followed in these photographs to quantify the shape recovery time.

To quantify the type of RBC shapes, the echinocytes were assigned scores of +1 to +5 (increasing values denote more severe crenation) (Bessis et al., 1973). Discocytes were scored at 0. The average score of the RBCs studied was defined as the morphological index (MI) (Daleke and Huestis, 1989). Additionally, the number of spicules visible on the top half of the cell was counted. Assuming the spicules were equally spaced, the total number of spicules per cell was calculated as twice the number of visible spicules. The shape recovery time was defined as the time interval between the time of exit of the echinocyte from the pipette and the time when no more spicules were observed on the discocytic RBC surface.

Calculation of the RBC membrane isotropic tension

About 5 s after the RBC was aspirated into the pipette, the RBC diameter of the cell portion outside the pipette and the pipette diameter were determined from the video recordings with an image analysis system. The RBC membrane isotropic tension T_o was calculated

$$T_o = (\Delta P \cdot d_p/4(1 - d_p/d_c)$$

where d_p and d_c are the pipette inner diameter and the diameter of the spherical portion of the RBC outside the pipette. ΔP is the aspiration pressure (Rand, 1964; Evans et al., 1976).

RESULTS

When a discocytic RBC was aspirated into a small-diameter glass pipette (d_p~1.5 μm) with the application of the negative pressure of −3.9 kPa, the length of the aspirated tongue inside the pipette increased rapidly with the induction of the aspiration pressure, and more gradually during the following seconds of aspiration. Within the duration of the 30-s aspiration, no further increase of tongue length was visible. At this time, occasionally a vesicle was formed at the end of the tongue and became detached from the cell. Also, in very rare cases, some cells were able to pass into the 1.5-μm pipette or were hemolyzed during the aspiration. These cells were not considered in our analysis. Permanent RBC deformations or "bumps" were not observed at the experimental conditions used in our experiments. After the sudden release of the aspiration pressure as a step function, the tongue length inside the pipette decreased with time. While there was still a tongue segment inside the pipette, the

outer nonaspirated part of the cell already formed spicules. After ~10 s, the cell was completely detached from the pipette, but not from the cover glass surface at the chamber bottom. As shown in Fig. 1, the RBC underwent a *discocyte-echinocyte shape transformation* after its exit from the pipette. At a suspension temperature of 20.9°C and with a pipette inner diameter d_p = 1.45 μm, t_h = 30 s, and ΔP = −3.9 kPa, the mean pipette-induced shape index was 3.2 ± 1.5 (mean ± SD, N = 47 RBCs), which corresponds to echinocyte III, and the mean total number of spicules per cell was 18.8 ± 3.3. The total number of spicules was positively and linearly correlated (r = 0.97) with the shape change induced with the pipette (Fig. 2). Some pre-investigations obtained on cells free in solution showed that the pipette-induced discocyte-echinocyte shape transformation also occurred. However it was hard to keep track of them after the pipetting.

To follow the shape recovery of a sufficient number of single RBCs after their release from the pipette, cells attached to the chamber bottom were used in all experiments. This enabled us to measure the shape recovery time of each individual cell. The average time needed to completely recover to the discocytic shape was 15.6 ± 8.0 min under the experimental conditions described above (20.9°C). As shown in Fig. 3 *a*, the shape recovery time showed a significant positive correlation with the pipette-induced total number of spicules (r = 0.73). The linear regression yielded a value of 0.88 min/spicule at 20.9°C.

The RBC membrane isotropic tension induced by the pipetting was calculated and correlated with the shape recovery time. The best-fit linear correlation gave r = 0.81 (Fig. 3 *b*). The slope of RBC shape recovery time versus the RBC isotropic membrane tension was 274 min/dyne/cm.

Fig. 4 *a* shows the effect of applying the same aspiration pressure (−3.9 kPa) to two RBCs for different durations: 10 s (*left*) versus 65 s (*right*). The isotropic membrane tension was T_o = 1.822 dyne/cm for the left cell and T_o = 1.860 dyne/cm for the right cell. When the left cell was released after the 10-s aspiration, it became an echinocyte I,

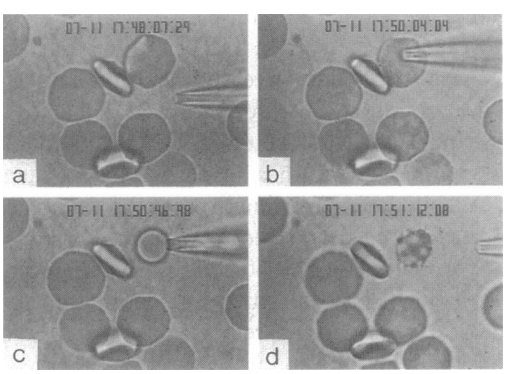

FIGURE 1 Micropipette-induced discocyte-echinocyte shape transformation (d = 1.3 μm, T = 20.9 ± 2°C, t_h = 30 s, isotropic membrane tension, T_o = 1.84 dyne/cm). The discocytic RBCs remained attached to the glass surface during the pipetting.

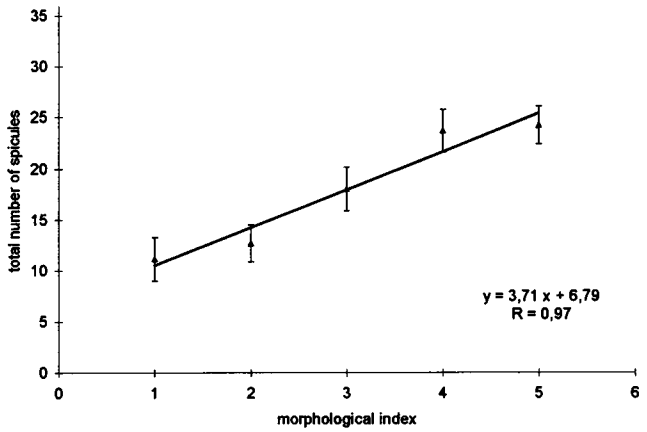

FIGURE 2 The total number of spicules was positively correlated with the degree of echinocytic transformation (positive morphological index) induced with the pipette under the same conditions as in Fig. 1.

and the right cell released after 65 s aspiration became an echinocyte III. Fig. 4, *b–i* show a sequence of photographs taken while the two cells were recovering their shapes. The echinocyte I (*left cell*) recovered after 14.1 min and the echinocyte III (*right cell*) after 32.4 min.

The shape recovery times found with these experiments were much longer than those needed to reverse the so-called "glass effect": RBCs suspended in PBS without albumin and attached to the glass bottom of the chamber became echinocytes which recovered to discocytes following the addition of 0.5 g % albumin; this shape recovery time was 2.5 min and rather uniform in all RBCs observed (Fig. 5).

To study the effect of temperature on shape recovery of pipette-induced echinocytes, studies were performed at suspension temperatures of 11.0, 29.5, and 37.4°C in addition to 20.9°C (1.76 dyne/cm < T_o < 1.86 dyne/cm). At 11°C the shape recovery time was significantly lengthened compared to those at higher temperatures. No significant difference was found among the shape recovery times at 20.9, 29.5, and 37.4°C (Fig. 6). The morphological index of the echinocytes at 11.0°C was 1.1, and this was significantly lower than those at other temperatures. In contrast to the echinocytes I induced at 11.0°C, echinocytes III were obtained at a temperature >20.9°C (MI = 3.2 at 20.9°C; MI = 3.7 at 29.5°C, and MI = 3.4 at 37.4°C).

Vanadate, known to inhibit the phospholipid translocase, did not affect the pipette-induced echinocytic shape transformation, but did effectively inhibit the shape recovery (Fig. 7). Pipette-induced echinocytes did not show any tendency to recover to their discocytic shapes in the presence of vanadate (200 μM) even after 1 h. Every single spicule remained at its position on the cell surface. At this concentration, the unpipetted RBCs remained biconcave.

In response to chlorpromazine (25 μM) normal RBCs undergo a shape transformation to sphero-stomatocytes in ~2 min (Fig. 8), whereas the pipette-induced sphero-echinocytes in the same microscopic field slowly passed through all stages of echinocytic shapes before they became

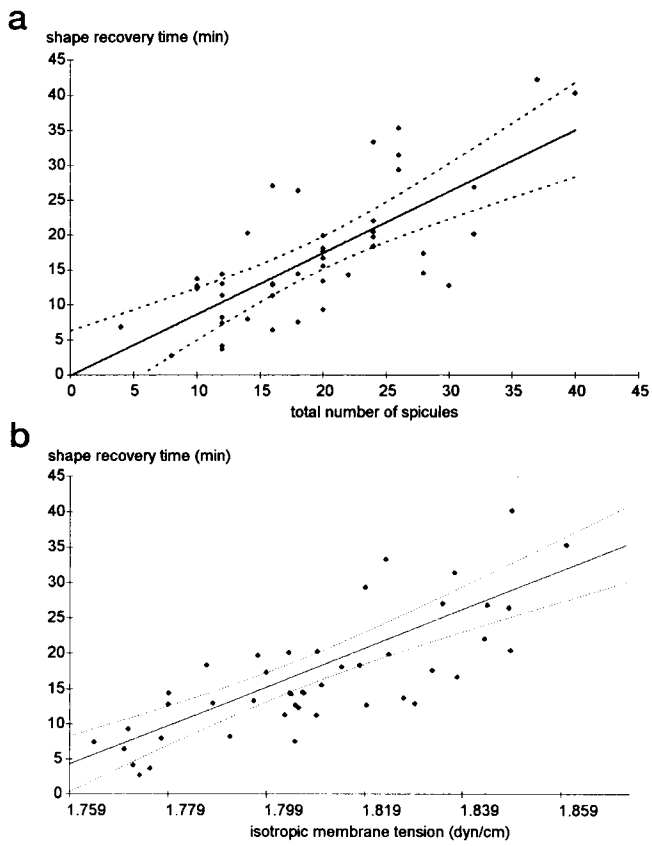

FIGURE 3 (*a*) Shape recovery time as a function of the total number of spicules induced by the pipetting (N = 47 RBCs, d = 1.4 μm, T = 20.9 ± 2°C, t = 30 s, ΔP = −3.9 kPa). The 95% confidence bands are shown. The recovery time correlates significantly (r = 0.73) with the total number of spicules induced with a slope of 0.88 min/spicule. (*b*) Shape recovery time as a function of the RBC membrane isotropic tension induced by the pipetting (for settings see *a*). The 95% confidence bands are shown. The recovery time correlates significantly (r = 0.81) with the RBC membrane isotropic tension with a slope of 274 min/dyne/cm.

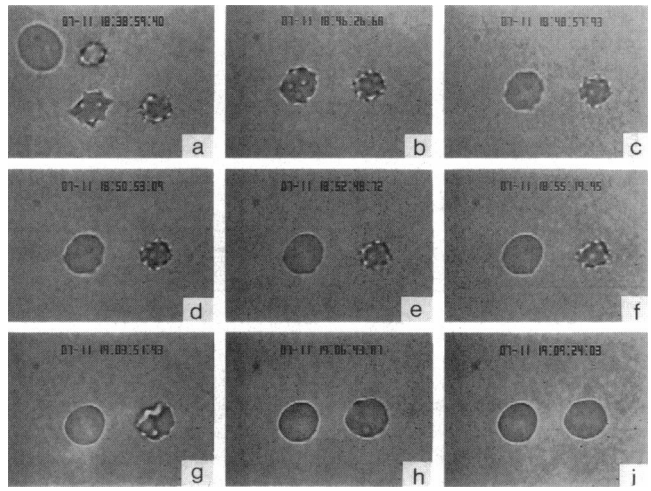

FIGURE 4 (*a–i*). Spontaneous RBC echinocytic-to-discocytic shape recovery during recovery following pipette aspiration at two different durations t_h: At t_h = 10 s and isotropic tension T_o = 1.822 dyne/cm (*left cell*, released from the pipette at 18:38:42) an echinocyte I was induced; at t_h = 65 s and T_o = 1.860 dyne/cm (*right cell*, released from the pipette at 18:37:01) an echinocyte III was induced [note that (*a*) was taken 17 s after the left RBC and 1 min 58 s after the right RBC was released from the pipette, respectively]. The echinocyte I recovered after 14.1 min (*e*), and the echinocyte III recovered after 32.4 min (*i*).

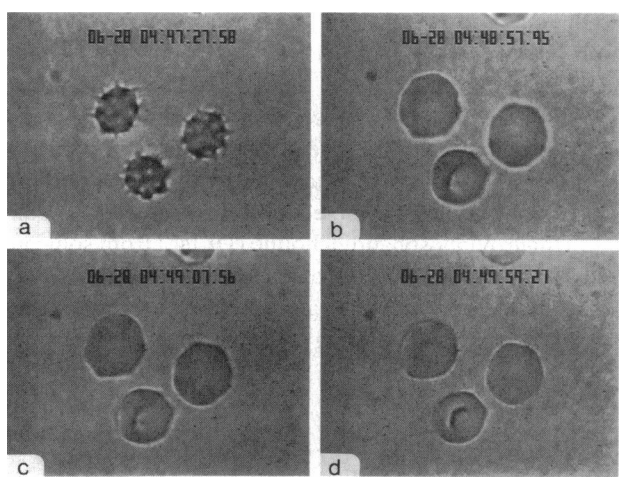

FIGURE 5 Sphero-echinocytes produced by the "glass effect" in PBS without BSA (*a*) were reversed to discocytes in PBS with BSA (final concentration 0.5 g %) at 20.9°C. The shape recovery took place in 2:30 min and was rather uniform among all RBCs (*b–d*). The BSA was added at time 4:47:29.

stomatocytic. The final occurrence of a sphero-stomatocyte for the pipetted RBC was delayed by ~10 min in comparison to the neighboring unpipetted RBC.

DISCUSSION

The present paper demonstrates the transformation of biconcave RBCs into echinocytes by pipette aspiration and subsequent removal of the RBCs from the pipette (Fig. 1). The hypothesis for the origin of this effect is as follows. During the aspiration of the RBC membrane into the pipette under a high negative pressure to create a spherical trailing segment outside the pipette, the cytosolic pressure inside the aspirated cell was increased and an isotropic tension was induced within the RBC membrane (Rand, 1964; Evans et al., 1976). This results in a net translocation of a small amount of phospholipids from the inner layer of the membrane to the outer layer. After the sudden release of tension by turning off the aspiration pressure, the tension normalized immediately. However, the translocated phospholipids remained temporarily "trapped" in the outer layer. The net number of phospholipid molecules translocated outward caused a slight area excess in the outer layer to induce the echinocytic shape (Sheetz and Singer, 1974; Sheetz et al., 1976). As a result of the phospholipid translocase activity, the outward translocated phospholipids, phosphatidylethanolamine and phosphatidylserine, were gradually flipped back to the inner layer. The area excess decreased and the RBC recovered its discocytic shape within the shape recov-

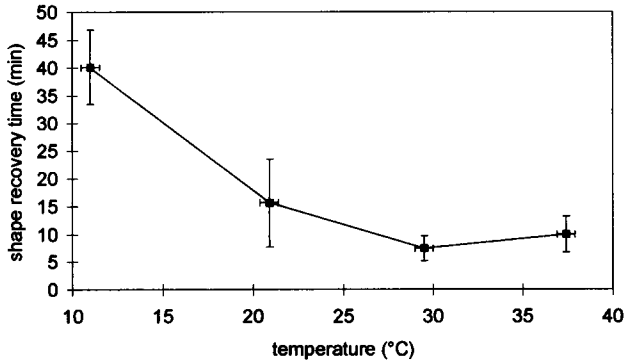

FIGURE 6 Average shape recovery time as a function of the suspension temperatures 11.0, 20.9, 29.5, and 37.4°C (d = 1.4 μm, ΔP = −3.9 kPa, 1.76 dyne/cm < T_o < 1.86 dyne/cm). At 11°C the shape recovery time was significantly elevated ($p < 0.001$) compared to those at the three higher temperatures. No significant difference was found among the shape recovery times at 20.9, 29.5, and 37.4°C.

ery times observed. The experiments reported in this paper were performed to test this hypothesis.

In the current study, the lipid excess in the outer layer is expressed by the RBC morphological index or the total number of spicules (Fig. 2). These were found to be accurate measures of the amount of foreign lipids incorporated into the outer layer and of the time course of lipid incorporation (Daleke and Huestis, 1989; Lin et al., 1994; Daleke and Huestis, 1985; Ferrel et al., 1985; Fujii and Tamura, 1983; Fujii et al., 1985). Since extremely small changes in stoichiometric bilayer balance lead to obvious morphological effects, this approach was applied to estimate the net number of phospholipid molecules translocated. The transfer of Dilauroylphosphatidylcholine (DLPC) from sonicated unilamellar vesicles into the outer leaflet of the RBC membrane caused echinocytic transformation, with the RBC morphological index varying approximately linearly with

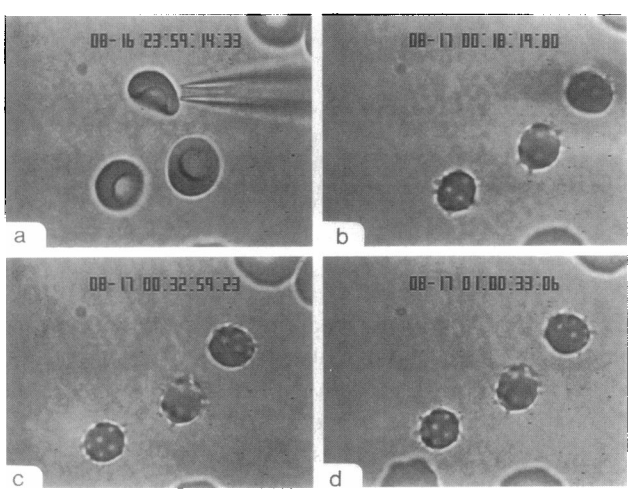

FIGURE 7 (a–d) Vanadate (200 μM) did not affect the pipette-induced echinocytic shape change (b), but did effectively inhibit the shape recovery over the 1-h period of observation (c, d).

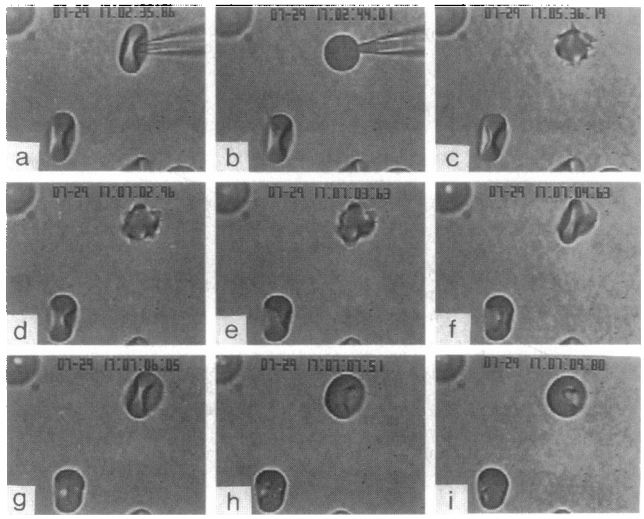

FIGURE 8 (a–i). Chlorpromazine inducing stomatocytes. a–c show the aspiration process (d = 1.1 μm, t_h = 120 s, and T_o = 1.832 dyne/cm) of a RBC in PBS (*top cell*), whereas the cell below was not pipetted. At time 17:05:36 (c) CP was applied to both RBCs at a final concentration of 25 μM. d–i show the RBC not pipetted transforming rapidly (within ~2 min) to a sphero-stomatocyte. In contrast, the pipette-induced sphero-echinocyte passed more slowly through different stages of echinocytes before it finally became stomatocytic. Its final shape was a sphero-stomatocyte (not shown).

the amount of DLPC (Daleke and Huestis, 1989; Fujii et al., 1985). Incorporation of 50 μmol DLPC per liter RBCs led to echinocytes stage +1, and 150 μmol DLPC per liter RBCs led to stage +3. These data correspond to 1.25% (stage +1) and 3.75% (stage +3) of DLPC per total RBC phospholipids, respectively (Daleke and Huestis, 1989; Fujii et al., 1985), or 1.25% per stage. Based on these data and assuming that the lipids translocated are similar in size to DLPC, we can estimate the net movement of lipid molecules from the inner layer to the outer layer during pipette-induced echinocytic transformation. According to the bilayer couple hypothesis, the shape is determined by *relative* area changes of the two leaflets. In the pipette-induced echinocytic transformation, as lipid molecules are translocated to the outer layer, the same number must have been removed from the inner layer. Therefore, the net number of lipids translocated per stage of echinocytic transformation is probably one-half of 1.25%, i.e., 0.625%. At a suspension temperature of 37.4°C we found an average pipette-induced echinocytic shape score of 3.4 ± 1.5. According to the above estimations, this would correspond to the flopping of ~2.1% of total RBC phospholipids as a result of the pipetting. Assuming that the total RBC phospholipids is 4.25 μmol/ml of packed RBCs (Broekhuyse, 1974) and 1 ml packed RBCs contains 10^{10} RBCs, the content per RBC is 4.25×10^{-16} mol. Therefore, the net number of phospholipids flopped during the pipetting was 8.93×10^{-18} mol, or 5.37×10^{6} molecules per RBC. The inner layer consists of 24% phosphatidylserine (PS), 48% phosphatidylethanolamine (PE), and 28% other lipid species (Verkleij et al.,

1973). This gives a total number of 1.29×10^6 PS molecules, 2.57×10^6 PE molecules, and 1.51×10^6 molecules of other lipid species that were translocated. The above estimation is for a net one-way-phospholipid flop. In view of the possibility of a two-way lipid flip-flop (lipid scrambling; Schrier, 1992b) due to the pipetting, the estimations represent the *minimum numbers* of these molecules appearing in the outer layer due to the pipetting.

The pipette-induced echinocytic RBC shape recovered spontaneously. The shape recovery times observed (Fig. 3 *a*) could be explained by the action of the ATP-driven phospholipid translocase, which is responsible for keeping the asymmetric lipid distribution in RBC membranes (Seigneuret and Devaux, 1984; Daleke and Huestis, 1985; Daleke and Huestis, 1989; Devaux and Zachowski, 1994). The significant and positive correlation of the shape recovery time with the total number of spicules (Fig. 3 *a*) supports the idea that the larger the amount of lipids translocated, the longer was the shape recovery time. The greater degree of echinocytic change and the faster shape recovery time in the cell pipetted for a longer period of time (Fig. 4 *a*) also support this concept.

Longer shape recovery times were also related to higher isotropic tensions (Fig. 3 *b*). This indicates that a higher isotropic membrane tension leads to a larger amount of phospholipids translocated and, consequently, longer shape recovery times. It should be noted, however, that the activity of the phospholipid translocase cannot be assumed to be the same in all RBCs investigated. The RBC population in the circulation is composed of RBCs of different ages. Older RBCs show a decreased activity of the phospholipid translocase (Zachowski and Devaux, 1990). At the same aspiration pressure, aspiration time and pipette inner diameter, older RBCs also show smaller diameters of the cell portion outside the pipette, indicating a high isotropic membrane tension (Linderkamp and Meiselman, 1982). This, together with the impaired activity of the phospholipid translocase, should cause the older cells to have very long shape recovery times.

The involvement of the aminophospholipid flippase in the shape recovery is supported by: a) a low temperature dependence, and b) vanadate sensitivity. The activity of the phospholipid translocase depends on the temperature (Zachowski et al., 1986). While the shape recovery time did not vary with temperature between 20.9°C and 37.4°C, at 11°C the shape recovery time was markedly increased (Fig. 6). The characteristics of the temperature dependency are similar to those found for spin-labeled phosphatidylserine (Zachowski et al., 1986; Zachowski and Devaux, 1990). It is possible that the PS-translocation predominates the shape recovery process.

We found that vanadate inhibited the shape recovery process of pipette-induced echinocytes (Fig. 7, *a–d*). This was most likely due to the inhibition of the phospholipid translocase (Boroske et al., 1981; Schrier et al., 1992a). At the concentration used, vanadate is broad-acting, affecting many different ATPases in the membrane. Vanadate could have stimulated the Gardos system via inhibition of the Ca^{2+}-pump and, thus, it could have mediated a Ca^{2+}-induced echinocytosis involving small traces of free Ca^{2+} present in the buffer. Since EDTA was added to it, a Ca^{2+}-effect was abolished (Fuhrmann et al., 1984; Lin et al., 1994). Furthermore, vanadate did not induce significantly different echinocytic shapes in the experiments where vanadate was present in the buffer compared to those carried out without. A possible vanadate-mediated inward transport acting against the pipette-induced lipid exchange during the pipetting therefore might have played a negligible role.

Chlorpromazine (CP) is known to have a stomatocytic effect on RBCs by causing an area excess of the inner layer (Bütikover et al., 1989; Daleke and Huestis, 1989; Schrier et al., 1992a). Consequently, CP should counteract the pipette-induced echinocytosis. This was in fact what happened (Fig. 8). The CP-induced stomatocytic shape change of the pipetted RBC (Fig. 8 *c*, *top cell*) was delayed in comparison to the unpipetted discocyte (Fig. 8 *c*, *bottom cell*). The pipetted cell returned to a discocytic shape 1.5 min after CP application, and then proceeded continuously with the CP-induced stomatocytic shape change (Fig. 8, *h* and *i*), whereas the unpipetted cell had already passed the discocytic stage and turned into a sphero-stomatocyte at this time. This result shows that the pipette-induced echinocytic shape partially was counteracted by the stomatocytogenic CP, as it is the case in echinocytes induced with echinocytogenic agents (Deuticke, 1968; Artmann et al., 1996).

Since in the pipette experiments, the RBC is in very close contact to the glass pipette, the glass effect might be involved in the formation of echinocytes (Trotter, 1956; Erikson, 1990). All experiments discussed here were carried out in PBS with 0.5 g % bovine serum albumin, which partially coats the RBC membrane but is primarily associated with the glass surface (Markle et al., 1983). RBCs suspended in buffer containing 0.5 g % albumin and sedimenting to a glass surface do not show a glass effect. The same experiment carried out without albumin, however, causes echinocytes (Fig. 5, *top left*). These "glass-induced" echinocytes were reversed to discocytes within ~2.5 min by adding albumin to the buffer (Fig. 5). Thus, the times required to reverse the "glass-effect" were much shorter than the shape recovery times of 15.6 min at room temperature observed in the pipette experiments. This mismatch in the shape recovery times suggests that there exist two different mechanisms leading to the shape recovery of echinocytes caused by the glass effect or of echinocytes induced by pipette experiments, respectively.

Shape changes of RBC can result from a mismatch between the two lipid bilayers and also from alterations in the cytoskeleton (Reinhardt et al., 1988; Sugihara et al., 1992; Mohandas and Chasis, 1993; Devaux and Zachowski, 1994). Therefore, we need to address the possibility that the pipette-induced shape changes could result from mechanical disruption of cytoskeletal protein bonds and the subsequent bond reformation during shape recovery. The RBC defor-

mation in the pipette aspiration experiments is asymmetric and a partial disruption of protein bonds may occur *locally* (Discher et al., 1994). This, however, should lead to an asymmetric RBC shape after the pipetting, but that was not observed (Fig. 1). Furthermore, vanadate, which inhibited the shape recovery process, is not known to inhibit the formation of protein bonds. Therefore, our results indicate that cytoskeleton reorganization is not the primary factor in the pipette-induced echinocytic-discocytic shape transformation. In contrast to Markle et al. (1983), permanent deformations or "bumps" were also not observed in our experiments. This might be due to the fact that the aspiration time chosen (typically 30 s) was too short to allow force relaxation and permanent deformation in the RBC membranes. The characteristic time constant for force relaxation according to Markle et al. was 62.8 min at 0.1 g % albumin and 120.7 min at 1.0 g % albumin, respectively. Thus, force relaxation and "bump" formation would be expected at much longer aspiration times.

In summary, the results in the present study provide evidence that a pipette-induced phospholipid translocation from the inner to the outer leaflet of the RBC membrane occurs at high aspiration pressures. A more direct proof, however, is still needed in future studies.

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