J. Membrane Biol. 107, 105–117 (1989)

The Journal of
Membrane Biology

Volume-Dependent Regulation of Ion Transport and Membrane Phosphorylation in Human and Rat Erythrocytes

Sergei N. Orlov, Nikolai I. Pokudin, Yuri V. Kotelevtsev, and Pavel V. Gulak
Central Research Laboratory of the Ministry of Public Health of the USSR, Moscow 121359, USSR

Summary. Osmotic swelling of human and rat erythrocytes does not induce regulatory volume decrease. Regulatory volume increase was observed in shrunken erythrocytes of rats only. This reaction was blocked by the inhibitors of Na^+/H^+ exchange. Cytoplasmic acidification in erythrocytes of both species increases the amiloride-inhibited component of ^{22}Na influx by five- to eightfold. Both the osmotic and isosmotic shrinkage of rat erythrocytes results in the 10- to 30-fold increase of amiloride-inhibited ^{22}Na influx and a two-fold increase of furosemide-inhibited ^{86}Rb influx. We failed to indicate any significant changes of these ion transport systems in shrunken human erythrocytes. The shrinking of quin 2-loaded human and rat erythrocytes results in the two- to threefold increase of the rate of ^{45}Ca influx, which is completely blocked by amiloride. The dependence of volume-induced ^{22}Na influx in rat erythrocytes and ^{45}Ca influx in human erythrocytes on amiloride concentration does not differ. The rate of ^{45}Ca influx in resealed ghosts was reduced by one order of magnitude when intravesicular potassium and sodium were replaced by choline. It is assumed that the erythrocyte shrinkage increases the rate of a nonselective $Ca_o^{2+}/(Na_i^+, K_i^+)$ exchange. Erythrocyte shrinking does not induce significant phosphorylation of membrane protein but increases the ^{32}P incorporation in diphosphoinositides. The effect of shrinkage on the ^{32}P labeling of phosphoinositides is diminished after addition of amiloride. It is assumed that volume-induced phosphoinositide response plays an essential role in the mechanism of the activation of transmembrane ion movements.

Key Words cell volume · cation transport · polyphosphoinositides · erythrocytes

Introduction

It is known that the primary change of a cell volume (V_C) induced by variation of the osmotic pressure (π) are satisfactorily described by the Boyle-van't Hoff law for an ideal osmometer $V_c = 1/\pi$. However, after a certain period the cells of most tissues tend to restore their volume to the initial value (Grinstein et al., 1984). It was shown that this cellular behavior, called regulatory volume increase (RVI) and regulatory volume decrease (RVD), is connected with the activation of ion transport systems. Thus, for example, RVD may be caused by the loss of potassium via the several-fold increase of K^+, Cl^--cotransport (erythrocytes of fish (Lauf, 1982), dog (Parker, 1983), and LK-erythrocytes of sheep (Lauf, 1984)), the opening of potassium and chloride channels (human lymphocytes (Grinstein et al., 1984), rat hepatocytes (Bakker-Grunwald, 1983), Ehrlich ascites tumor cells (Hoffman, Simonsen & Lambert, 1984)) or the increase of the rate of K_i^+/H_o^+ exchange (*Amphiuma* erythrocytes (Cala, 1983)). As a rule, RVI is caused by the increase of the rate of Na_o^+/H_i^+ exchange (human lymphocytes (Grinstein et al., 1984; Grinstein & Rothstein, 1986)) or Na_o^+, K_o^+, $2Cl_o^-$-cotransport (avian erythrocytes (Schmidt & McManus, 1977)).

The data on the volume-dependent regulation of ion fluxes in human and rat erythrocytes are limited and contradictory. Duhm and Göbel (1982) had not found any changes of potassium (^{86}Rb) transport in osmotically shrunken human erythrocytes but have indicated a 1.5-fold increase of the furosemide-insensitive ^{86}Rb influx in swollen cells. The analysis of ^{86}Rb influx carried out in erythrocytes of a large group of patients established the positive correlation between the furosemide-inhibited component of the isotope flux and mean cellular hemoglobin content (Duhm & Göbel, 1984*a*) as well as between ^{86}Rb influx and the average cell volume (O'Neill & Mikkelsen, 1987). The osmotic swelling of human erythrocytes increased the furosemide-inhibited component of ^{86}Rb influx only by 30–40% (O'Neill & Mikkelsen, 1987). A negligible increase (10–20%) of the rate of ^{86}Rb influx was observed in osmotically shrunken rat erythrocytes (Duhm & Göbel, 1984*b*). Adragna and Tosteson (1984) demonstrated a reduction in furosemide-sensitive potassium efflux with isosmotic, but not osmotic, cell swelling and an increase in furosemide-sensitive sodium ef-

flux with isosmotic shrinkage of human erythrocytes treated with 2,5-chloromercuribenzoate. On the contrary, the N-ethylmaleimide-stimulated but not the basal ^{86}Rb influx was found to be volume-dependent in intact human erythrocytes (Lauf, Perkis & Adragna, 1985).

Here we report that RVD is practically absent both in human and rat erythrocytes. The nearly equal $\Delta\mu_{H^+}$ (electrochemical proton gradient)-induced Na^+/H^+ exchange has been indicated in erythrocytes of both species. However, a strong activation of Na^+/H^+ exchange by cell shrinking as well as RVI was observed in rat erythrocytes only. We also disclosed that the shrinking of human and rat erythrocytes is accompanied by the two- to threefold increase of the rate of ^{45}Ca influx that is probably due to the activation of noneselective $Ca_o^{2+}/(Na^+, K^+)_i$ countertransport. The volume-dependent calcium influx is completely blocked by amiloride, similar to the Na^+/H^+ exchange. The trigger mechanisms of RVI and RVD are unknown. In this connection we studied the possibility of volume regulation by membrane protein and polyphosphoinositide phosphorylation.

Materials and Methods

Erythrocytes

Donor blood (NIP, SNO) and blood of 14–22 week-old male Kyoto-Wistar rats were used. The samples of blood containing heparin (30–50 IU/ml) were kept on ice no more than 2–3 hr. After centrifugation (2,000 × *g*, 10 min) plasma and white blood cells were removed and erythrocytes were washed twice with saline solution containing 5 mM sodium phosphate (pH 7.4) and once more with medium A, consisting of (in mM): 130 NaCl, 3 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 1 K_2HPO_4, 10 glucose, 30 HEPES-Tris (pH 7.4, 37°C). It was shown with ^3H-inulin that the volume of extracellular water in packed rat and human erythrocytes did not exceed 2–3%.

Erythrocyte Ghosts

Hemolysis of erythrocytes was carried out with 20 vol of 5 mM sodium phosphate solution (pH 8.0, 0–2°C). After sedimentation (25,000 × *g*, 20 min) the membrane pellet was washed twice and adjusted by the hemolysis medium to protein concentration of 3–4 mg/ml. Protein content in samples was determined by Lowry's methods.

Erythrocyte Shrinking

To decrease cell volume by the osmotic method, erythrocytes were transferred in hypertonic medium B, which contained the same components as medium A and 0.35 M sucrose. The isosmotic method used in this study was based on the loss of intracellular potassium chloride due to valinomycin (2.5 μM)-induced membrane hyperpolarization.

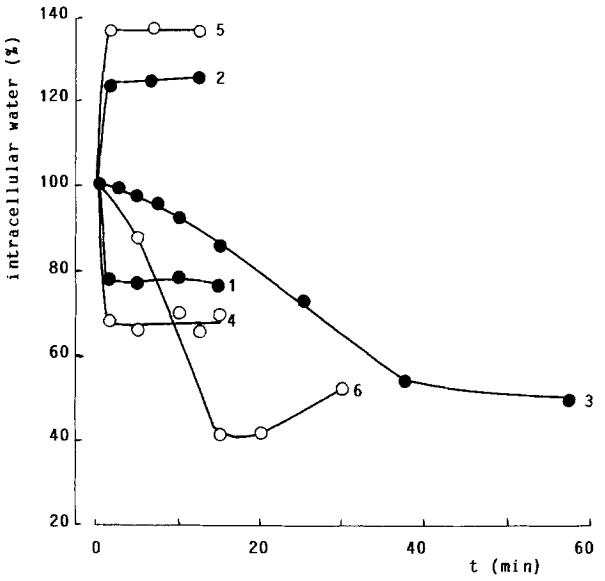

Fig. 1. The volume of the intracellular water of human (●) and rat (○) erythrocytes incubated in medium B (*1*, *4*), C (*2*, *5*) or in medium A containing 2.5 μM of valinomycin (*3*, *6*)

Erythrocyte Swelling

Erythrocytes were transferred into hyposmotic medium C, which contained the same components as medium A, but NaCl was 85 mM.

Cell Volume Control

Light scattering of the erythrocyte suspension was continuously monitored. Additionally, the intracellular water volume was determined at the fixed times of incubation. The light scattering was measured in erythrocyte suspension with hematocrit (H_k) 0.05% on MPF-4 spectrofluorimeter (Hitachi) at light wavelengths and slits 600 and 5 nm, respectively. To determine the relative cell volume changes erythrocytes were incubated at 37°C (H_k = 10%) in the control medium (medium A), in the same medium containing 2.5 μM valinomycin, and in medium B or C. In all media 2.4 μCi/ml of ^3H_2O was added. At fixed incubation times 1 ml of suspension was centrifuged, supernatant was carefully removed and sediment was treated by subsequent additions of 0.5 ml of 0.5% triton X-100 and 10% of TCA solutions. After centrifugation 0.8 ml of the protein-free supernatant was transferred into Bray's solution. The relative volume change was calculated as $(A_1/A_2) \times 100$ where A_1 and A_2 are the radioactivities of the control (medium A) and experimental samples, respectively. As seen from Fig. 1 the erythrocyte volume changes induced by cell transferring into media B or C are completed after 1 min. This observation is in accordance with the data on high permeability of erythrocyte membranes for water (Macey, 1977). On the contrary, the completion of the valinomycin-induced shrinkage of rat and human erythrocytes is observed at 15 and 40 min, respectively. Using anion transport inhibitor (125 μM DIDS), it was shown that the rate of valinomycin-induced eryth-

rocyte shrinking is limited by the rate of chloride efflux, which is increased by two- to threefold in rat erythrocytes as compared to human (Orlov, Pikudin & Postnov, 1988).

^{22}Na and ^{86}Rb Influx

200 μl of packed erythrocytes was transferred into 1 ml of medium A and preincubated 30 min at 37°C. The erythrocyte suspension was centrifuged for 1–2 min at 5,000 rpm (centrifuge FP-9, Finland), and cells were mixed with 0.5 ml medium A, B or C containing 4 μCi/ml ^{22}NaCl or ^{86}RbCl and in some cases 0.2 mM ouabain, 0.25 mM furosemide, 1 mM amiloride, 1 mM quinidine and 2.5 mM valinomycin. At fixed times of the incubation 200 μl of suspension was transferred into 1 ml of cold medium and centrifuged, and erythrocytes were washed twice under the same conditions. The washing medium was composed of 150 mM choline chloride and 10 mM Tris-HCl (pH 7.4; 0–2°C). To determine the radioactivity the erythrocyte pellet was treated by triton X-100 and TCA solutions as mentioned above. The kinetics of ^{22}Na and ^{86}Rb uptake were linear for 40–50 min. Sodium (^{22}Na) or potassium (^{86}Rb) influx (V) was calculated as

$$V = (A_1 - A_2)/amt$$

where A_1 and A_2 are the radioactivity of m liters of cells at 45 and 15 min of incubation (cpm), respectively, a is the specific radioactivity of the incubation medium (cpm/μmol), and t is the incubation time (0.5 hr).

To determine the total sodium and potassium content the erythrocyte pellet was washed as discribed above, lysed in 30 vol of 0.15 N HCl, centrifuged and the supernatant was diluted with water by 100-fold. The measurement of cation content was carried out with atomic absorption spectrophotometer AA-855 (Nippon Jarrel).

^{45}Ca-Influx in Quin 2-Loaded Erythrocytes

Unlike the majority of tissues, erythrocytes are not able to accumulate sufficient quantities of ^{45}Ca both because of the Ca^{2+} pump and a lack of intracellular structures containing exchangeable calcium. To increase intracellular calcium content we used the procedure of erythrocyte loading with highly selective calcium chelator described by Lew et al. (1982).

Erythrocytes were loaded with quin 2 by methods described previously (Pokudin & Orlov, 1986) with slight modification. The packed erythrocytes were diluted to H_k = 20% with medium A, containing 1% bovine serum albumin and 100 μM quin 2 AM. This suspension was incubated during 90 min at 37°C, 800 μl of suspension was centrifuged, the supernatant was removed and 1 ml of medium A was added. After 30 min preincubation the samples were centrifuged and erythrocyte pellets were mixed with 600 μl of medium A, B or C containing 4 μCi/ml $^{45}CaCl_2$. In some cases these media also contained 125 μM DIDS, 2.5 μM valinomycin as well as ion transport inhibitors at concentrations noted above. At the fixed incubation times 200 μl of cells were transferred into 1 ml of cold medium containing 150 mM NaCl, 5 mM sodium phosphate (pH 7.4) and 0.1 mM EDTA, centrifuged and washed twice in the same solution. The radioactivity accumulated by erythrocytes was determined after their treatment by triton X-100 and TCA solutions. The kinetics of ^{45}Ca uptake by quin 2-loaded erythrocytes of human and rat were linear for 60 and 20–30 min, respectively. To determine the rate of calcium influx, the incubation time was limited to 30 min.

Preliminary experiments revealed that the increase of quin 2 AM concentration in the loading medium from 50 to 200 μM did not alter the rate of ^{45}Ca influx in human erythrocytes but enhances this parameter in rat erythrocytes by two- to threefold. It was also shown that after 90 min incubation of erythrocytes with 100 μM of ^{3}H-quin 2 AM the intracellular quin 2 content was equal to 500–700 μmol per liter of packed cells. The kinetics of ^{22}Na and ^{86}Rb influx in intact and quin 2-loaded erythrocytes did not differ (*data not presented*).

^{45}Ca Influx in Resealed Ghosts

To investigate the dependence of the rate of ^{45}Ca influx on the ratio of intracellular concentrations of monovalent cations, the resealed ghosts of human erythrocytes were used. 0.5 ml of packed erythrocytes was transferred into 8.5 ml of hemolysis medium containing (in mM) 5 glucose, 1 ATP Na_2, 2 $MgCl_2$, 1 EGTA, 0.5 $CaCl_2$, 10 HEPES-Tris (pH 7.4; 0–2°C) and shaken. Two min later membrane fragments were resealed by addition of 1 ml of NaCl, KCl and choline chloride at a total concentration of 1.5 M. After 30 min incubation at 37°C the resealed ghosts were sedimented (3000 × g; 10 min), and the supernatant was removed. To determine the rate of ^{45}Ca influx, 0.2 ml of ghost suspension was transferred into 1 ml of medium A, centrifuged and mixed with 0.8 ml of the same medium containing 4 μCi/ml $^{45}CaCl_2$. To minimize the nonspecific leakage of the resealed ghosts the rate of ^{45}Ca influx was determined as a difference between the values of radioactivity obtained after 5 min incubation of vesicles at 37 and 0–2°C.

Setting of an Electrochemical Proton Gradient ($\Delta\mu_{H^+}$)

The methods described by Escobales and Canessa (1986) were used with slight modification. Two ml of packed intact or quin 2-loaded erythrocytes were placed into 8 ml of medium A. After 30 min preincubation at 37°C, pH_o values were adjusted to 6.20 and 8.00 by 0.2 N HCl and NaOH solutions containing 150 mM NaCl, respectively. To minimize the transmembrane proton exchange, 200 μM DIDS was added. Then erythrocytes were sedimented and the supernatant was removed. To determine pH_i, 200 μl of packed erythrocytes were lysed in 10 vol water. The pH_i values obtained in these experiments at pH_o 6.20, 7.40 and 8.00 were equaled to 6.50–6.70, 7.15–7.30 and 7.65–7.85, respectively. There were no significant differences between human and rat erythrocytes as well as between intact and quin 2-loaded cells (*data not presented*). The rates of ^{22}Na and ^{45}Ca influx in erythrocytes with different pH_i values were determined as described above in medium A at pH_o 8.0. It's known that producing of $\Delta\mu_{H^+}$ in erythrocytes is accompanied by a change of cell volume (Escobales & Canessa, 1986). To eliminate these differences sucrose was added to the incubation medium.

The rate of proton efflux from valinomycin-treated erythrocytes was determined by methods described by Orlov et al. (1988).

^{32}P-Labeling of Protein and Phospholipids in Erythrocytes

150 μl of packed erythrocytes were washed in medium A without K_2HPO_4 (medium E) and incubated at 37°C in the same medium

containing 0.2–0.5 mCi/ml ^{32}P-orthophosphate. After 2 hr erythrocytes were sedimented and 2 M sucrose solution in 130 mM NaCl or water were added to adjust osmolarities of medium B and C correspondingly. In some samples aliquots of medium E (as a control) or medium E containing protein kinase activators (dibutyryl-cAMP, dibutyryl-cGMP, and TPA) at concentrations noted at the caption for Fig. 5 were added. After 30 min incubation the samples were transferred to 7 ml of cold hemolysis medium (5 mM sodium phosphate, pH 8.0) and centrifuged (30,000 × *g*, 20 min). The supernatant was discarded, the membrane pellet was washed, resuspended in 0.5 ml of the same medium, and kept at −70°C for no more than 20 hr.

^{32}P-Labeling of Proteins and Phospholipids in Erythrocyte Ghosts

500 μl of erythrocyte ghosts were mixed with 475 μl of medium G containing 50 mM Tris-HCl (pH 7.4, 37°C), 2.5 mM $MgCl_2$, 170 mM KCl, 200 μM ATP Na_2, 62.5 μM Na_3VO_4 and 25 μCi/ml ^{32}P-γ-ATP. Some samples contained protein kinase activators or amiloride. After 30 min of incubation at 37°C samples were placed into 23 ml of cold medium G without radioactivity and centrifuged (25,000 × *g*, 30 min). The membranes were resuspended in 0.5 ml of the same medium and kept as mentioned above.

Resolving of Membrane Proteins

One volume of ^{32}P-labeled erythrocyte ghosts was mixed with 3 vol of medium containing 10 mM Tris-HCl (pH 7.0), 1% SDS, 2% β-mercaptoethanol, 20% glycerin and kept for 5 min in a boiling bath. After cooling, 30 μl of protein suspension was placed on the plates with 5–13% gradient of polyacrylamide gel. The separating buffer consisted of 40 mM Tris-HCl (pH 8.3), 0.1% SDS, 2 mM EGTA. The electrode buffer consisted of 40 mM Tris-HCl (pH 8.3), 20 mM sodium acetate and 0.1% SDS. Electroblotting was carried out by methods described by Towbin, Staehelin and Gordon (1979) using Trans-Blot Bio-Rad (120 V; 1.5 hr; 10°C). To visualize the protein bands, 0.05% Coomasie brilliant blue R250 solution was used. The nitrocellulose sheets were autoradiographed on X-ray film at −70°C.

Resolving of Polyphosphoinositides

Acid phospholipids were extracted from erythrocyte ghosts by methods described by Irvine, Letcher and Dawson (1984) with some modifications. One volume of ^{32}P-labeled erythrocyte ghosts was mixed with 3.75 vol of solvent containing chloroform : methanol : HCl = 125 : 250 : 1 (here and below the volume ratios are given). After addition of 1.2 vol of chloroform this cocktail was centrifuged (10 min, 500 × *g*), the upper phase was removed and the lower phase was washed twice with chloroform : methanol : 1 N HCl (3 : 48 : 47) and then evaporated. Acid phospholipids were resolved by thin-layer chromatography (Jolles et al., 1981) on Kieselgel F_{254} plates (Merck). The lipid film was dissolved in chloroform : methanol : H_2O (75 : 35 : 2) and placed on plates as a 8 × 2 mm bar. Chromatography was carried out in chloroform : aceton : methanol : acetic acid : H_2O (40 : 15 : 13 : 12 : 8). Mono- (PI), di- (PIP), and triphosphoinositides (PIP_2) (Sigma) as well as ^{14}C-phosphatidic acid (Amersham) were used as markers. Plates were dried on air and exposed on X-ray films. PIP and PIP_2 fractions were collected into vials containing toluene scintillator. The total phosphorus content in acid phospholipids was determined by the method of Petiton, Tay and Rosenfeld (1977).

Reagents

NaCl, KCl, $MgCl_2$, Na_2HPO_4, NaH_2PO_4, K_2HPO_4, Na_3VO_4, HCl, Tris—BDH (UK); HEPES, choline chloride, triton X-100, sucrose, ouabain—Serva (FRG); glucose—Merck (FRG); ATP Na_2, valinomycin, dibutyryl-cAMP, dibutyryl-cGMP, cAMP—Boehringer (FRG); TPA (12-O-tetradecanoilphorbol 13 acetate), α phorbol ester (4 α-phorbol 12,13-didecanoate), furosemide, phloretin, amiloride, quinidine, quin 2 AM—Sigma (USA); DIDS (4,4′-diisothiocyanostilbene-2,2′ disulfonic acid)—Calbiochem (USA); $^{45}CaCl_2$, ^3H_2O, ^{32}P-orthophosphate, ^{32}P-γ-ATP, ^{3}H-quin 2 AM—Amersham (UK); ^{22}NaCl, ^{86}RbCl—Isotope (USSR). Other chemicals were obtained from Soyuzchimreaktiv (USSR).

Results

Kinetics of the Cell Volume Changes Induced by Erythrocyte Shrinking and Swelling

As shown in Fig. 2, the transfer of erythrocytes in hypertonic (curve *1*) or hypotonic (curve *2*) media was accompanied by the rapid decrease or increase of the light scattering responding to the shrinking and swelling of cells, respectively (*see* Fig. 1). Neither human (*a*) nor rat (*b*) erythrocytes changed their volumes after osmotic swelling. This observation means that RVD is absent in erythrocytes of both species. On the contrary, after 10–15 min of incubation in the hypertonic medium the volume of rat shrunken erythrocytes was increased and at 70–80 min its volume reached the normal value. RVI was nearly completely blocked by such inhibitors of Na^+/H^+ exchange as amiloride (Haggerty et al., 1985) and quinidine (Lowe et al., 1985) (curves *3* and *4*). Inhibitors of Na^+, K^+ cotransport (furosemide) and Na^+/Na^+ countertransport (phloretin) did not change the kinetics of RVI (*data not presented*). We failed to indicate any significant RVI in human shrunken erythrocytes.

$\Delta\mu_{H^+}$-Induced Na^+/H^+ Exchange

The first evidence for Na_o^+/H_i^+ exchange in human erythrocytes was presented by Escobales and Canessa (1986). In this study it was shown that generation of $\Delta\mu_{H^+}$ results in an increase of the amiloride-inhibited component of ^{22}Na influx. In our experiments (pH_i = 6.58; pH_o = 8.00) this compo-

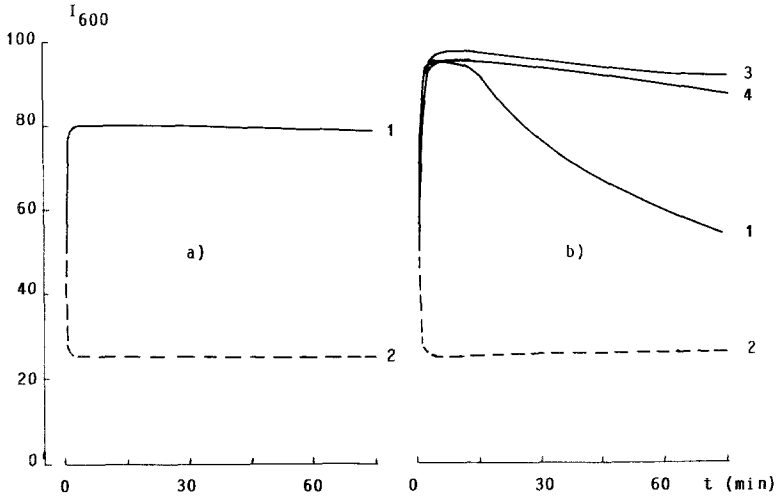

Fig. 2. The effect of hypertonicity (medium B, curves *1, 3, 4*) and hypotonicity (medium C, curve *2*) on the light scattering of human (*a*) and rat (*b*) erythrocyte suspensions. The reactions were initiated by additions into medium A of aliquots of 2 M sucrose solution (medium B) or water (medium C). *3*—the incubation medium contains 1 mM of amiloride; *4*—the incubation medium contains 1 mM of quinidine

nent was about 4 and 14 mmol per liter of cells per hr in human and rat erythrocytes, respectively (Table 1). At the physiological value of $\Delta\mu_{H^+}$ (pH_i = 7.18; pH_o = 7.40) Na^+/H^+ exchange was quenched in erythrocytes of both species.

Volume-Dependent Regulation of Sodium, Potassium, and Proton Transport

As can be seen from Table 2 shrinking of erythrocytes was accompanied by a moderate decrease in activity of Na^+, K^+-ATPase (the decrease of the ouabain-dependent component of ^{86}Rb influx by 20–40%). The significant modification of the ouabain-insensitive components of ^{22}Na and ^{86}Rb fluxes by osmotic shrinking was revealed in rat erythrocytes only. The seven- to eightfold increase in the rate of ^{22}Na influx in shrunken rat erythrocytes was blocked by the inhibitors of Na^+/H^+ exchange (amiloride and quinidine), whereas the twofold increase in the rate of ^{86}Rb influx was blocked by the inhibitor of Na^+, K^+-cotransport (furosemide). The last observation is in accordance with previously reported data (Duhm & Gobel, 1984*b*). In our experiments the increment of ^{86}Rb influx in shrunken erythrocytes was also abolished by amiloride and quinidine and diminished by phloretin (Table 2).

A more pronounced shrinking of erythrocytes may be induced by K^+-ionophore valinomycin (Fig. 1). Under these conditions a drastic increase of the intracellular sodium content was observed in rat erythrocytes only (Fig. 3). The sodium influx in rat erythrocytes was blocked by amiloride to an equal degree (Table 3). This table also displays that proton efflux from valinomycin-treated erythrocytes in sodium-free medium was markedly reduced.

Table 1. The ^{22}Na influx at different values of pH_i and pH_o

	pH_i	pH_o	Amiloride	V (μmol per liter of cells per hr)
	7.18	7.40	–	1081 ± 118
Human	7.18	7.40	+	1042 ± 65
erythrocytes	6.58	8.00	–	5730 ± 530
	6.58	8.00	+	1370 ± 201
	7.18	7.40	–	2180 ± 215
Rat	7.18	7.40	+	2443 ± 207
erythrocytes	6.58	8.00	–	17913 ± 561
	6.58	8.00	+	3670 ± 383

Regulation of ^{45}Ca Influx

The osmotic shrinking of human and rat erythrocytes was accompanied by two- to threefold increase in the rate of ^{45}Ca influx (Table 4). This effect was blocked by amiloride and quinidine and does not depend on the presence of other ion transport inhibitors. The osmotic swelling of erythrocytes does not modify the rate of ^{45}Ca influx. The addition of valinomycin (isosmotic shrinking) results in the increase of ^{45}Ca influx by 50–70%. Unlike the osmotic shrinking, the effect of valinomycin is blocked by the anion transport inhibitor (DIDS).

The rate of ^{45}Ca influx in quin 2-loaded erythrocytes does not depend on the pH_i value (*data not presented*). The selective modification of potassium and sodium content in intact erythrocytes is difficult. Taking into account this circumstance, we performed experiments using resealed ghosts. The results of this study are displayed in Fig. 4. It can be seen that the rate of ^{45}Ca influx does not depend on the Na_i^+/K_i^+ ratio, but is drastically decreased if in-

Table 2. The effect of hypertonicity (medium B) on sodium and potassium influx in human(a) and rat(b) erythrocytes (μmol per liter of cells per hr)

Additions in the incubation medium (mM)	^{22}Na influx				^{86}Rb influx			
	Medium A		Medium B		Medium A		Medium B	
	a	b	a	b	a	b	a	b
None	—	—	—	—	1326 ±125	3748 ±207	981 ±85*	5097 ±410*
Ouabain (0.20)	1001 ±94	2140 ±203	1313 ±120	15090 ±620*	247 ±48	2044 ±183	344 ±64	3971 ±294*
Ouabain and furosemide (0.25)	—	2180 ±218	—	15030 ±720*	—	2305 ±207	—	1866 ±216
Ouabain and phloretin (0.25)	—	1995 ±191	—	12001 ±306*	—	2144 ±201	—	2774 ±313
Ouabain and amiloride (1.00)	—	2483 ±233	—	1891 ±181	—	2044 ±211	—	1908 ±199
Ouabain and quinidine (1.00)	—	1999 ±201	—	1740 ±210	—	1799 ±274	—	1261 ±301

* $P < 0.05$.

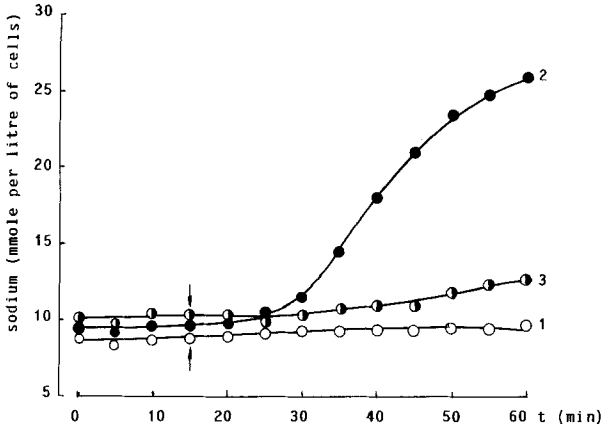

Fig. 3. Sodium content of human (*1*) and rat (*2, 3*) erythrocytes. At arrow, valinomycin (2.5 μM) was added. *3*—the incubation medium contains 1 mM of amiloride

tracellular sodium and potassium were replaced by choline. It may be noted from this figure that the affinities of Ca^{2+}-transport system for K_i^+ and Na_i^+ do not differ.

The Dependence on Amiloride Concentration

The data presented above show that the rate of ^{45}Ca influx in shrunken human and rat erythrocytes is increased by two- to threefold (Table 4). It was also observed that in shrunken rat erythrocytes the rates of ^{22}Na and ^{86}Rb influx are increased by seven- to eight- and twofold, respectively (Table 2). All these processes are blocked by amiloride. The dependences of influx of ^{45}Ca and ^{22}Na on amiloride concentration do not differ (K_i 20–30 μM). Unlike these cation fluxes, the ^{86}Rb influx is decreased at higher concentrations of this inhibitor (>100 μM; *data not presented*).

Phosphorylation of Membrane Proteins

Data on the phosphorylation of membrane proteins after 2.5 hr incubation of human and rat erythrocytes with ^{32}P-orthophosphate are presented in columns *I* of Figs. 5 and 6, respectively. Without additions of protein kinase activators the major amount of label is incorporated in band 4.1 and 4.9 proteins of erythrocytes of both species. The basal phosphorylation of band 3 protein is neglegible in rat erythrocytes and practically absent in human where ^{32}P incorporation into a M_r 100-kDa protein takes place.

The addition of dibuytyryl-cAMP in human erythrocyte suspension results in a significant increase of the band 4.9 labeling and in smaller increases of the phosphorylation of bands 2.1, 4.1, and 4.8 proteins as well as proteins M_r 120, 100, 35, and 28 kDa (Fig. 5, columns *II*). As can be seen from the gel pattern (Fig. 5*a*) the last four proteins are minor membrane components with unknown function. Data on protein kinase A-induced phosphorylation of 2.1, 4.1, and 4.9 proteins were obtained in previous studies (Avruch & Fairbanks, 1974; Rubin, 1975; Hosey & Tao, 1976; Horne, Leto & Marchesi, 1985). To our knowledge this is

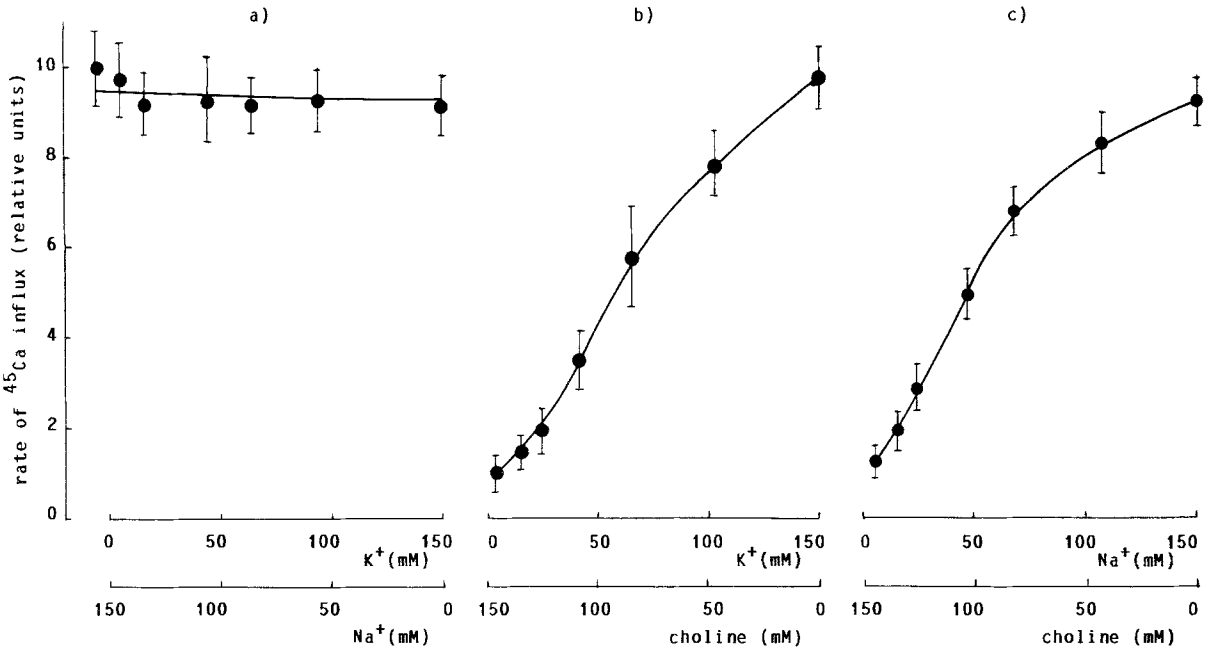

Fig. 4. The dependence of the rate of ^{45}Ca influx in human erythrocyte resealed ghosts on the ratio of intravesicular concentrations of NaCl/KCl (*a*), KCl/choline chloride (*b*), and NaCl/choline chloride (*c*)

Table 3. The effect of valinomycin on the rate of sodium influx and proton efflux in rat erythrocytes

Additions in the incubation medium (mM)	Concentrations in the incubation medium (mM)		Sodium influx (mmol per liter of cells per hr)	Proton efflux (mmol per liter of cells per hr)
	Sodium	Choline		
Valinomycin (0.0025)	130	0	42.10 ± 7.81	38.69 ± 5.70
Valinomycin and amiloride (1.0)	—	—	4.30 ± 0.93	5.61 ± 1.01
Valinomycin	0	130	—	4.01 ± 0.73

The rates of sodium influx and proton efflux were determined between 15 and 30 min after valinomycin addition (*see* Fig. 3).

Table 4. The effect of hypertonicity (medium B) on the rate of ^{45}Ca influx (μmol per liter of cells per hr) in Quin 2-loaded human (a) and rat (b) erythrocytes

Additions in the incubation medium (mM)		Medium A		Medium B		Medium A and valinomycin (2.5 μM)
		a	b	a	b	a
None		18.5 ±2.7	53.3 ±7.2	55.0 ±4.3*	102.5 ±9.6*	30.1 ±5.1*
Amiloride	(1.0)	10.1 ±2.0	36.9 ±6.3	14.5 ±3.7	41.0 ±5.3	16.0 ±3.0
Quinidine	(1.0)	17.4 ±1.9	47.5 ±6.0	19.3 ±2.6	41.0 ±4.3	—
DIDS	(0.125)	18.0 ±2.7	—	50.9 ±5.6*	—	21.9 ±3.0

* $P < 0.05$.

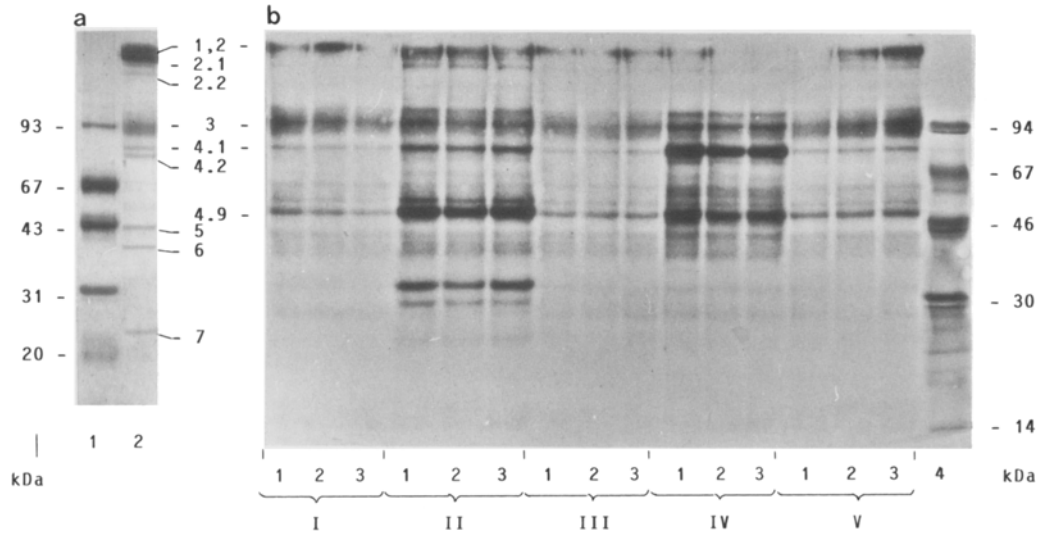

Fig. 5. Electrophoretogram (*a*) and autoradiogram (*b*) of human erythrocyte membrane proteins obtained after incubation of cells with ^{32}P-orthophosphate. (*a*) *1*—M_r standards; *2*—protein bands stained by Coomassie brilliant blue R250. (*b*) *I*—control; *II*—1 mM dibutyryl-cAMP was added; *III*—1 mM dibutyryl-cGMP was added; *IV*—1 μM TPA was added; *V*—shrinking (medium B). *1*—control; *2*—amiloride (1 mM); *3*—quinidine (1 mM); *4*—M_r ^{14}C standards. In the middle of the figure the numeration of bands in accordance with Steck's (1974) nomenclature are given

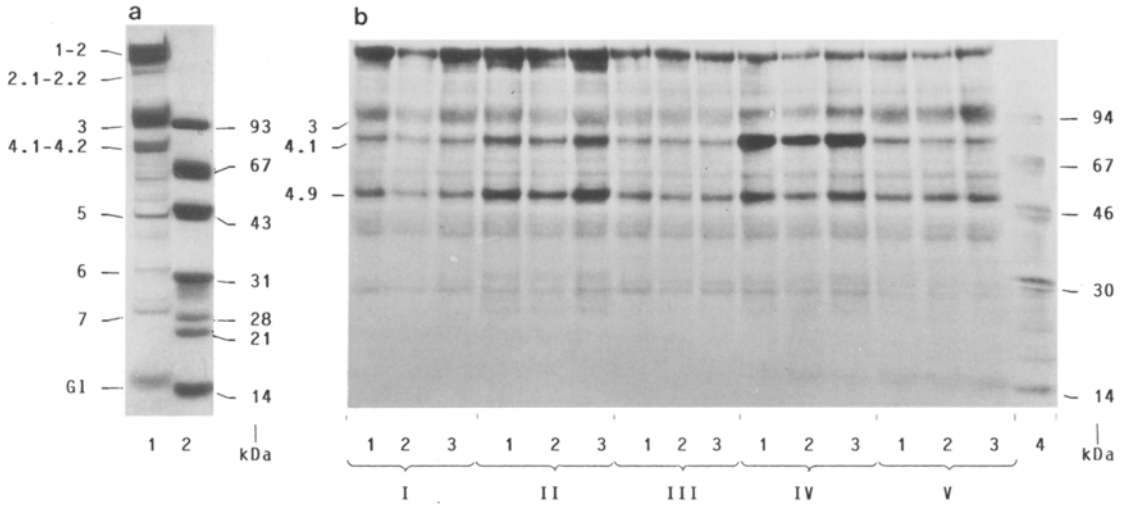

Fig. 6. Electrophoretogram (*a*) and autoradiogram (*b*) of rat erythrocyte membrane proteins obtained after incubation of cells with ^{32}P-orthophosphate. (*a*) *1*—protein bands stained by Coomassie brilliant blue R250; *2*—M_r standards. (*b*) *I*—control (medium A); *II*—1 mM dibutyryl-cAMP was added; *III*—1 mM dibutyryl-cGMP was added; *IV*—1 μM of TPA was added; *V*—shrinking (medium B). *1*—control; *2*—amiloride (1 mM); *3*—quinidine (1 mM); *4*—M_r ^{14}C standards

the first demonstration of cAMP-dependent phosphorylation of 35 and 28 kDa molecular weight proteins.

The addition of protein kinase C activator (TPA) to human erythrocytes (Fig. 5*b*, columns *IV*) results in predominant labeling of band 4.1 protein. Extensive incorporation of ^{32}P in band 4.9 and M_r 120 and 100 kDa proteins is also observed. One may also note the minor phosphorylation of bands lying between 4.1 and 4.9 and a protein with a molecular weight close to actin. This observation is in accordance with previously reported data (Horne et al., 1985; Palfrey & Waseem, 1985; Faquin et al., 1986).

The data on regulation of membrane protein phosphorylation in rat erythrocytes are limited by two observations where TPA- (Postnov, Gulak & Orlov, 1986; Orlov, Pokudin & Kotelevtsev, 1987) and dibutyryl-cAMP (Orlov et al., 1987) -induced phosphorylation of band 4.1 and 4.9 proteins has been revealed. As can be seen from Figs. 5 and 6,

the distribution of label between these proteins in TPA- and cAMP-treated human and rat erythrocytes does not differ. Unlike human erythrocytes, only one additional protein (Band 2.1) was noted as a substrate of those protein kinases in rat cells.

Neither dibutyryl-cGMP (Figs. 5 and 6; columns *III*) nor the inactive analogue of TPA (α-phorbol ester—*data not presented*) modified phosphorylation of erythrocyte membrane proteins. We were also not able to identify any modification of membrane protein phosphorylation in shrunken erythrocytes (Figs. 5 and 6, columns *V*).

The ^{32}P distribution obtained after incubation of erythrocyte ghosts with ^{32}P-γ-ATP (Fig. 7) is essentially different from intact erythrocytes incubated with ^{32}P-orthophosphate (Figs. 5 and 6). These differences may be induced by alterations of membrane structure or redistribution of protein kinases during hypotonic hemolysis. Unlike the intact erythrocytes, the addition of TPA and dibutyryl-cAMP to erythrocyte ghosts results in the increase of ^{32}P-labeling of all membrane proteins resolving on gel. This effect is expressed more prominantly in human erythrocyte ghosts, a fact probably due to high content of membrane-bound protein kinase A and C. Recently it was shown that membrane content of protein C is dependent on the concentration of Ca_i (Wolf et al., 1985), pretreatment of erythrocytes with TPA (Palfrey & Waseem, 1985), and the activity of exogenous proteinases (Melloni et al., 1985). It may be assumed that the latter case is the main reason for a more pronounced effect of TPA on ^{32}P-incorporation in rat erythrocyte ghosts obtained by hemolysis in the medium containing the proteinase inhibitor PMSF (Postnov et al., 1986).

Figures 5 and 6 show that 30 min incubation of erythrocytes with amiloride results in the decrease both of the basal and protein kinase activator-induced phosphorylation of membrane proteins (*compare* columns *1* and *2*). Not long ago the same results were obtained for cells of other tissues (Besterman et al., 1985; Davis & Czech, 1985). The other inhibitor of Na^+/H^+ exchange, quinidine, decreases the phosphorylation level of membrane proteins in human erythrocytes and does not modify or negligibly increase ^{32}P incorporation in rat erythrocytes (columns *3*). We believe that the decrease of ^{32}P-labeling after amiloride addition is due to inhibition by this compound of protein kinases rather than activation of a phosphoprotein phosphatase. Indeed, the simultaneous addition of amiloride and ^{32}P-γ-ATP to erythrocyte ghosts results in practically complete inhibition of phosphorylation of all proteins resolved on gel (Fig. 7). This result is in accordance with data on amiloride inhibition of purified protein kinase A (Ralph et al., 1982) and C (Besterman et al., 1985).

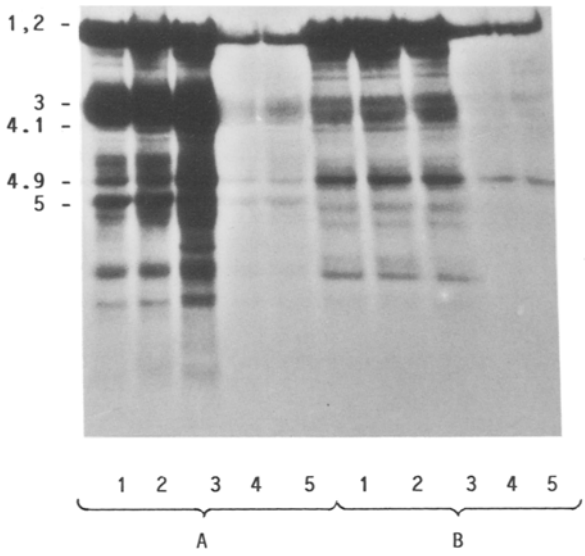

Fig. 7. Autoradiogram of human (*A*) and rat (*B*) erythrocyte membrane proteins after incubation of ghosts with ^{32}P-γ-ATP. *1*—control; *2*—TPA (1 μM); *3*—cAMP (1 mM); *4*—amiloride (1 mM); *5*—TPA + amiloride

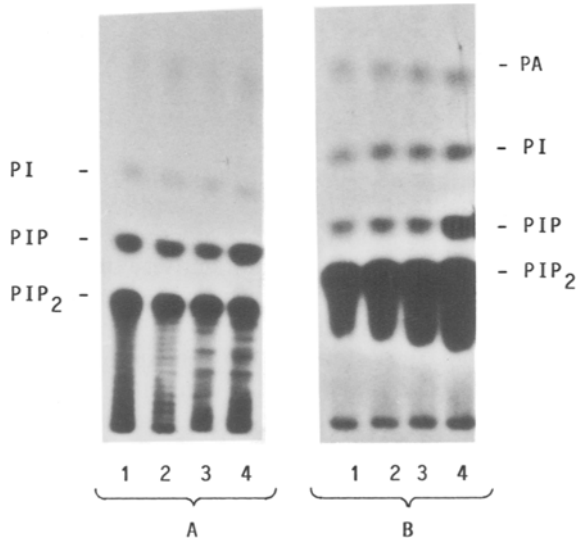

Fig. 8. Autoradiogram of human (*A*) and rat (*B*) erythrocyte membrane polyphosphoinositides obtained after incubation of cells with ^{32}P-orthophosphate. *1*—control (medium A); *2*—TPA (1 μM); *3*—dibutyryl-cAMP (1 mM); *4*—shrinking (medium B)

Phosphorylation of Phosphoinositides

Unlike membrane proteins, ^{32}P incorporation into phosphoinositides does not depend on addition of protein kinase activators but is modified in shrunken erythrocytes (Fig. 8). The data on total (A_t-cpm) and relative (A_r-cpm per nmol of acid lipid phosphorus) radioactivity of PIP and PIP_2 are presented in Table 5. It may be seen that in human

Table 5. The effect of protein kinase activators and hypertonicity (medium B) on PIP and PIP_2 phosphorylation in human (a) and rat (b) erythrocytes

	PIP				PIP_2			
	A_t (cpm)		A_r (cpm per nmol of P_{al})		A_t (cpm)		A_r (cpm per nmol of P_{al})	
	a	b	a	b	a	b	a	b
Control (medium A)	1246	485	113	46	4057	7313	368	692
Dibutyryl-cAMP	1081	604	127	42	3067	7946	361	545
TPA	1120	714	124	51	3080	8498	342	604
Medium B	2548	2332	300	161	2331	14008	274	963

Table 6. The effect of amiloride on phosphorylation of PIP and PIP_2 in human (a) and rat (b) erythrocyte ghosts

	PIP				PIP_2			
	A_t (cpm)		A_r (cpm per nmol of P_{al})		A_t (cpm)		A_r (cpm per nmol of P_{al})	
	a	b	a	b	a	b	a	b
Control	8206	9254	391	366	1444	5956	69	238
Amiloride (1 mM)	1554	1365	69	74	450	764	20	41

and rat shrunken erythrocytes both the total and relative radioactivity of PIP is increased by two- to threefold and four- to fivefold, respectively. Under the same condition the total radioactivity of PIP_2 is increased by twofold in rat erythrocytes and is decreased by 40% in human.

Based on the steady-state phosphorylation level, one may conclude that the content of PIP_2 in intact human and rat erythrocytes is three- to fourfold and 15-fold higher than the content of PIP (Table 5). As can be seen from Table 6, the PIP_2/PIP ratio in erythrocyte ghosts is decreased. As in the intact erythrocytes, the activators of protein kinases do not modify ^{32}P incorporation in phosphoinositides of erythrocyte ghosts (Fig. 9). The simultaneous addition of amiloride and ^{32}P-γ-ATP to erythrocyte ghosts results in a four- to sixfold decrease of ^{32}P labeling of PIP and PIP_2 (Table 6), indicating the strong inhibition of phosphoinositide kinase activities.

Experiments described above were carried out on erythrocytes of 14-week-old rats. The results presented in Table 7 were obtained on 23-week-old rats. This table shows that shrinking of erythrocytes is accompanied by three- and twofold increase of total and relative radioactivity in PIP, correspondingly. Data on age-dependent regulation of phosphoinositide metabolism in rat erythrocytes was presented by Kiselev et al. (1981). Here it is interesting to note that amiloride inhibits the volume-dependent modification of PIP phosphorylation and decreases the basal incorporation of ^{32}P in PIP_2. We failed to indicate any modification of PIP and PIP_2 labeling in swollen erythrocytes (Table 7; lines 5 and 6).

Discussion

We failed to reveal RVD in swollen erythrocytes of human and rat (Fig. 2). Thus it may be concluded that hyposmotic swelling does not induce any significant alteration of net-ion fluxes across plasma membrane.

Both osmotic (medium B) and isosmotic (addition of valinomycin) shrinkage induces RVI in rat erythrocytes only (Fig. 2). The following data show that the activation of Na^+/H^+ exchange is the basic mechanism of RVI.

a) In rat erythrocyte RVI is blocked by inhibitors of Na^+/H^+ exchange (amiloride, quinidine) (Fig. 2).

Table 7. The effect of hypertonicity (medium B) and hypotonicity (medium C) on phosphorylation of PIP and PIP_2 in rat erythrocytes

	PIP		PIP_2	
	A_t (cpm)	A_r (cpm per nmol of P_{al})	A_t (cpm)	A_r (cpm per nmol of P_{al})
Control (medium A)	2176	176	28057	2415
Medium A and amiloride (1 mM)	2666	135	23499	1292
Medium B	6161	308	35845	1853
Medium B and amiloride (1 mM)	4122	193	33044	1626
Control (medium A)	3241	179	44844	2518
Medium C	2847	162	41094	2373

b) Both isosmotic and osmotic shrinkage of rat erythrocytes results in the increase of the rate of ^{22}Na influx blocked by the same compounds (Tables 2 and 3). On the contrary, shrinkage of human erythrocytes does not increase ^{22}Na influx (Table 2).

c) The rates of amiloride-dependent sodium influx and proton efflux in valinomycin-treated rat erythrocytes are nearly equal (Table 3). This observation is in accordance with numerous data on eqimolarity and electroneutrality of Na^+/H^+ exchange (Aronson, 1985; Grinstein & Rothstein, 1986).

As can be seen from Table 2, the shrinkage of rat erythrocytes is also accompanied by an increase of ^{86}Rb influx. However, this increment is not above 2 mmol per liter of cells per hr, which is less than the increment of Na^+/H^+ exchange observed under the same conditions (11–13 mmol per liter of cells per hr).

Unlike the Na^+/H^+ exchange, the shrinkage increases the rate of ^{45}Ca influx both in rat and human erythrocytes (Table 4). The mechanism of transmembrane calcium movement along its electrochemical gradient in these cells is unknown. There are only a few reports on the increase of calcium influx in human erythrocytes upon their membrane hyperpolarization (Szasz, Sarkadi & Gardos, 1977) and particularly after valinomycin addition (Yingst & Hoffman, 1984; Pokudin & Orlov, 1986). Concerning these data, it was assumed that the erythrocyte membrane contains a hypothetical carrier and this carrier in complex with calcium is positively charged. However, as can be seen from Table 4, the inhibitor of anion transport blocks valinomycin-induced calcium influx. Recently it was shown that this compound blocked erythrocyte shrinkage but does not modify membrane hyperpolarization induced by valinomycin (Orlov et al., 1987, 1988). Thus it may be concluded that valinomycin-induced

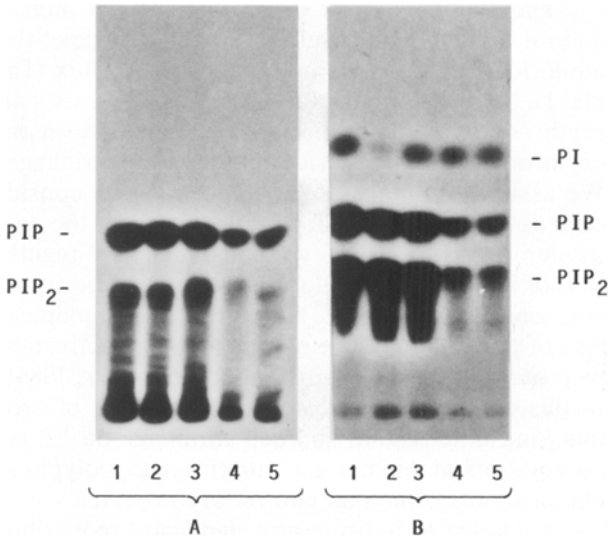

Fig. 9. Autoradiogram of human (*A*) and rat (*B*) erythrocyte membrane polyphosphoinositides obtained after incubation of ghosts with ^{32}P-γ-ATP. *1*—control; *2*—TPA (1 μM); *3*—cAMP (1 mM); *4*—amiloride (1 mM); *5*—TPA + amiloride

erythrocyte shrinkage, but not hyperpolarization, is a main mechanism of calcium influx activation.

Data obtained in studies of ^{45}Ca influx in human erythrocytes with Ca-ATPase inhibited by orthovanadate suggest that the calcium carrier operates as Ca_o^{2+}/K_i^+ or Ca_o^{2+}/H_i^+ antiporter (Vareska & Carafoli, 1982). We failed to observe the effect of the transmembrane electrochemical proton gradient ($\Delta\mu_{H^+}$) on the rate of ^{45}Ca influx in human erythrocytes (*data not presented*). As can be seen from Fig. 4*a*, replacing the intracellular potassium by sodium has no effect on ^{45}Ca influx. On the contrary, replacing of potassium (Fig. 4*b*) or sodium (Fig. 4*c*) by choline decreases the rate of ^{45}Ca influx in hu-

man resealed ghosts by one order of magnitude. Thus it may be assumed that the hypothetical volume-dependent calcium carrier operates as a nonselective $Ca_o^{2+}/(K_i^+, Na_i^+)$ antiporter.

We failed to observe any saturation of ^{45}Ca influx up to 150 mM of Na_i^+ or K_i^+ (Fig. 4). These data suggest that the two- to threefold increase of calcium influx in human and rat erythrocytes revealed in hyperosmotic medium is conditioned by the increase of the intracellular concentration of univalent cations due to the loss of cellular water. On the contrary, under isosmotic shrinkage induced by valinomycin the intracellular concentration (not content) of univalent cation must not be essentially changed. Indeed, the valinomycin-induced increment of ^{45}Ca influx is only about 60% in human erythrocytes (Table 4) and absent in rat (*data not presented*).

The acidification of cytoplasm both of human and rat erythrocytes results in an appearance of the amiloride-dependent component of ^{22}Na influx (Table 1). It means that Na^+/H^+ exchange exists in erythrocytes of both species, but that only in rat erythrocytes is this carrier activated by shrinkage. We assume that this circumstance may be considered as a starting point for bringing to light the molecular mechanisms of volume-dependent regulation of ion fluxes. It is known that the majority of hormones regulates Na^+/H^+ exchange via modification of phosphoinositide metabolism and activation of protein kinase C (Grinstein & Rothstein, 1986). In this connection we compared the effect of protein kinase activators and cell shrinkage on ^{32}P incorporation in membrane proteins and polyphosphoinositides of human and rat erythrocytes.

We failed to indicate any significant redistribution of ^{32}P between membrane proteins of human and rat erythrocytes being under osmotic shrinkage (Figs. 5 and 6). Two alternative explanations of these data are:

a) activation of protein kinases and membrane protein phosphorylation are not involved in the volume-dependent regulation of ion transport;

b) erythrocyte shrinking results in phosphorylation of some minor membrane proteins, which are not seen in electrophoretograms (for example, ion carriers themselves).

It is known that in vivo protein kinase C is activated by diacyl glycerol—the product of triphosphoinositide hydrolysis by phospholipase C. In cells this reaction is coupled with polyphosphoinositide resynthesis that is accompanied by ^{32}P incorporation in PIP and PIP_2 (Berridge, 1984). As can be seen from Fig. 8 and Table 5, the shrinkage in erythrocytes drastically modifies the phosphorylation of these acid phospholipids. The effect of shrinkage on PIP phosphorylation is more pronounced in rat erythrocytes capable of RVI. In erythrocyte ghosts phosphorylation of both membrane proteins and polyphosphoinositides is inhibited by amiloride (Figs. 7 and 9). Moreover, both RVI (Fig. 2) and the shrinking-induced increase of PIP phosphorylation are also blocked by amiloride (Table 7). On the contrary, the swelling of erythrocytes does not influence the level of PIP and PIP_2 ^{32}P-labeling (Table 7). Under the same conditions we failed to observe RVD (Fig. 2) and any significant modifications of the rates of transmembrane sodium fluxes (*data not presented*). Taking into account these data, we assume that the shrinkage-stimulated phosphoinositide response plays a key role in the increase of Na_o^+/H_i^+ exchange which induced the RVI in rat erythrocytes.

Separate fragments of this manuscript were reported at the 3d European Meeting of Hypertension (Milan, June 14–17, 1987), 12th All-Union Symposium on transport ATPase (Irkutsk, July 13–16, 1987) and the 3d Joint USSR-FRG Symposium on excitable membranes (Kiev, October, 11–16, 1987).

We are grateful to Mrs. Natalya Serebryakova for her excellent assistance in the study of phosphoinositide phosphorylation.

References

Adragna, N.C., Tosteson, D.C. 1984. Effect of volume changes on ouabain-insensitive net outward cation movements in human red cells. *J. Membrane Biol.* **78:**43–52

Aronson, P.S. 1985. Kinetics properties of the plasma membrane Na^+-H^+ exchanger. *Annu. Rev. Physiol.* **47:**545–560

Avruch, J., Fairbanks, G. 1974. Phosphorylation of endogenous substrates by erythrocyte membrane protein kinase. I. A monovalent cation-stimulated reaction. *Biochemistry* **13:**5507–5514

Bakker-Grunwald, T. 1983. Potassium permeability and volume control in isolated rat hepatocytes. *Biochim. Biophys. Acta* **731:**239–242

Berridge, M.J. 1984. Inositol triphosphate and diacylglycerol as a second messenger. *Biochem. J.* **220:**345–360

Besterman, J.M., May, W.C., LeVine, H., III., Cragoe, E.J., Cuatrecasas, P. 1985. Amiloride inhibits phorbol ester stimulated Na^+/H^+ exchange and protein kinase C. An amiloride analogue selectively inhibits Na^-/H^- exchange. *J. Biol. Chem.* **260:**1155–1159

Cala, P.M. 1983. Cell volume regulation by *Amphiuma* red blood cells. The role of Ca^{2+} as a modulator of alkali metal/H^+ exchange. *J. Gen. Physiol.* **82:**761–784

Davis, R.J., Czech, M.P. 1985. Amiloride directly inhibits growth factor receptor tyrosine kinase activity. *J. Biol. Chem.* **260:**2543–2551

Duhm, J., Göbel, B.O. 1982. Sodium-lithium exchange and sodium-potassium cotransport in human erythrocytes: I. Evaluation of simple uptake test to assess the activity of the two transport systems. *Hypertension* **4:**468–476

Duhm, J., Göbel, B.O. 1984*a*. Role of furosemide-sensitive Na^+/K^+ transport system in determining the steady-state Na^+ and K^+ content and volume of human erythrocytes in vitro and in vivo. *J. Membrane Biol.* **77:**243–254

Duhm, J., Göbel, B.O. 1984*b*. Na^+-K^+ transport and volume of rat erythrocytes under dietery K-deficiency. *Am. J. Physiol.* **246:***C*20–*C*26

Escobales, N., Canessa, M. 1986. Amiloride-sensitive Na^+ transport in human red cells: Evidence for Na/H exchange system. *J. Membrane Biol.* **90:**21–28

Faquin, W.C., Chahwala, S.B., Cantley, L.C., Branton, D. 1986. Protein kinase C of human erythrocytes phosphorylates band 4.1 and 4.9. *Biochim. Biophys. Acta* **887:**142–149

Grinstein, S., Rothstein, A. 1986. Mechanisms of regulation of the Na^+/H^+ exchanger. *J. Membrane Biol.* **90:**1–12

Grinstein, S., Rothstein, A., Sarkadi, B., Gelfand, E.W. 1984. Responses of lymphocytes to anisotonic medium: Volume regulation behaviour. *Am. J. Physiol.* **246:***C*204–*C*215

Haggerty, J.C., Cragoe, E.J., Slayman, C.W., Adelberg, E.A. 1985. Na^+/H'^+ exchange activity in the pig kidney epithelial cell line LLC-PK_1: Inhibition by amiloride and its derivatives. *Biochem. Biophys. Res. Commun.* **127:**759–767

Hoffman, E.K., Simonsen, L.O., Lambert, I.H. 1984. Volume-induced increase of K^+ and Cl^- permeabilities in Ehrlich ascites tumor cells. Role of internal Ca^{2+}. *J. Membrane Biol.* **78:**211–222

Horne, W.C., Leto, T.L., Marchesi, V.T. 1985. Differential phosphorylation of multiple sites in protein 4.1 and protein 4.9 by phorbol ester-activated and cyclic AMP-dependent protein kinases. *J. Biol. Chem.* **260:**9073–9076

Hosey, M.M., Tao, M. 1976. An analysis of the autophosphorylation of rabbit and human erythrocyte membranes. *Biochemistry* **151:**1561–1568

Irvine, R.F., Letcher, A.J., Dawson, P.M.C. 1984. Phosphatidylinositol-4,5-biphosphate phosphodiesterase and phosphomonoesterase activities of rat brain. *Biochem. J.* **218:**177–185

Jolles, J., Zwiers, H., Dekker, A., Wirtz, K.W.A., Gispen, W.H. 1981. Corticotropin-(1-24)tetracosapeptide affects protein phosphorylation and phosphoinositides metabolism in rat brain. *Biochem. J.* **194:**283–291

Kiselev, G., Minenko, A., Moritz, V., Ochme, P. 1981. Polyphosphoinositide metabolism in erythrocytes of spontaneously hypertensive rats. *Biochem. Pharmacol.* **30:**833–837

Lauf, P.K. 1982. Evidence for chloride-dependent potassium and water transport induced by hyposmotic stress in erythrocytes of the marine teleost, *Ospames tau. J. Comp. Physiol.* **146:**9–16

Lauf, P.K. 1984. Thiol-dependent passive K^+/Cl^- transport in sheep red blood cells: VI. Functional heterogeneity and immunologic identity with volume-stimulated $K^+(Rb^+)$ fluxes. *J. Membrane Biol.* **82:**167–178

Lauf, P.K., Perkis, C.M., Adragna, N.C. 1985. Cell volume and metabolic dependence of NEM-stimulated K^+-Cl^- flux in human red blood cells. *Am. J. Physiol.* **249:***C*124–*C*128

Lew, V.L., Tsien, R.Y., Miner, C., Bookchin, R.M. 1982. Physiological Ca_i^{2+} level and pump-leak turnover in intact red cells measured using an incorporated Ca chelator. *Nature (London)* **289:**478–481

Lowe, A., Lin, H.Y., Yee, V.J., Warnock, D.G. 1985. Regulation of the activity of the Na^+/H^+ antiporter in brush-border membrane vesicles from proximal tubule. *Ann. N.Y. Acad. Sci.* **456:**229–232

Macey, R.I. 1977. Transport of water and nonelectrolytes across red cell membranes. *In:* Membrane Transport in Biology. Vol. 2, pp. 1–58. D.S. Tosteson, editor. Springer-Verlag, Berlin–Heidelberg–New York

Melloni, E., Pontremoli, S., Sacco, O., Sparatore, B., Salamino, F., Horecker, B.L. 1985. Binding of protein kinase C to neutrophil membranes in the presence of Ca^{2+}-requiring proteinase. *Proc. Natl. Acad. Sci. USA* **82:**6435–6439

O'Neill, W.C., Mikkelsen, R.B. 1987. Furosemide-sensitive Na^+ and K^+ transport and human erythrocyte volume. *Biochim. Biophys. Acta* **896:**196–202

Orlov, S.N., Pokudin, N.I., Kotelevtsev, Yu.V. 1987. Transport of K^+, anions and the activity of Na^+-pump in erythrocyte membranes: Three different mechanisms of regulation by intracellular calcium. *Biochemistry (Moscow)* **52:**1373–1386

Orlov, S.N., Pokudin, N.I., Postnov, Yu.V. 1988. Transport of sodium and proton and hypotonic haemolysis in the valinomycin-treated erythrocytes of rats with spontaneous hypertension. *J. Hypertens.* **6:**351–359

Palfrey, H.C., Waseem, A. 1985. Protein kinase C in human erythrocytes. Translocation to the plasma membrane and phosphorylation of bands 4.1 and 4.9 and other membrane proteins. *J. Biol. Chem.* **260:**16021–16029

Parker, J.C. 1983. Volume-responsive sodium movements in dog red blood cells. *Am. J. Physiol.* **244:***C*324–*C*330

Petiton, M., Tuy, F., Rosenfeld, C. 1978. A simplified procedure for organic phosphorus determination from phospholipids. *Anal. Biochem.* **91:**350–353

Pokudin, N.I., Orlov, S.N. 1986. Ca^{2+} transport in human erythrocytes: The study on cells loaded with a highly selective calcium chelators. *Biol. Membr. (Moscow)* **3:**108–117

Postnov, Yu.V., Gulak, P.V., Orlov, S.N. 1986. Evidence for altered phosphorylation of the erythrocyte membrane skeleton in spontaneously hypertensive rats. *J. Hypertens.* **4(Suppl. 6):***S*367–*S*369

Ralph, R.K., Smart, J., Wojcik, C.J., McQuillan, J. 1982. Inhibition of mouse mastocytoma protein kinase by amiloride. *Biochem. Biophys. Res. Commun.* **104:**1054–1059

Rubin, C.S. 1975. Adenosine 3′:5′-monophosphate-regulated phosphorylation of erythrocyte membrane proteins. Separation of membrane-associated cyclic adenosine 3′:5′-monophosphate-dependent protein kinase from its endogenous substrates. *J. Biol. Chem.* **250:**9044–9052

Schmidt, W.F., McManus, T.J. 1977. Ouabain-insensitive salt and water movements in duck red cells. I. Kinetics of cation transport under hypertonic conditions. *J. Gen. Physiol.* **70:**59–79

Steck, T.L. 1974. The organization of proteins in the human red blood cell membranes. *J. Cell Biol.* **62:**1–19

Szasz, I., Sarkadi, B., Gardos, G. 1977. Mechanism of Ca^{2+}-dependent selective rapid K^+-transport induced by propranolol in red cells. *J. Membrane Biol.* **35:**75–93

Towbin, H.H., Staehelin, T., Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76:**4350–4354

Vareska, L., Carafoli, E. 1982. Vanadate-induced movements of Ca^{2+} and K^+ in human red blood cells. *J. Biol. Chem.* **257:**7414–7421

Wolf, M., Le Vine, H., III, May, S., Cuatrecasas, P., Sahyoun, N. 1985. A model for intracellular translocation of protein kinase C involving synergism between Ca^{2+} and phorbol esters. *Nature (London)* **317:**545–549

Yingst, D.R., Hoffman, J.F. 1984. Passive Ca transport in human red blood cell ghosts measured with entrapped Arsenazo III. *J. Gen. Physiol.* **83:**1–17

Received 15 December 1987; revised 27 September 1988