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Volume-Activated Na/H Exchange Activity in Fetal and Adult Pig Red Cells: Inhibition by Cyclic AMP

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Summary. Hyposmotic swelling of pig red cells leads to a selective increase in K permeability, whereas hyperosmotic cell shrinkage augments the Na permeability. In this regard, the ouabain-resistant (OR) Na flux of red cells of newborn and adult pigs is characterized in detail. A reduction in cell volume by approximately 18% leads to an increase in the OR Na efflux of fetal and adult cells by 15- and fourfold, respectively. The OR Na influx in both cell types is equally influenced by cell shrinkage. Depletion of cellular K does not influence the volume-activated OR Na efflux. Nor does OR Na influx require external K. Both OR Na efflux and influx activated by shrinkage are inhibited by the diuretics furosemide and amiloride. The rank order of decreasing anion sensitivity for diuretic-sensitive Na efflux was acetate > chloride > gluconate > nitrate. Cell shrinkage induced by the addition of hypertonic salts results in an acidification of the unbuffered and CO_2-free media, provided that both Na and DIDS are present. The acidification process can be reversed by either of the diuretic agents. These findings suggest that the shrinkage-activated OR Na flux is primarily mediated by a Na/H exchanger rather than by a Na/K/Cl cotransporter. Once loaded with either cAMP or cGMP, cell swelling can no longer activate the Na/H exchanger. The Na/H exchanger activity is detectable in the fetal cells of normal volume but quiescent in adult cells, indicating that the exchanger undergoes a developmental change during the transition from the fetal to adult stage.

Key Words Na/H exchange · erythrocyte · cell volume · cyclic nucleotides · developmental changes

Introduction

Pig red blood cells produced during the fetal stage are glucose permeable (Kim & Luthra, 1977), but are replaced during early postnatal development by cells, which are impermeable to glucose and therefore nonglycolytic (Kim, McManus & Bartlett, 1973). Recent studies have shown that plasma inosine, which is present at the micromolar concentration, substitutes for glucose as an energy source (Watts et al., 1979; Jarvis et al., 1980; Kim et al., 1980; Zeidler et al., 1985).

This inosine-dependent erythrocyte has ATP levels amounting to 1–2.5 μmol/ml packed cells (Kim & McManus, 1971*a*,*b*; Young et al., 1986), which support the active cation transport as evidenced by the presence of ouabain-sensitive Na efflux and K influx components (Sorenson, Kirschner & Barker, 1962; Whittaker, Hawkins & Swaminathan, 1983; Lauf, Zeidler & Kim, 1984; Brand & Whittam, 1985). The ouabain binding capacity and the Na + K ATPase activity in pig erythrocytes have been reported to range from approximately two-thirds to nearly comparable levels seen in human erythrocytes (Whittaker et al., 1983; Kim et al., 1984). However, the ouabain-sensitive Na efflux of pig cells was significantly lower than that of human cells (Whittaker et al., 1983).

During the course of reticulocyte maturation, the Na + K pump activity undergoes a drastic change. Lauf et al. (1984) reported that nearly 90% of the pump activity is lost as the reticulocyte matures. Furthermore, the ouabain-resistant Cl-dependent Rb flux component decreases as well. This is of interest in that a significant decrease in cell volume, which takes place during the reticulocyte maturation, is accounted for primarily by the loss of cellular K rather than Na (Zeidler & Kim, 1982).

Although small in magnitude, the ouabain-resistant K flux in the adult pig erythrocytes is composed of roughly equal Cl-dependent and independent components (Lauf et al., 1984). The Cl-dependent K pathway can be stimulated nearly 15-fold by NEM (N-ethylmaleimide) in the mature red cell (Lauf et al., 1984) and exhibits properties similar to the thiol-dependent K/Cl cotransporter in sheep erythrocytes (Lauf, 1983). Moreover, cell swelling activates the ouabain-resistant chloride-dependent K flux. Increased cyclic AMP content, which can be accomplished by incubation of cells

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with exogenously added cyclic AMP, results in an augmentation of ouabain-resistant chloride-dependent K flux in normal and swollen cells (Kim et al., 1989).

In contrast to the ouabain-sensitive and insensitive K fluxes, little is known about the nature of Na flux in general, and the ouabain-resistant Na pathways, in particular. In this communication, we report the presence of a Na/H exchange pathway in pig erythrocytes. While Na/H exchange activity is present in fetal erythrocytes, it is quiescent in adult cells. Upon cell shrinkage, the Na/H exchange pathway is activated in both cell types, although to a much lesser extent in the adult erythrocytes, indicating that the exchanger undergoes developmental modifications. Furthermore, cell shrinkage can no longer activate the Na/H exchange activity in cells loaded with either cyclic AMP or cyclic GMP. A preliminary report of this work has been presented elsewhere (Sergeant & Kim, 1987).

Materials and Methods

Materials

Nystatin, ouabain, amiloride, harmaline and DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid) were purchased from Sigma Chemical. Choline chloride, also from Sigma, was routinely recrystallized from hot ethanol in petroleum ether and acetone. Sucrose was obtained from Fisher Scientific. $MgCl_2$ and tetramethylammonium chloride (TMA) were purchased from Aldrich Chemical. Furosemide was a gift from Hoechst-Roussel Pharmaceuticals (Somerville, NJ), but was also purchased from Aldrich. Tris and Na salts of cyclic AMP and cyclic GMP were obtained from Sigma. Bumetanide was a generous gift from Dr. Peter Sorter of Hoffman-LaRoche (Nutley, NJ). ^{22}Na was purchased from New England Nuclear. ^{24}Na was obtained from the University of Missouri-Columbia Research Reactor.

Methods

Collection of Blood

Blood was drawn using a heparinized syringe from the jugular vein of mature pigs (6 mo. or older) housed at the University of Missouri-Columbia School of Veterinary Medicine or collected in heparinized containers (2.5 U/ml blood) from pigs at a local abattoir (Wilson Meat Processing Plant, Marshall, MO). In either case, the blood was used within 1½ hr after collection. Blood of fetal origin was obtained from one-day-old piglets purchased from Eldon Kreisel Farms (Rocheport, MO). Blood was collected from anesthetized animals (sodium pentobarbital, i.p.) in heparinized syringes by cardiac puncture. Plasma and buffy coat were aspirated and discarded following centrifugation of whole blood at 5°C for 5 min at 3,000 × *g* (Beckman, model J-21C). Erythrocytes were then washed three or four times in ice-cold 154 mM NaCl.

The Na and K content of original cells was determined by first washing the cells several times with a Na/K-free buffer (MSH) composed of 75 mM $MgCl_2$, 85 mM sucrose and 5 mM HEPES-Tris, pH 7.2. Cells were then lysed in a hemolyzing solution composed of 18.5 mM NH_4OH, 4 mM CsCl and Triton X-100 (31.25 μl/liter H_2O). The cation contents in lysates were determined by an atomic absorption spectrophotometry (Perkin-Elmer, model 303). The mean corpuscular hemoglobin concentration (MCHC) of original, swollen and shrunken cells was calculated from hematocrit and hemoglobin measurements.

Alteration of Cellular Na and K Content

Freshly drawn pig red cells have Na and K content of 4.74 ± 0.56 μmol/ml packed cells and 108.9 ± 6.0 μmol/ml packed cells, respectively (mean ± SD, $n = 23$).

To prepare cells for flux measurements, sodium and potassium content of erythrocytes was first altered by the nystatin method similar to that described by Cass and Dalmark (1973) and Cavieres (1982). This method was found to be more effective in our hands in altering cation content of pig cells compared to the PCMBS (P-chloromercuribenzenesulphonic acid) method of Garrahan and Rega (1967). Nystatin was prepared in methanol as a 5 mg/ml stock solution, which was cleared by centrifugation just prior to use. For efflux studies, cells were enriched with Na at the expense of K by suspending at 4–6% hematocrit in an isotonic loading buffer (LB) composed of 70 mM KCl, 50 mM NaCl, 10 mM choline chloride, 10 mM sodium phosphate buffer pH 6.7, 27 mM sucrose, 3 mM ribose, 1 mM inosine, 0.5 mM adenosine and approximately 40 μg nystatin/ml. Cells were then incubated at 4°C for 30 min in the dark. Cells to be used for influx experiments were depleted of Na by incubation in LB of similar composition for efflux experiments except that NaCl and choline chloride were replaced by 130 mM KCl and potassium phosphate buffer was used instead of sodium phosphate buffer. Nystatin treatment was carried out at 4°C to facilitate the action of the ionophore. In either case, the nystatin incubation was terminated by centrifugation of cells at 25°C, 2,000 × *g* for 7 min (Sorvall, model RT6000). Nystatin was removed by a series of eight washes (20–30 volumes per wash) of nystatin-free LB in the following sequence: twice in LB, pH 6.7 at 25°C; twice in LB, pH 7.0 at 25°C; and four times in LB, pH 7.2 at 37°C.

Na and K Efflux

Cation efflux was measured by the appearance of Na and K in a Na/K-free flux medium composed of 75 mM $MgCl_2$, 5 mM HEPES (titrated to pH 7.2 with Tris base), 3 mM ribose, 0.5 mM adenosine, 0.1 mM ouabain ± 1.0 mM furosemide and a variable concentration of sucrose depending on the desired osmolarity of the media. Typically, 105 and 185 mM sucrose was added to give flux media of approximately 330 and 410 mOsM, respectively, as determined by osmometry (Advanced Instruments, model 3R). A neutralized stock solution of furosemide (50 mM) was prepared daily by first dissolving furosemide in a small volume of 1 M Tris and then diluting with water.

Just prior to the efflux measurement, nystatin-free cells were quickly washed three times with large volumes of ice-cold Na/K-free wash buffer (MSH; 75 mM $MgCl_2$ *x* mM sucrose 5 mM HEPES-Tris, pH 7.2 and metabolic substrates) to reduce extracellular Na and K accompanying the cell pellet. The cells were then suspended to 50% hematocrit with cold MSH. Flux suspen-

sions were prepared, in duplicate, by the addition of 0.72 ml of 50% cell suspension to 5.28 ml ice-cold flux medium (final hematocrit = 6%) in 12 ml polyallomer centrifuge tubes (Nalgene 3109-0120). Efflux was initiated by placing the flux tubes in a 37°C shaker water bath (New Brunswick, model G76). The flux was terminated by centrifugation of tubes at 4°C for 2 min at 12,000 × *g*. The supernatant fluid was transferred to plastic tubes for cation and hemoglobin measurements. Samples for the determination of cell Na, K and hemoglobin contents were taken at the beginning of the flux. Cells were washed once with cold MSH and then lysed with 8 ml of hemolyzing solution. Na and K contents in lysates and supernatant fluids were quantitated using atomic absorption spectrophotometer following appropriate dilution of samples in hemolyzing solution. All efflux data are corrected for hemolysis, which was usually less than 1%.

It is now known that Na and K fluxes, which are mediated by the Na/K/Cl cotransport pathway, are inhibited by high Mg^{2+} concentrations (Wiley & Cooper, 1974; Ellory, Flatman & Stewart, 1980, 1983). To test for an inhibitory effect of Mg^{2+}, Na and K effluxes in $MgCl_2$ and tetramethyl-ammonium chloride (TMA) media were compared. In preliminary experiments there was virtually no difference in Na and K efflux whether Mg^{2+} was present or not. Therefore, $MgCl_2$ was used in flux buffers throughout these studies.

Na Influx

Unidirectional Na influx was measured using ^{22}Na or ^{24}Na radioisotope. Nystatin-free and Na-depleted cells were washed once in ice-cold phosphate buffered saline (PBS) composed of 125 mM NaCl, 5 mM KCl, 10 mM sodium phosphate buffer pH 7.6 *x* mM sucrose, 3 mM ribose, 1 mM inosine and 0.5 mM adenosine. The osmolarity of the flux media was adjusted with 27 mM and 140 mM sucrose, which typically give solutions of 300 and 430 mOsM, respectively. Following the PBS wash, cells were suspended to 50% hematocrit with cold PBS and kept on ice. The flux suspension was prepared by adding 1 volume of this suspension to 4 volumes of ice-cold PBS containing 0.12 mM ouabain ± various drugs as indicated in figure legends (final hematocrit = 10%) and 1.5 μCi [^{22}Na]/ml or 3 μCi [^{24}Na]/ml suspension. Influx was initiated upon incubation at 37°C in a shaker water bath. At predetermined times, 1 ml aliquots of the flux suspension were added to 5 ml of ice-cold PBS and centrifuged. The cells were washed two more times in cold PBS and lysed with 1 ml of hemolyzing solution. Radioactivities in lysates and aliquots of the flux suspension were determined by a Gamma Trac gamma counter (model 1191). The initial 50% cell suspension was used for the determination of hemoglobin content by optical density measurements at 527 nm and hematocrit measurements from which MCHC was calculated. Influx is expressed as μmol Na/gHb · hr.

Hydrogen Ion Efflux

The method of monitoring pH of cell suspensions was similar to that described by Jennings, Douglas and McAndrew (1986). Cells were suspended at 20% hematocrit in a buffered salt solution containing 150 mM NaCl, 3 mM ribose, 1 mM inosine, 0.5 mM adenosine and 10 mM HEPES-Tris, pH 7.4. The cell suspension was incubated at 37°C for 1½ to 2 hr. Cells were then washed at 4°C with a buffered salt solution composed of 150 mM KCl, 5 mM HEPES-Tris, pH 7.4, and metabolic substrates. The cells were stored as packed cells on ice prior to pH measurements. To initiate the proton flux, 1 ml of packed cells was rapidly washed at 4°C in a 50-ml polycarbonate tube with 30-ml unbuffered 150 mM KCl, which had been bubbled with nitrogen to remove CO_2. The cells were then rapidly resuspended to a final hematocrit of 10% in 9 ml of the unbuffered and CO_2-free 150 mM KCl and placed in a shaking water bath at 37°C. After resuspension, the pH was initially unstable but stabilized between 7.35 to 7.4 within a few minutes in most cases. In some preparations, the pH stabilized to a lower value. To initiate Na flux, cells were shrunken in 410 mM media by the addition of 2 M NaCl or KCl. The pH was monitored continuously with a pH meter (Fisher Accumet, model 810) using a combination electrode with a flat surface membrane (Fisher, model E-5D) and a chart recorder (Linear Instruments, model 282).

Cyclic Nucleotide Loading of Erythrocytes

Pig erythrocytes were loaded with cyclic AMP and cyclic GMP by the method described elsewhere (Sergeant & Kim, 1985; Kim et al., 1989). Briefly, cells were incubated with 1 mM cyclic nucleotides in order to increase the intracellular cyclic nucleotide content. Because no precautions were taken to prevent degradation of the cyclic nucleotides, cyclic nucleotides were present in both loading buffers used for the nystatin treatment and flux media at a concentration of 1 mM.

Results

Time Course of Cation Efflux

Figure 1 shows the unidirectional efflux of Na or K from normal volume (MCHC = 33–34%) erythrocytes of newborn pigs suspended in Na/K-free media. Na efflux was found to be linear for up to 1 hr of incubation. It is evident that the erythrocytes of the fetal origin are more permeable to Na than to K, even though the cells contained approximately equal amounts of each cation after the nystatin treatment. In agreement with the findings of Sorenson et al. (1962), the ouabain-sensitive (OS) Na efflux accounted for a large fraction of the total Na flux. Unlike Na efflux, which is composed of active and other passive components, the magnitude of total K efflux was reduced by the opposing pump activity. Thus, K efflux in the presence of ouabain was actually higher than in ouabain-free media. Although Na + K pump would not be expected to operate in a Na/K-free medium, it has not been possible to achieve a true Na/K-free solution. Typically, a background Na concentration of 8–12 μM and 2–5 μM K in the Mg-sucrose flux medium was found. Apparently, a trace concentration of substrate was sufficient to activate the pump. Beaugé and Adragna (1971) have shown that at low or in the absence of extracellular Na, the pump has a higher affinity for K. In the presence of ouabain, a portion

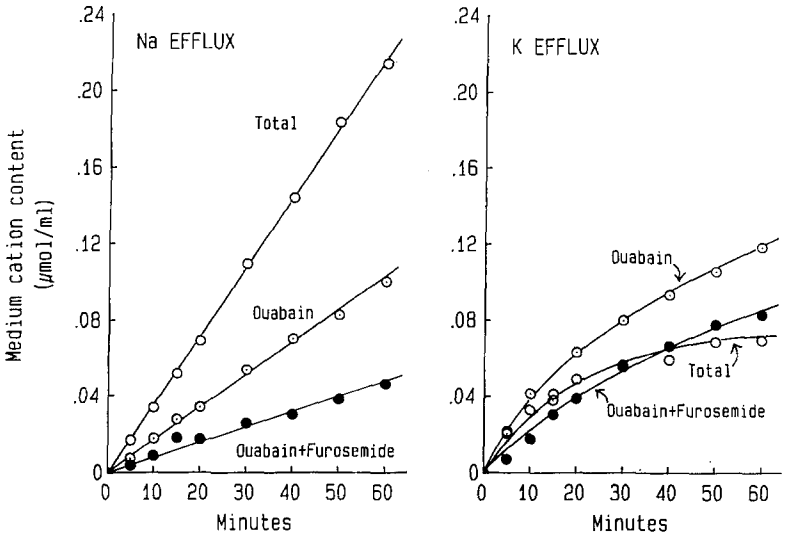

Fig. 1. Time course of Na and K efflux in pig fetal erythrocytes. In a representative experiment of this series (n = 6), fetal cells having altered cation content of 172 μmol Na/gHb and 192 μmol K/gHb were suspended in the isotonic MSH flux media at 37°C. For the composition of the MSH flux media, *see* the Methods section. Cation efflux was measured by the appearance of Na or K in media. All efflux data were corrected for hemolysis, which was generally less than 1%. No inhibitors, (○); 0.1 mM ouabain (⊙); ouabain + 1 mM furosemide, (●)

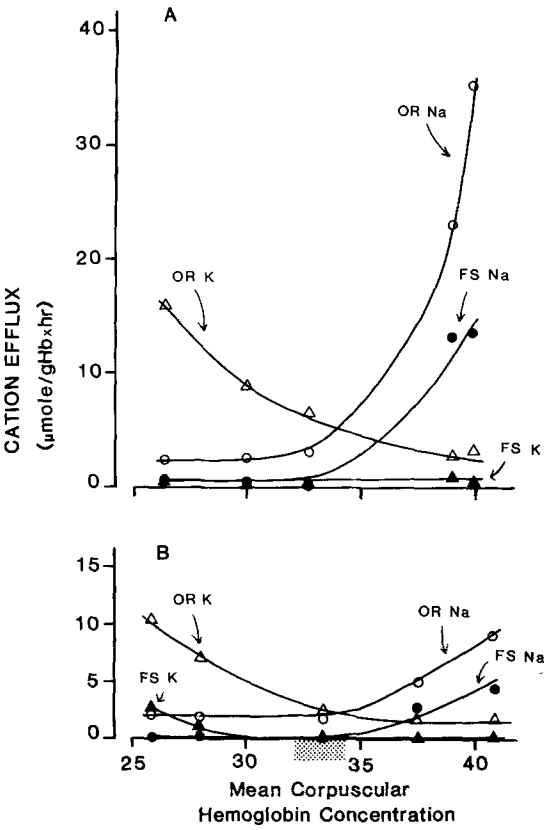

Fig. 2. Effects of cell volume on ouabain-resistant Na and K efflux in (*A*) fetal and (*B*) adult pig erythrocytes. Following treatment with nystatin, fetal (168 ± 4 μmol Na/gHb, 190 ± 2 μmol K/gHb) and adult (203 ± 5 μmol Na/gHb, 194 ± 4 μmol K/gHb) erythrocytes were suspended to give 5–6% hematocrit in $MgCl_2$-sucrose media of varying osmolarities (240–410 mOsM) containing 0.1 mM ouabain either with or without 1 mM furosemide at 37°C, pH 7.2. The furosemide-sensitive (FS) efflux refers to the difference between efflux measured with or without furosemide. The data shown are representative of two similar experiments. Ouabain-resistant efflux (○, OR Na, △, OR K); furosemide-sensitive efflux (●, FS Na; ▲, FS K). The shaded bar along the x-axis represents the normal volume of erythrocytes

of both Na and K effluxes was inhibited by furosemide.

Volume Dependence of Cation Efflux

To determine whether these ion fluxes were affected by changes in cell volume, Na and K efflux was measured in nominally Na/K-free media of varying osmolarity. Figure 2*A* shows that in response to cell shrinkage, ouabain-resistant (OR) Na efflux increased dramatically in fetal cells. It is clear that a part of the shrinkage-induced OR Na efflux was furosemide sensitive (FS). Unlike OR Na efflux, OR K efflux did not respond to cell shrinkage.

Shown in Fig. 2*B* are volume-activated K and Na fluxes in adult cells. As in fetal cells, adult cells lost Na but not K upon shrinkage and lost K but not Na upon enlargement. The results of a detailed investigation on K flux of hypotonically swollen cells have been presented elsewhere (Kim et al., 1989). We found that the K flux is chloride dependent and stimulated by increased cyclic AMP content.

The magnitude of the OR Na and the FS Na fluxes in shrunken adult cells was two to four times less than those in fetal cells. In contrast to fetal red cells, FS Na efflux was not readily detectable in adult erythrocytes of normal volume. These findings, taken together, show that volume-activated ion fluxes undergo developmental changes.

Furosemide-Sensitive Na Efflux Independent of K

Although a furosemide-sensitive K efflux could not be detected in shrunken cells (Fig. 2), it is conceivable that the cell cation composition in these experi-

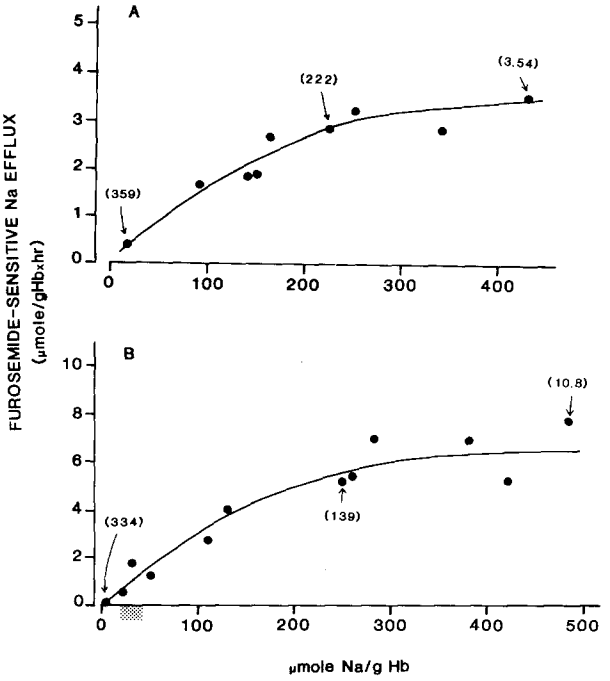

Fig. 3. Effects of intracellular cation content on furosemide-sensitive Na efflux in fetal and adult pig erythrocytes. Cellular Na and K content was varied reciprocally using a series of loading buffers in which the sum of Na, K and choline concentrations was 130 mM. (*A*) Na efflux in fetal cells of normal volume (MCHC = 33.3–34.8%) and (*B*) shrunken adult cells (MCHC = 38.9–42.3% adult cells), was measured at 37°C in $MgCl_2$-sucrose-ouabain media either with or without 1 mM furosemide. The flux media for fetal and adult cells contained 105 mM and 185 mM sucrose, respectively. Values given in parentheses indicate the cell K content (μmol/gHb). A typical result of two experiments is shown. The shaded bar along the *x*-axis indicates the normal Na content of pig erythrocytes with an MCHC of 33–34% (7 to 10 mmol Na/liter cell water)

ments was not appropriate for unmasking the presence of the K flux component. Cell Na and K content was varied reciprocally in order to determine whether Na efflux in pig erythrocytes was K dependent. Figure 3 shows that furosemide-sensitive Na efflux from both normal volume fetal cells (panel *A*) and shrunken adult cells (panel *B*) exhibited saturation kinetics when ploted as a function of cellular Na content. In Fig. 3, efflux in adult cells was greater than that of fetal cells due to the experimental manipulation, which resulted in an extensive shrinkage of adult cells. In both cell types, furosemide inhibited Na efflux at all variations of intracellular Na and K content. In cells in which nearly all of the K was depleted (right side of upper and lower plots), FS Na efflux did not decrease, suggesting that the ubiquitous Na/K/Cl cotransport system is not present in pig red cells as is also the case for rabbit erythrocytes (M.L. Jennings, *personal communication*).

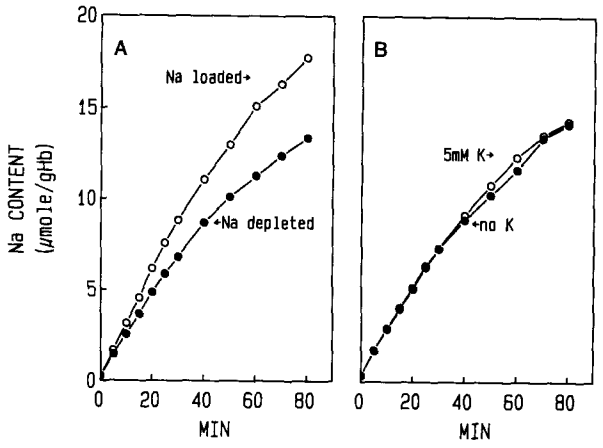

Fig. 4. Comparison of Na influx: (*A*) effects of intracellular Na depletion and (*B*) effects of the removal of external K. Na influx was measured using ^{22}Na as a tracer in hypertonic flux media of 410 mOsM containing 40 mM NaCl and 0.1 mM ouabain. Cellular Na content was altered by the nystatin procedure. The resulting Na content of Na-loaded and Na-depleted cells was 78 μmol/gHb and 0.24 μmol/gHb, respectively. In experiments in which the external K was withheld, sucrose was used to maintain the flux media of 410 mOsM

Na/Na Exchange Component

The measurement of Na influx can be complicated by the presence of a Na/Na exchange pathway. To determine effects of intracellular Na on the Na influx, Na influx in cells containing Na was compared with that of cells depleted of Na. In both cases, cells were shrunken to the same extent. The results summarized in Fig. 4*A* indicate that Na influx was less in Na-depleted cells than in cells containing Na, revealing the presence of a Na/Na exchange pathway. Figure 4*B* shows the effect of external K on the volume-activated Na influx. It is clear that the presence of external K did not influence Na influx, suggesting that pig cells lack a Na/K/Cl cotransporter.

To minimize the complication arising from the Na/Na exchange component, all subsequent Na influx experiments were performed with Na-depleted cells.

Inhibition of OR Na Flux by Diuretics

The bidirectionality of volume-activated Na flux is evident in Fig. 5 in which cell shrinkage progressively stimulated Na influx in a manner analogous to Na efflux described in Fig. 2. Again, the magnitude of volume-activated Na influx in adult cells was much less than in fetal cells. In both cell types, amiloride was found to be much more potent than furosemide in inhibiting Na flux.

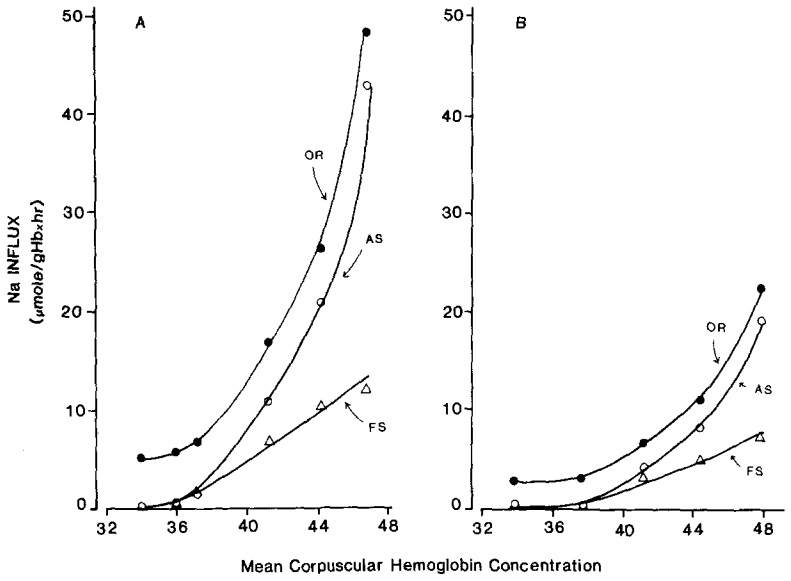

Fig. 5. Effects of cell volume on Na influx in (*A*) fetal and (*B*) adult pig erythrocytes. Red cells, depleted of Na described for Fig. 4, were suspended in PBS solution varying in osmolarity from 250–440 mOsM, and Na influx using ^{22}Na was measured at 37°C in the presence of 0.1 mM ouabain with or without 1 mM amiloride or furosemide. The diuretic-sensitive influx refers to the difference between the influx measured in the absence or presence of 1 mM furosemide or amiloride. The data shown are representative of two experiments. Ouabain resistant (●, OR), amiloride sensitive (○, AS) and furosemide sensitive (△, FS)

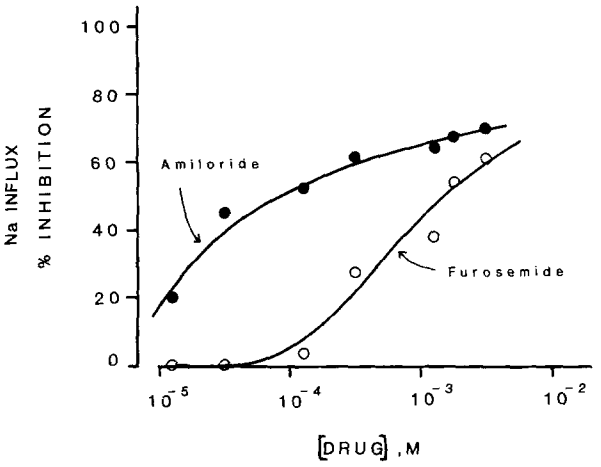

Fig. 6. Dose-dependent inhibition of Na influx by diuretics in Na-depleted adult pig erythrocytes. Na-depleted cells were washed once in ice-cold hypertonic PBS containing 140 mM sucrose to shrink the cells (MCHC = 41.4% for amiloride curve and 44.5% for furosemide curve). Na influx using ^{24}Na was measured at 37°C in hypertonic flux buffer in the presence of 0.1 mM ouabain with various concentration of diuretics. The IC_{50} for each drug was estimated by nonlinear regression. The data shown are representative of two experiments

Figure 6 shows a dose response curve for the inhibition of Na influx by diuretic agent in Na-depleted shrunken adult cells. Both amiloride and furosemide inhibited nearly 70% of the OR Na influx when present at concentrations greater than 1 mM. Amiloride exhibited an apparent IC_{50} of 32 μM compared to 760 μM for furosemide. Effects of furosemide and amiloride when present together in the incubation medium were not additive (*data not shown*), suggesting that these diuretics inhibit the same transport system. In addition, Na influx in pig erythrocytes was inhibited nearly 50% by 1 mM harmaline (*data not shown*), a hallucinogen that has been shown to inhibit Na/H exchange activity in renal microvillus membrane vesicles (Aronson & Bounds, 1980).

Anion Dependence

The inhibition of Na flux by amiloride suggests the presence of a Na/H exchanger, which is activated by cell shrinkage. Net salt movement takes place when the Na/H exchanger operates in parallel with a Cl/HCO_3 anion exchanger (Cala, 1980). Alternatively, the volume-activated Na flux could be mediated by a Na/Cl cotransporter, which has been demonstrated in epithelial cells (Binder & Rawlins, 1973; Nellans, Frizell & Schultz, 1973; Frizzell, Dugas & Schultz, 1975) and in Ehrlich ascites tumor cells (Hoffmann et al., 1983). The Na/Cl cotransporter is also inhibited by loop diuretics like furosemide and bumetanide.

To distinguish between these possibilities, we examined the Cl dependence of the OR Na flux in fetal erythrocytes. Replacement of Cl by another anion would be expected to reduce furosemide-sensitive Na flux if it were mediated by a Na/Cl cotransporter. Figure 7 shows the results of Na efflux, which was measured in fetal erythrocytes of varying Cl content. Nitrate, a readily permeable anion in red blood cells, was used to replace Cl. As the Cl content in these cells decreased, the rate of Na efflux decreased in a nonlinear fashion both in the presence or absence of furosemide. This decrease

Table. Effect of anion replacement on Na influx in shrunken Na-depleted pig erythrocytes[a]

Anion	*n*	Ouabain resistant	% of Rate in Cl medium		Ouabain resistant, furosemide sensitive	Ouabain resistant, amiloride sensitive
			Ouabain + furosemide resistant	Ouabain + amiloride resistant		
Chloride	6	100	100	100	100	100
Nitrate	2	76 ± 6	98 ± 21	168 ± 30	52 ± 7	27 ± 3
Gluconate	3	44 ± 4	21 ± 1	53 ± 5	64 ± 4	43 ± 7
Acetate	4	141 ± 7	122 ± 30	88 ± 5	170 ± 10	170 ± 7

[a] Influx was measured in hypertonic PBS containing 130 mM Na and 0.1 mM ouabain in the presence or absence of the 1 mM furosemide or amiloride. Since influx rates were exquisitely sensitive to slight changes in MCHC, ranging from 42 to 47 in this series of experiments, it was difficult to make a direct comparison of Na influx rates in different experiments. To alleviate this problem, Na influxes in the presence of various anions are expressed as percentages (mean ± SEM) of the Cl control rate at the same MCHC for a given experiment. The control rates were estimated from a volume curve (Fig. 5) at the MCHC of that for the test anion. The 100% values for the Cl controls represent a range of rates for the 42–47% MCHC range as follows: ouabain resistant, 7.55–18.7 μmol Na/gHb · hr; ouabain + furosemide resistant, 4.40–11.60; ouabain + amiloride resistant, 2.55–3.60; furosemide sensitive, 3.15–7.10; and amiloride sensitive, 5.00–15.10. The effects of ion replacement on ouabain-resistant and diuretic-sensitive fluxes were significantly different ($P = 0.01$) from control values as determined by analysis of variance.

in efflux could not be attributed to a variation in cell size because MCHC was held within a narrow range (36 to 38%). A component of Na flux persisted even in cells nearly depleted of Cl. However, the FS Na efflux was abolished with the removal of cell Cl. These results suggest that the furosemide-sensitive Na transport could be a Cl-dependent process and, therefore, may be mediated by a Na/Cl cotransporter. However, an alternative interpretation of these results is also possible in that nitrate is known to inhibit Na/H exchange in dog (Parker, 1983), rabbit (Jennings et al., 1986), trout red cells (Borgese, Garcia-Romeu & Motais, 1986) and *Amphiuma* (Cala, 1983) erythrocytes.

To distinguish between Cl dependence and nitrate inhibition, Na influx was measured in the presence of various anions. In the Table, effects of various anions on the ouabain-resistant Na influx in shrunken cells are summarized. If OR Na influx is mediated by a Na/Cl cotransporter, one would expect more inhibition by an impermeant anion. This was not observed. In point of fact, nitrate was more stimulatory than gluconate to ouabain- and diuretic-resistant Na flux. That gluconate alters the Cl distribution and, thus, the membrane potential in red cells may account for the dramatic inhibitory effect on the ouabain- and diuretic-resistant flux. Compared to Cl, acetate actually stimulated the OR influx as well as the diuretic-sensitive Na flux pathway. Moreover, the pattern of anion dependence of the OR Na influx in pig erythrocytes is similar to

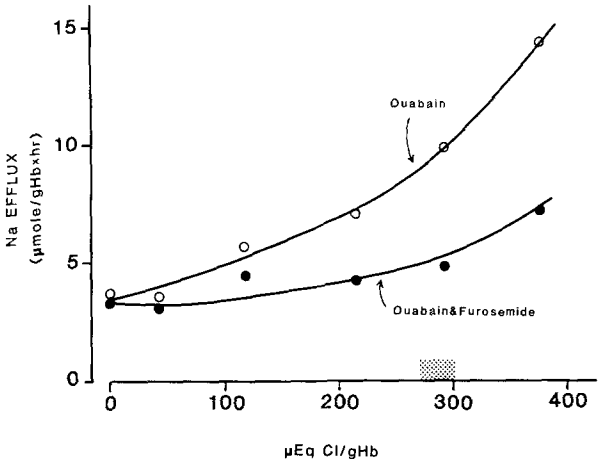

Fig. 7. Effects of intracellular chloride content on Na efflux in pig fetal erythrocytes. Ionic content of cells was altered such that Na (176 ± 3 μmol Na/gHb) and K (199 ± 3 μmol K/gHb) were held constant, but varied in Cl concentration using nitrate as the replacement anion. Cl content was measured using a Buchler-Cotlove chloridometer in perchloric acid extracts of cells lysed with deionized water. Na efflux was measured at 37°C in the presence (●) or absence (○) of 1 mM furosemide in Mg-sucrose-ouabain media having the same Cl/NO_3 ratio as that of the loading buffers in which the cells were prepared. MCHC ranged from 36.2–38.1%. The shaded bar along the *x*-axis represents the usual chloride content (140–150 mmol/liter cell water) of pig erythrocytes of normal volume. The results shown were representative of three other experiments. For each experiment, the Na efflux in the presence of ouabain and furosemide was significantly less ($P = 0.016$) than the efflux on the presence of ouabain alone, as determined by a type of nonparametric permutation test

that reported for the Na/H exchanger in other cells (Cala, 1983; Parker, 1983; Jennings et al., 1986).

Hydrogen Ion Flux

The operation of a Na/H exchanger obligates the movement of internal protons in exchange for external Na. Under conditions in which Na influx in erythrocytes is activated, one would expect to observe an acidification of the incubation medium due to the efflux of hydrogen ions. The extrusion of protons into the extracellular medium was continuously measured and recorded as shown in Fig. 8 by a method similar to that described for rabbit erythrocytes (Jennings et al., 1986). Following resuspension in unbuffered 150 mM KCl, there was an initial rapid rise in pH, which stabilized between pH 7.35 and 7.40 within 3 to 4 min. The addition of ouabain and an aliquot of 2 M NaCl or KCl to shrink the cells caused a sharp drop in pH, which rapidly stabilized between pH 7.3 and 7.5 (*data not shown*). The start of traces shown in Fig. 8 marks the pH of media after the cells had been shrunken and pH had stabilized.

The cells used in trace *A* of the upper panel of Fig. 8 were shrunken by the addition of an aliquot of 2 M NaCl, thereby providing an extracellular substrate for the Na/H exchanger. Upon the addition of the anion transport inhibitor DIDS, as indicated by the arrow, a rapid acidification of the external pH took place. Amiloride added 6 to 7 min later resulted in an instantaneous and partial reversal of pH, which was maintained thereafter. The sudden reversal of the pH after the amiloride addition may reflect residual anion exchange activity. In trace *B* of Fig. 8, cells were first treated with 0.66 mM amiloride in a Na-free medium and then shrunken with 2 M NaCl. Following the addition of DIDS, the acidification of the amiloride containing media (trace *B*) was much slower compared to that of the amiloride-free media (trace *A*). Further addition of amiloride was without effect. This observation is consistent with the suggestion of Parker (1986) that Na and amiloride may compete for the same external binding site, since the addition of Na was unable to overcome the inhibitory effect of amiloride.

In KCl hypertonic media (trace *C*), the DIDS-induced acidification was significantly reduced compared to that of the NaCl hypertonic media (trace *A*). The presence of Na in isotonic media (trace *D*) produced a slow acidification, which was indistinguishable from that of KCl hypertonic media (trace *C*). Moreover, the addition of amiloride had virtually no effect in both cases. These results lend strong support to the conclusion that the major component of the volume-sensitive Na flux in pig erythrocytes is mediated by a Na/H exchanger.

The lower panel of Fig. 8 shows that furosemide (trace *F*) was also able to halt the DIDS-induced acidification when cells were shrunken with 2 M NaCl. Furosemide, if acting as an anion exchange inhibitor, would be expected to have little influence on the acidification of medium resulting from DIDS-treated cells. Instead, furosemide appeared to be as effective as amiloride (trace *E*) in arresting the medium acidification. Moreover, furosemide was also able to blunt the acidification response when added before cell shrinkage. When furosemide was added to a suspension of shrunken cells instead of DIDS, it did not cause a rapid acidification of the medium (*data not shown*). Thus, it seems likely that furosemide inhibits the Na/H exchanger rather than the Cl/HCO_3 exchanger.

Effect of Cyclic Nucleotides on OR Na Flux

Mature mammalian erythrocytes lack a functioning receptor-coupled adenylate cyclase system. However, we (Sergeant & Kim, 1985; Kim et al., 1989) and others (Garay, 1982) have shown that the cyclic AMP content of erythrocytes can still be raised far in excess of basal levels by incubation of cells with exogenously added cAMP. Since pig erythrocytes can also be loaded with cyclic nucleotides, it was of interest to determine whether cyclic nucleotides play a role in the control of the Na/H exchanger. In a typical experiment, control cell cAMP content was 36.75 ± 1.54 pmol/ml cells, while the content of cAMP-loaded cells increased to 8.48 ± 0.28 nmol/ml cell, as determined by radioimmunoassay. Figure 9 shows that the volume-activated Na flux was drastically reduced in cells loaded with either cAMP or cGMP. The inhibitory effect of the cyclic nucleotides on Na flux was more pronounced as cells shrank. Since the inhibition of OR Na influx by cAMP (Fig. 9*A*, closed circles) was nearly complete, the addition of amiloride (Fig. 9*C*, open circles) did not cause further inhibition. As a result, the amiloride-sensitive flux in cAMP-loaded cells was largely undetectable even in extensively shrunken cells as can be seen in Fig. 9*B*. The furosemide-sensitive Na flux was similarly affected by cyclic nucleotides (*data not shown*).

Discussion

It is now well established that the regulation of cell volume depends on selective changes in ion permeabilities in a variety of cells. For example, nucleated red cells (Kregenow, 1971; McManus & Schmidt, 1978) and numerous others (Hoffmann, 1986) exhibit the ability to restore their volume in

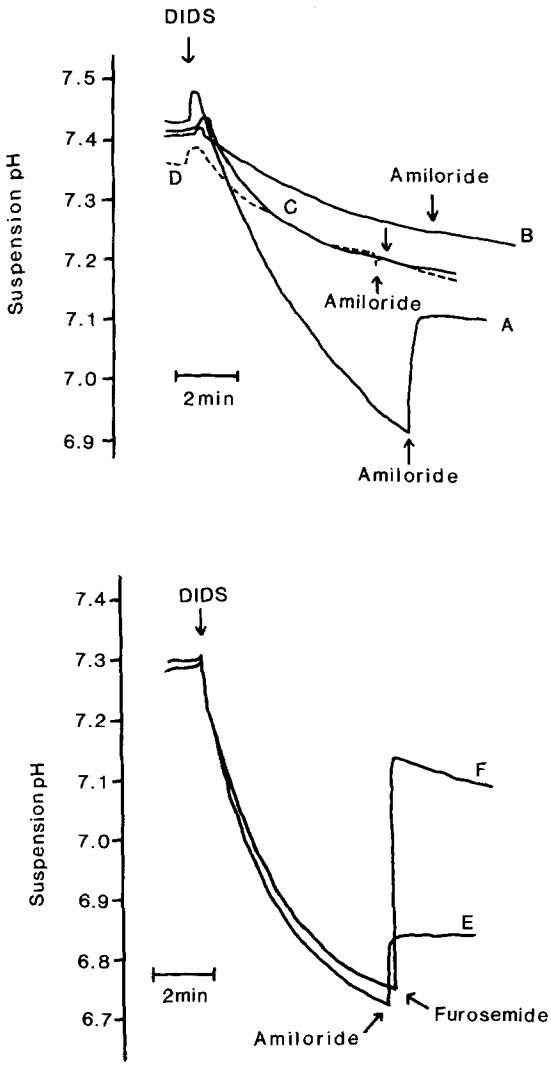

Fig. 8. Effects of osmotic shrinking of adult pig erythrocytes on extracellular pH. Cells were prepared for the measurement of hydrogen ion efflux as described in the Methods section. In the upper panel, the start of traces marks the pH of media after the pH had stabilized following the additions of ouabain and either 2 M NaCl (trace *A*) or 2 M KCl (trace *C*) to give 409 mM salt media. In trace *B*, amiloride was added at a final concentration of 0.66 mM to the cell suspension prior to the addition of 2 M NaCl and again added at the time indicated by an arrow. In trace *D* (dashed line), the cells were resuspended in isotonic 154 mM NaCl instead of 150 mM KCl and were not shrunken. At the times indicated by the arrows, DIDS and amiloride were added to give a final concentration of 4 μM and 0.66 mM, respectively. In the lower panel, cells were shrunken by the addition of 2 M NaCl. After the pH had stabilized, DIDS was added at a final concentration of 4 μM. Amiloride (trace *E*) or furosemide (trace *F*) was added as indicated by the arrows to give a final concentration of 0.66 mM or 1 mM, respectively. Similar results were obtained in two other experiments

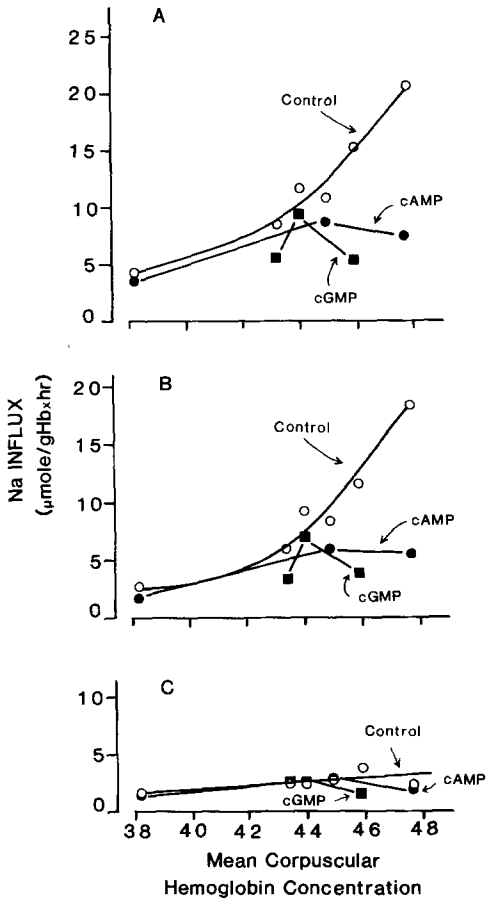

Fig. 9. Effects of cyclic nucleotides on Na influx in Na-depleted pig erythrocytes. To load cells with cyclic nucleotides, 1 mM cAMP or cGMP was present during the nystatin treatment and flux measurements. Na influx was measured using either ^{22}Na or ^{24}Na as tracer at 37°C in PBS of varying osmolarity containing 0.1 mM ouabain. (*A*) shows effects of cyclic nucleotide on Na influx as a function of cell volume. (*B*) depicts effects of cyclic nucleotides on amiloride-sensitive Na influx. (*C*) shows Na influx in the presence of 1 mM amiloride in cyclic nucleotide-loaded cells

response to swelling induced by osmotic challenge, the process of which is referred to as the regulatory volume decrease (RVD). The loss of K rather than Na has invariably been found to account for this (Lauf, 1985; Eveloff & Warnock, 1987). The transport pathways, which are thought to mediate the extrusion of KCl and osmotically obligated water, include the KCl cotransporter (Lauf, 1982; Thornhill & Laris, 1984), the conductive Cl flux coupled to conductive K flux (Grinstein et al., 1982; Hoffmann, 1985) and the coupled K/H and Cl/HCO_3 exchangers (Cala, 1980; Cala, Mandel & Murphy, 1986).

Cell shrinkage is an equally effective signal by which several ion transport pathways can be stimulated (Hoffmann, 1986; Eveloff & Warnock, 1987). The ensuing restoration of cell volume, which is otherwise known as the regulatory volume increase (RVI) depends on a number of OR Na transport pathways, including the Na/K/Cl cotransporter (Schmidt & McManus, 1977*a*; Kregenow, 1981),

the Na/Cl cotransporter (Hoffmann, 1985) and the coupled Na/H and Cl/HCO_3 exchangers (Cala, 1980; Cala et al., 1986). In renal epithelia endowed with both Na/K/Cl and Na/Cl cotransporters, the expression of one cotransporter over the other is apparently determined by the cell volume (Eveloff & Warnock, 1987).

RVI can not only be demonstrated by initially shrinking cells in hypertonic media, but also in isotonic media if supplemented with catecholamines to cause β receptor activation. In isotonic media to which catecholamine has been introduced, the activation of a Na/K/Cl cotransport accounts for duck red cells undergoing gradual swelling (Schmidt & McManus, 1977*b*; Kregenow, 1981), whereas the stimulation of an Na/H exchanger coupled to a Cl/HCO_3 exchanger is said to be the underlying mechanism responsible for swelling of trout red cells (Borgese, Garcia-Romeu & Motais, 1987). The operation of the latter mechanism for RVI can also be demonstrated in circulating mononuclear cells, which were initially swollen in hypotonic solution and then restored in isotonic media (Grinstein, Clarke & Rothstein, 1983).

Although the regulatory volume changes have not unequivocally been demonstrated in anucleated mammalian red cells, the cell volume is, nonetheless, a key determinant of cation permeability of certain red cells. Selective increases in the permeability of Na over K or of K over Na, depending upon the volume of pig cells, bear remarkable similarities to the well-characterized system investigated by Parker and Hoffman (1976) on dog red cells. In keeping with the observation in dog and rabbit red cells (Jennings et al., 1986), the increased Na movement triggered by shrinkage of pig cells exhibits features which are consistent with the activation of a Na/H exchanger. The Na movement across the pig cell membrane does not appear to be mediated by a Na/K/Cl cotransporter. The conclusion that pig cells are devoid of a Na/K/Cl cotransport stems from the findings on the lack of a mutual dependency of cotransported ion species: (*i*) the swelling-activated, chloride-dependent K efflux is uninfluenced by depletion of cellular Na (Kim et al., 1989); (*ii*) the K influx does not require external Na (Kim et al., 1989); (*iii*) the Na efflux activated by shrinkage is not affected by K depletion (Fig. 3); and (*iv*) the Na influx does not require external K (Fig. 4). These results are incongruous with the finding reported by Hall and Willis (1987), who reported that pig red cells have a Na/K cotransport system. In that study, the bumetanide-sensitive K uptake was taken as the sole measure of Na/K cotransport without testing for the requirement of cotransported Na ion.

From a developmental point of view, it is interesting that Na/H exchanger activity is present in normal fetal cells, but quiescent in adult cells. However, the Na/H exchanger is augmented in both cell types upon reduction in cell volume, indicating that the Na/H exchanger is not discarded but undergoes a modification during the transition from the fetal to the adult stage.

In addition to the well-known inhibitory action of furosemide on the Na/K/Cl cotransport, several studies have now shown that furosemide exhibits a broad specificity. The growing list of transport processes, which are influenced by furosemide, include the glucose (Jacobs, Mookerjee & Jung, 1984) and adenosine (Ford, Sharp & Rovetto, 1985) carriers, Na + K pump (Ellory et al., 1982), epithelial acid-base pump (Ehrenspeck & Voner, 1985), Cl/HCO_3 exchanger (Brazy & Gunn, 1976) and Na/H exchanger (Grinstein et al., 1983; Baroin et al., 1984). Although the inhibitory effect of furosemide on Na flux in pig cells could be explained if furosemide inhibits the Cl/HCO_3 exchanger as described in human cells (Brazy & Gunn, 1976), results shown in Figs. 2, 5 and 8 taken together argue in favor of the notion that furosemide inhibits the Na/H exchanger.

The manner by which cAMP influences ion transport is complex. In some cells, cAMP elicits an inhibition of the Na/H exchanger (Kahn et al., 1985; Reuss & Petersen, 1985; Semrad & Chang, 1986), whereas in others the same transporter is stimulated by cAMP (Borgese et al., 1987). This dual regulation by cAMP is also true for the Na/K/Cl cotransporter (McManus & Schmidt, 1978; Garay, 1982).

In addition to eliciting either stimulation or inhibition of a given ion transport system depending on the cell type, cAMP also causes either stimulation or inhibition of different ion transport pathways in a given cell. Thus, in pig cells, swelling-activated, chloride-dependent K flux is stimulated by cAMP, as opposed to an inhibition seen by cAMP of the Na/H exchanger. Since mature erythrocytes can not ordinarily produce cyclic nucleotides through the agonist-stimulated adenylate cyclase system, the physiological significance in pig and other mammalian red cells is less clear. However, it is tempting to speculate that the cyclic nucleotide inhibition could reflect the remnant of a regulatory control mechanism for the Na/H exchanger in earlier stages of erythroid development in which an intact and functional β-adrenergic receptor system undergoes a modification (Farfel & Cohen, 1984).

The mechanism by which cAMP acts is poorly understood. Phosphorylation of a 230,000 Da protein has been suggested as a potential site of action

involved in the regulation of Na/K/Cl cotransport by cAMP in turkey erythrocytes (Palfrey, Alper & Greengard, 1980).

In conclusion, it is clear that neither the ubiquitous Na/K/Cl cotransporter nor the Na/Cl cotransporter is present in the pig cells and that a major component of the ouabain-resistant, volume-sensitive Na movement is mediated by a Na/H exchanger. The properties of Na transport through this system in pig red cells share similarities with well-characterized Na/H exchange systems in other cell types. In addition, the observed postnatal developmental changes in and the influence of cyclic nucleotides on the volume-activated Na flux warrant further investigation on (*i*) the regulation of the Na/H exchanger in reticulocytes, which possess hormone-responsive adenylate cyclase and (*ii*) the potential role of this transport system in cell volume regulation in pig erythrocytes.

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