Comparative aspects of Na+/K+ pump-mediated uncoupled Na+ efflux in red blood cells and kidney proteoliposomes

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ABSTRACT   Ouabain-sensitive uncoupled Na+ efflux has been studied in human, pig, and rat red cells and in vesicles containing reconstituted kidney Na+/K+ pumps obtained from these same species. The red cells from the different species gave qualitatively similar results; the uncoupled Na+ efflux was 15–30% of the Na+/K+ exchange rate, and this flux was inhibited at 5 mM extracellular Na+ (Na_o^+). At higher levels of Na_o^+ there was a monotonic increase in the Na+ efflux. As has previously been observed in human red cells, the uncoupled efflux from pig red cells consists of Na+ and anion cotransport, suggesting that anion cotransport may be a general characteristic of uncoupled Na+ efflux in red cells. The uncoupled Na+ efflux carried out by pig and rat kidney Na+/K+ pumps differs from the red cell activity in that it represents no more than 2–4% of the Na+/K+ exchange rate and that 5 mM Na_o^+ does not inhibit this efflux. Furthermore, the efflux does not appear to be dependent on anion cotransport. Vesicles containing human kidney Na+/K+ pumps differ from vesicles derived from pig or rat kidneys in that the Na+ efflux is not inhibited or stimulated by Na+ present on the opposite side; it thus appears that the Na+,K+-ATPase in these vesicles may be incapable of Na+/Na+ exchange. These results indicate that the ligand and kinetic properties of the uncoupled Na+ efflux mode of red cells are markedly different from kidney-derived Na+/K+ pumps reconstituted into proteoliposomes. The basis for these differences may be inherent in the Na+/K+ pumps themselves or represent differences between the two types of preparations studied.

The Na+/K+ pump in red blood cells is capable of working in several modes depending on the ionic composition of the internal and external media (1). Under physiological conditions, three intracellular Na+ ions (Na_i^+) are exchanged for two extracellular K+ ions (K_o^+); however, if the cells are suspended in media free of Na_o^+ and K_o^+, it is possible to measure what is referred to as an "uncoupled" efflux of Na_i^+ since it occurs in the absence of Na_o^+ and K_o^+. This uncoupled efflux is not only pump mediated, it is ATP dependent and inhibited by cardiotonic steroids, such as ouabain (1–6). If the concentration of Na_o^+ is increased to 5 mM this efflux is inhibited by 60–80%, but at higher levels of Na_o^+ there is a stimulation of Na+ efflux via Na_i^+/Na_o^+ exchange. It has been shown that in human red cells the uncoupled Na+ efflux is electroneutral and that the charge compensation of Na+ movement is achieved by a cotransport of intracellular anions: the Na_o^+-inhibitable efflux is compensated by an efflux of cytoplasmic Cl− or SO_4^{2-} (7) while the Na_o^+-insensitive portion (20–40%) is coupled to an efflux of P_i that is directly donated, via the pump's phosphointermediate, from the γ-phosphate of ATP (8).

Parallel studies of uncoupled Na+ efflux have also been carried out in lipid vesicles containing reconstituted Na+/K+

pumps that were isolated from rectal gland or kidney tissue; however, the characteristics of uncoupled efflux as seen in these vesicle preparations are different from those seen in red cells (cf. ref. 7). Thus, Cornelius (9) has found that the uncoupled Na+ flux carried out by shark rectal gland Na+/K+ pumps is electrogenic and he has shown that this uncoupled Na+ efflux is not inhibited at any concentration of Na_o^+. Goldshleger et al. (10), however, found that with pig kidney pumps, the uncoupled Na+ flux is electroneutral between pH 6.5 and 7, and because they could not detect any influence of anions, they concluded that this efflux may be balanced by H+ uptake. One possible explanation for the discrepancies between the results displayed by the red cell and vesicle preparations is that they represent species- and/or tissue-specific differences; another is that the differences may lie in the types of preparation studied. In this work, we have attempted to address these questions by comparing in the same species some of the properties of uncoupled efflux of Na+ in red cells with their respective kidney Na+/K+ pumps reconstituted into proteoliposomes. Our results indicate that the uncoupled efflux in red cells from humans, pigs, and rats all have the same qualitative characteristics, whereas the flux measured in the various vesicle preparations has markedly different properties. These results may help explain some of the inconsistencies in the interpretation of ion flux data between red cell and reconstituted kidney Na+/K+ pumps.

MATERIALS AND METHODS

Solutions Used. First wash solution: 150 mM NaCl, 20 mM Hepes, 5 mM adenosine, 10 mM inosine, and 1 mM MgCl2 adjusted to pH 7.4 at 37°C. The Na+ efflux solution used for pig red cells (Fig. 1) is the same as the first wash solution except that choline chloride was substituted for NaCl. Various amounts of Na+ and K+ were used to replace an equimolar amount of the choline chloride. Second wash solution: 115 mM Na2SO4, 10 mM Tris2SO4, 5 mM inosine, 5 mM adenosine, and 0.5% Na-free bovine serum albumin (BSA). This solution was made to 330 mosM (determined osmometrically) by addition of solid Na2SO4, bubbled with hydrated N2 gas at pH 5.5 to remove traces of bicarbonate, and then titrated to pH 7.4 at 0°C with H2SO4. Anion efflux solution: 244 mM N-methylglucamine (NMG), 5 mM inosine, 5 mM adenosine, 0.5% Na-free BSA. To make (NMG)2SO4, the solution is first titrated to pH 5.5 at 20°C with H2SO4 and to 330 mosM with solid NMG and then bubbled with hydrated

N_2 gas before being finally brought to pH 7.4 at 37°C. In some experiments with rat red blood cells, $MgSO_4$ was substituted isosmotically (330 mosM) for $NMG(SO_4)_2$ as indicated in the relevant legend.

Measurement of Na^+ Efflux from Human, Pig, and Rat Red Blood Cells. Human blood was obtained by venipuncture from healthy volunteers, pig blood was obtained from an abattoir, and rat blood was collected from pentobarbital-anesthetized animals by syringe either from the heart or from the bifurcation of the femoral artery. The samples were stored at 0–4°C for no more than 5 hr with sufficient heparin to prevent clotting. The blood was then washed three times at 12,000 × g for 5 min at 0°C, each with 10 vol of the first wash solution. The washed red cells (buffy coat removed) were then loaded with $^{22}NaCl$ by incubation at 37°C in the solutions as specified in the legends for 3 hr at a 50% hematocrit. The cells were then washed five times each with 10 vol of their respective loading solutions (isotope-free) at 0°C. At the end of the washes an aliquot of the red cells was removed for determination of Na_i^+, K_i^+, and water content (see ref. 7). The remaining cells were diluted to a 1–2% hematocrit in the specified efflux solutions with various amounts of Na^+ and K^+, with or without 1 mM ouabain. The $^{22}Na^+$ flux was measured as described (7, 8) by incubating the suspension mixture at 37°C for up to 1 hr. Aliquots were removed at different times and rapidly cooled to 0°C in an iced bath, and the radioactivity present in the supernatant was determined after centrifuging the cells at 12,000 × g for 1 min. The time 0 radioactivity was measured by counting the supernatants from aliquots of the suspension that had been kept at 0°C. In addition, the radioactivity of the total suspension mixture was also determined. The efflux was calculated from the fractional loss of $^{22}Na^+$ from the cells that occurred during the timed incubation intervals. The ouabain-sensitive efflux of Na^+ was taken as the difference in the rates in the absence and presence of ouabain (0.1 or 0.5 mM for human and pig red cells; 1 mM for rat red cells).

Measurement of Phosphate, Sulfate, and Na^+ Effluxes in Red Blood Cells. The cells were washed as described in the second wash solution before being suspended to a 5% hematocrit and incubated for 30 min at 37°C. The SO_4^{2-}-loaded cells were then divided into three portions: one was incubated for up to 4 hr at 37°C with $^{35}SO_4^{2-}$, another was incubated with $^{22}Na^+$ as described above, and the third was kept at 0°C to be used for measurements of P_i effluxes. (In some experiments with rat red cells, a fourth portion was set aside for $^{86}Rb^+$ influx determinations.) The three portions were washed three times with the second wash solution without BSA and incubated with 50 μM 4,4′-diisothiocyanos-tilbene-2,2′-disulfonic acid (DIDS) for 5 min at 37°C. The cells were then washed again three times with the second wash solution and resuspended in this solution (pH 7.5 at 20°C) with various concentrations of Na_2SO_4 and K_2SO_4 substituted for $NMG(SO_4)_2$ plus or minus ouabain; the effluxes ($^{35}SO_4^{2-}$, $^{22}Na^+$, and P_i) were measured by incubation at 37°C for up to an hour. The $^{22}Na^+$ and $^{35}SO_4^{2-}$ effluxes were usually carried out at a 1–2% hematocrit and were measured as described above for the Na^+ effluxes (cf. refs. 7 and 8). The P_i effluxes were measured at a 10–15% hematocrit by determining the changes in the concentration of P_i in the supernatant at various times over a 1-hr incubation interval as described (8). Since the P_i efflux is inhibited by K_o^+, care was taken to ensure that the P_i efflux was not significantly influenced by changes in K_o^+ (see below and ref. 8).

Measurement of Rb^+ Influx in Rat Red Blood Cells. Because Rb^+ can be taken as a congener of K^+, the influx of $^{86}Rb^+$ was measured in rat red cells at various concentrations of K_o^+ and Na_o^+. The influx of $^{86}Rb^+$ by the cells was estimated from the rate of uptake of $^{86}Rb^+$ by the cells incubated under comparable conditions as referred to above for P_i efflux regarding

the hematocrit and the primary composition of the incubation medium. K_o^+ and Na_o^+ in the latter solution were varied by substitution for equivalent amounts of either $NMG(SO_4)_2$ or $MgSO_4$. The Na^+/K^+ pump-mediated Rb^+ influx was taken as the difference in the influx obtained in the absence and presence of 1 mM ouabain.

Isolation of Kidney Na^+/K^+ Pumps. Pig and Sprague–Dawley rat kidneys were obtained from Pel-Freez Biologicals; fresh frozen human kidneys were kindly provided by Michael Kashgarian of the Pathology Department. These were the excised kidneys from patients receiving kidney transplants. Na^+,K^+-ATPase was isolated from the outer medulla of the kidneys following the shorter of the two procedures described by Jørgensen (11). The activity of the preparation was assessed by measuring the release of phosphate (12) from ATP after a 10-min incubation at 37°C and the protein was measured as described by Peterson (13). For both the rat and the pig kidney preparations the Na^+,K^+-ATPase activity ranged from 13 to 20 μmol·mg^{-1}·min^{-1}; the human kidney preparations ranged from 5 to 8 μmol·mg^{-1}·min^{-1}.

Reconstitution of Kidney Na^+/K^+ Pumps. Kidney Na^+/K^+ pumps were reconstituted into proteoliposomes by the freeze/thaw sonication procedure using cholate solubilized enzyme as described by Karlish and Pick (14). The optimal protein/phospholipid ratio (1 part protein/15 parts azolectin) was determined by measuring the Na^+ uptake rate with $^{22}Na^+$ as described below, at various lipid/protein ratios. Except where noted, the vesicles were loaded with either 200 mM KCl or 200 mM Tris·HCl plus 15 mM imidazole and 0.6 mM EDTA, pH 7.0. The Na^+ fluxes were assayed by prewarming 20 μl of vesicles at 20°C for 1.5 min and then adding 20 μl of a solution containing, except where noted, 20 mM NaCl, 200 mM Tris·HCl, 100 μCi of $^{22}NaCl$ per ml (1 Ci = 37 GBq), and either 0.4 mM ATP and 1.87 mM $MgCl_2$ or 1.52 mM $MgCl_2$. After 1 min at 20°C the vesicles were applied to the top of a column containing 1.75 ml of Dowex 50Wx8, 50/100 mesh (Fluka). The vesicles were immediately washed through the column with 1.5 ml of 250 mM sucrose (1–4°C) and the radioactivity of the eluent was subsequently determined. The transport activity of the pump, which was found to be essentially constant over the first 2 min after mixing, was taken to be the ATP-sensitive uptake of $^{22}Na^+$ into the vesicles. Because these values are dependent on (i) the concentration of vesicles, (ii) the relative content of successfully reconstituted pumps, and (iii) the leak rate of the vesicles, the results on $^{22}Na^+$ uptake rates are reported as the 2-min fractional increase in stimulation of Na^+ uptake due to ATP. The fractional increase (FI) is defined as FI = (A − B)/B where A is the uptake of $^{22}Na^+$ with ATP and B is the uptake of $^{22}Na^+$ without ATP. This normalizes the results and provides a direct way of comparing the results obtained with the different kidney preparations.

RESULTS

Uncoupled Na^+ Effluxes. In human red cells, the ouabain-sensitive Na^+ efflux in the absence of Na_o^+ (uncoupled Na^+ efflux) has been shown to be inhibited by the addition of Na_o^+, with the maximum inhibition occurring at ≈5 mM Na_o^+ (1, 8). This same effect is observed in red cells from other species. Fig. 1 (*Upper*) shows the results of experiments in which the Na^+ efflux in pig and rat red cells was measured at various concentrations of Na_o^+ in the absence and presence of ouabain. Fig. 1 (*Lower*) shows the ouabain-sensitive differences between the two paired curves (*Upper*), where it is evident that as the concentration of Na_o^+ is raised the response is biphasic as is also the case in human red cells (2, 8). Uncoupled Na^+ efflux is inhibited by 5 mM Na_o^+, but at higher values of Na_o^+ the increase in the Na^+ efflux has been shown to represent a different process—namely, Na_i^+ for
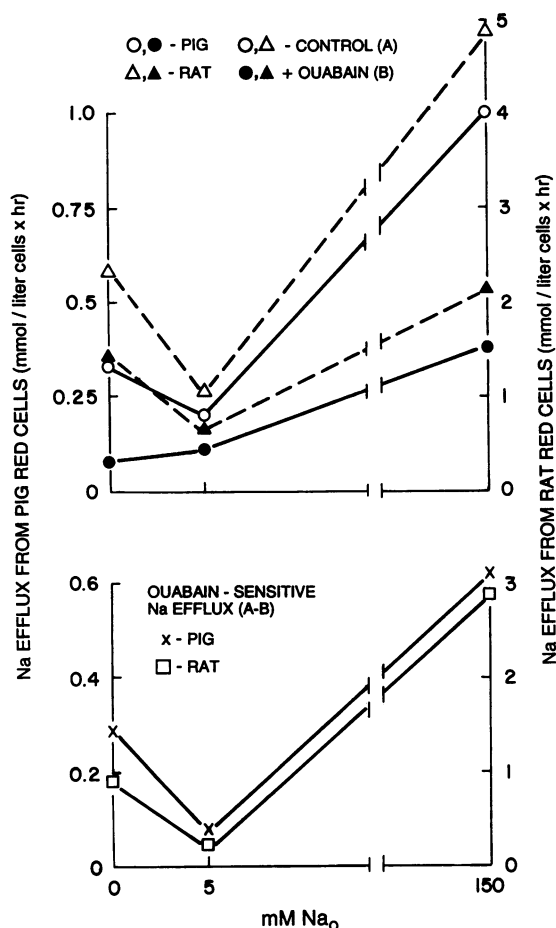
FIG. 1. Effect of Na_o^+ on efflux of Na^+ from DIDS-treated pig and rat red cells. Na_o^+ was varied by substitution of the increased concentrations of Na_o^+ for an osmotically equivalent concentration of either choline chloride or $MgSO_4$ in the measurements involving, respectively, pig and rat red cells (see *Materials and Methods* and legend to Table 2). (*Upper*) Effluxes of Na^+ in the absence (open symbols; A) and presence (solid symbols; B) of 0.5 or 1.0 mM ouabain for pig and rat red cells, respectively. It should be noted that in other experiments with rat red cells there was no difference between the level of ouabain inhibition obtained at zero and 5 mM Na_o^+ (as with pig red cells). The difference (solid triangles) may indicate here that complete inhibition of the Na^+ efflux was not obtained with 1 mM ouabain. (*Lower*) Ouabain-sensitive components, taken as the difference in the appropriate upper curves (A − B). The Na^+ effluxes are presented in units of mmol per liter of cells × hr where Na_i^+ was 5.7 and 3.1 mmol per liter of cells, respectively, for pig and rat red cells. The variances (SEM; $n = 3$) in the effluxes are less than the size of the graphed symbols.

Na_o^+ exchange (1, 2, 8). This inhibitory action of Na_o^+ on Na_i^+ efflux was obtained in five additional experiments with both pig and rat red cells (results not shown) and extends the list of red cell species that show this effect. Note that in both species the uncoupled Na^+ efflux, as in human red cells (8), is inhibited more by ouabain than it is by 5 mM Na_o^+. This has been the basis for separating the uncoupled efflux of Na^+ into two components where one is Na_o^+-sensitive and the other is Na_o^+-insensitive (see below).

The next question was to test the extent to which Na^+/K^+ pumps purified from different mammalian kidneys display this same behavior. The uncoupled Na^+ flux was measured in vesicles prepared from pig, rat, and human kidneys. In these preparations, we measured only the Na^+ uptake by those Na^+/K^+ pumps that can be activated by ATP from the outside surface of the vesicle; in this respect, the orientation of the pumps that are activated is the reverse of that in red

cells. Thus, as is evident from the results presented in Table 1, when vesicles reconstituted with kidney Na^+/K^+ pumps are loaded with 200 mM KCl and incubated in the presence of 10 mM Na_o^+, an ATP-dependent uptake of Na_o^+ occurs that is taken to represent Na_o^+ for K_i^+ exchange. When the vesicles are K^+-free (containing Tris instead) there is still an ATP-dependent uptake of Na^+ that is now taken to represent the pump-mediated uptake of Na^+ in its uncoupled mode. Note that the ATP-dependent uncoupled uptake of Na^+ as well as the exchange of Na_o^+ for K_i^+ are inhibited by strophanthidin. (Strophanthidin is used here because in penetrating to the vesicle interior it inhibits those pumps that are activated by external ATP.) These results with the pig kidney enzyme are similar to those reported by others (10). The uncoupled uptake of Na^+ into kidney vesicles differs from the uncoupled Na^+ efflux seen in red cells in that, at least in pig and rat kidney preparations, it is composed of a much smaller proportion of the Na_o^+ for K_i^+ exchange rate. As noted before, the uncoupled Na^+ efflux in red cells is 15–30% of the K^+-stimulated rate, but in vesicles reconstituted with pig kidney Na^+ pumps, the uncoupled rate is <4% of the flux observed in the presence of K^+. In rat kidney preparations, it is ≈1% of the rate but ≈14% of the K_i^+-stimulated rate in human kidney preparations. Another more dramatic difference is shown in Fig. 2. Here uncoupled Na^+ efflux is not inhibited by 5 mM Na_i^+, although there is an increase in the uncoupled Na^+ flux rate as Na_i^+ is raised in vesicles containing reconstituted pig and rat kidney pumps. Human kidneys are different in that the Na_o^+ uptake rate is not affected by Na_i^+, at least in the two different vesicle preparations used in this study. Whether or not the characteristics of these human vesicle preparations and their lower specific activity are the result of their pathological origin is not known.

Anion Fluxes. It was shown (7, 8) that not only is uncoupled Na^+ efflux in human red cells electroneutral but that cellular anions are cotransported with Na^+ and that the fluxes of these anions are inhibited by Na_o^+, K_o^+, and ouabain. In view of these results, it was of interest to determine to what extent the Na^+/K^+ pumps of pig and rat red cells mediate similar effluxes of anions. Table 2 shows the results of experiments in which the effluxes of Na^+, SO_4^{2-}, and P_i from pig and rat red cells were measured under conditions where the medium contained either 0 or 5 mM Na_o^+ or 5 mM K_o^+. It is evident that like human red cells (see refs. 7 and 8) the uncoupled efflux of Na^+ is accompanied by a Na_o^+-sensitive efflux of SO_4^{2-} and a Na_o^+-insensitive efflux of P_i. The presence of K_o^+ converts uncoupled Na^+ efflux to Na_i^+ for K_o^+ exchange and inhibits the efflux of anions. The stoichiometry of Na^+ to total anion efflux (in the absence of Na_o^+ and K_o^+) in red cells from both the pig and rat is similar to that of humans since the ratios of the ouabain-sensitive effluxes of Na^+ to the sum of [SO_4^{2-} + P_i] are, respectively, 1.89, 2.31, and 1.96, approximating 2 monovalent cations per divalent anion. It should be mentioned that because these measurements were carried out on intact cells that contained high K_i^+ there is the possibility that some K_i^+ was lost during the flux period that could influence the Na^+ efflux and the attendant stoichiometries, especially when the hematocrit was high (i.e., 10%). As has been previously discussed (see ref. 8) the rat red cell offered a unique circumstance to test the possible effect of K_i^+ leakage on the Na_i^+ efflux because of the relative insensitivity (that is, marked sigmoidicity) of this cell's Na^+/K^+ pump to activation by K_o^+ (15). Thus, the influx of Rb^+, a congener of K^+, was measured on cells suspended at a 10% hematocrit where K_o^+ was intentionally held at ≈0.2 mM. In this instance, the ouabain-sensitive uptake of Rb^+, in mmol of Rb^+ per liter of cells × hr ± SEM ($n = 3$), was found (one experiment) to be 0.24 ± 0.03 and 0.26 ± 0.02, respectively, when Na_o^+ was either 0 or 5 mM, indicating that if any Na_i^+/K_o^+ exchange

Table 1. $^{22}Na^+$ uptake into liposomes reconstituted with purified Na^+,K^+-ATPase from pig, rat, and human kidneys

Vesicle content	Outside medium	FI in ATP-dependent $^{22}Na^+$ uptake into vesicles		
		Pig	Rat	Human
KCl	NaCl	26.64 ± 0.96	51.91 ± 1.92	2.85 ± 0.21
KCl + strophanthidin	NaCl	4.24 ± 0.11	—	0.00 ± 0.06
Tris·HCl	NaCl	1.40 ± 0.38	0.43 ± 0.05	0.39 ± 0.31
Tris·HCl + strophanthidin	NaCl	0.53 ± 0.41	—	0.11 ± 0.30
Tris·HCl	NaSCN	—	0.55 ± 0.10	—
$Tris_2$ tartrate	NaCl	—	0.40 ± 0.03	—
$Tris_2$ tartrate	NaSCN	—	0.53 ± 0.05	—
$Tris_2$ tartrate	Na_2 tartrate	—	0.56 ± 0.08	—

The KCl- and Tris·HCl-loaded vesicles contained 200 mM KCl or Tris·HCl together with 15 mM imidazole and 0.65 mM EDTA at pH 7.0. These vesicles were suspended in a medium that contained a tracer quantity of $^{22}NaCl$ together with 200 mM Tris·HCl, 1.53 mM $MgCl_2$, or 1.87 mM $MgCl_2$ + 0.4 mM ATP. Tartrate-loaded vesicles contained 142.8 mM tartaric acid adjusted to pH 7.0 with 304 mM Tris·OH together with 15 mM imidazole and 0.65 mM EDTA. The medium containing NaSCN or disodium tartrate had the same composition as the NaCl medium except that 142.8 mM tartaric acid + 304 mM Tris·OH were substituted for the 200 mM Tris·HCl, and 20 mM disodium tartrate or 20 mM NaSCN was substituted, respectively, for the 20 mM NaCl. The pig and human kidney vesicles were treated, respectively, with 85 and 125 μM strophanthidin (controls treated with ethanol alone) and incubated at 20°C for 10 min prior to the flux assay. The flux assay was initiated by mixing an equal volume of the vesicle suspension with an equal volume of medium followed by incubation for 2 min and separation as described in *Materials and Methods*. Uptake rate of $^{22}Na^+$ by the vesicles was measured in the presence (A) and absence (B) of ATP. Results are presented as FI in ATP-dependent $^{22}Na^+$ uptake by the vesicles according to the relation FI = (A − B)/B. Values represent means ± SEM, where n = 4. Similar results were obtained on two other preparations of pig and rat and one of human kidneys.

occurred it was negligible because the affinity of K_o^+ was not affected by Na_o^+. It should also be added that the inhibition in rat red cells of uncoupled Na^+ efflux by 5 mM Na_o^+, as in Fig. 1, is prevented by the presence of 0.2 mM K_o^+ (results not shown).

In marked contrast, kidney vesicles differ from red cells in that the uncoupled Na^+ flux in these preparations is not affected by anions as shown before in pig kidney (10) and by the results presented in Table 1. Dissing and Hoffman (7) have shown that the uncoupled Na^+ efflux is inhibited in red cell ghosts containing tartrate in place of Cl^- or SO_4^{2-}, but as

FIG. 2.   Uptake rate of Na^+ by vesicles, reconstituted with Na^+/K^+ pumps from either pig, rat, or human kidneys, containing various concentrations of intravesicular Na^+. Vesicular Na^+ was set by preincubation of the vesicles with 200 mM Tris·HCl, 15 mM imidazole, and 0.65 mM EDTA (pH 7.0) at 20°C, where the indicated concentrations of Na^+ were substituted for Tris·HCl. As described in *Materials and Methods*, the uptake rate of Na^+ was measured in the presence (A) and absence (B) of ATP, where the medium concentration of Na^+ was 20 mM. Results are presented as FI in ATP-dependent $^{22}Na^+$ uptake by the vesicles according to the relation FI = (A − B)/B. Mean values ± SEM are plotted, where n = 4.

shown in Table 1, the uncoupled Na^+ flux in vesicles prepared with rat kidney pumps is not affected by the anion composition. There was no change in the uptake rate of Na^+ when tartrate replaced Cl^- on both sides of the vesicles or when SCN was substituted for Cl^- in the external medium.

DISCUSSION

It is clear from the above results that the uncoupled efflux of Na^+ as seen in pig, rat, and human red cells displays different properties from those seen in vesicles reconstituted with Na^+,K^+-ATPase isolated from the kidneys from the same three species. Thus, while uncoupled Na^+ efflux in each of the three kinds of red cells is inhibited by Na_o^+ (Fig. 1; Table 2; refs. 7 and 8), this is not the case in their respective kidney vesicle preparations (Fig. 2; refs. 9 and 10). As also pointed out, another difference lies in the fractional size of the uncoupled Na^+ efflux component relative to the size of Na^+/K^+ exchange—it is much smaller in the kidney vesicle preparations than in the red cells of the same species. This difference presumably does not represent any inherent differences in pump mechanism but rather could reflect differences in critical rate constants that define the turnover rates of the pump's intermediates during uncoupled Na^+ efflux. On the other hand, the small size of uncoupled Na^+ efflux in the kidney vesicles may be responsible for the minimal detectability of this flux when kidney Na^+/K^+ pumps have been inserted into intact dog red cells (16). Because dog red cells lack endogenous Na^+/K^+ pumps, this approach optimizes study of the Na^+/K^+ pumps of the kidney in an intact membrane. Perhaps when the sensitivity of the system is increased it will be possible to evaluate whether or not the new membrane environment substantively alters any of the characteristics as seen here of the inserted pumps with regard to uncoupled Na^+ efflux (see below).

Perhaps the most important difference between uncoupled Na^+ efflux in red cells and their kidney vesicle counterparts lies in the effect of anions and their cotransport with Na^+. As pointed out before (Table 2; refs. 7 and 8) Na^+ efflux is accompanied in all three species of red cells by the movement of anions from two different intracellular sources, one being
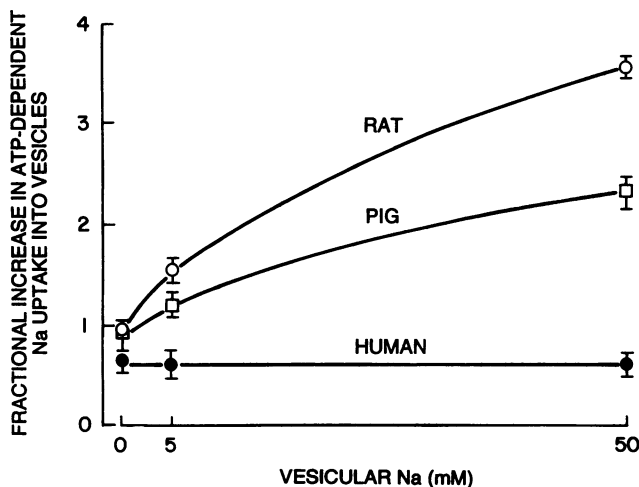
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Table 2.   Efflux of Na^+, SO_4^{2-}, and P_i from pig, rat, and human red cells during uncoupled Na^+ efflux

Species	Na_o^+, mM	K_o^+, mM	$^oM_{Na}^{ouab}$	$^oM_{SO_4}^{ouab}$	$^oM_{P_i}^{ouab}$
	0	0	1.14 ± 0.01	0.525 ± 0.051	0.078 ± 0.001
Pig ($n = 5$)	5	0	0.23 ± 0.01	0.150 ± 0.051	0.085 ± 0.002
	0	5	5.23 ± 0.03	—	0.004 ± 0.001
Rat ($n = 3$)	0	0	0.932 ± 0.036	0.344 ± 0.043	0.060 ± 0.005
Human ($n = 3$)	0	0	0.869 ± 0.033	0.387 ± 0.043	0.056 ± 0.002

Results are expressed as mmol per liter of cells × hr. Measurements were carried out at 37°C, as described, on DIDS-treated, SO_4^{2-}-loaded cells that had been labeled with $^{22}Na^+$ (Na^+ efflux) and $^{35}SO_4^{2-}$ (SO_4^{2-} efflux). P_i efflux was determined on cells that had not been prelabeled but were otherwise treated in the same way. The efflux medium used with pig red cells contained 244 mM $(NMG)_2SO_4$, 10 mM $Tris_2SO_4$, 5 mM adenine, 5 mM inosine, and 0.5% BSA. When present Na_2SO_4 or K_2SO_4 was substituted for an osmotic equivalent concentration of $(NMG)_2SO_4$. The efflux medium used with rat and human red cells contained 220 mM $MgSO_4$ and 10 mM $Tris_2SO_4$. In all cases, the hematocrit used in the efflux determinations of Na^+ and SO_4^{2-} was 1–2% and ≈10% in the P_i efflux determinations. The concentration of ouabain, when present, was 0.5 mM for pig, 1 mM for rat, and 0.1 mM for human red cells. For the experiments reported, Na_i^+ was 26.7, 12.1, and 16.4 mmol per liter of cells, respectively, for pig, rat, and human red cells. The ouabain-sensitive efflux of Na^+, SO_4^{2-}, and P_i is taken as the difference between the effluxes in the absence and presence of ouabain and is symbolized by $^oM_{Na}^{ouab}$, $^oM_{SO_4}^{ouab}$, and $^oM_{P_i}^{ouab}$, respectively, in units of mmol per liter of cells × hr ± SEM. Results on human red cells were taken from table V (experiment C) in ref. 8 and are presented here for comparison. Results similar to those presented where Na_o^+ and K_o^+ were both zero were obtained in other experiments of the same type with all three species of red cells.

cytoplasmic (SO_4^{2-}), the other being the terminal PO_4 from substrate ATP. Substitution of SO_4^{2-} by impermeant anions such as tartrate (7) inhibits the uncoupled efflux of Na^+ in human red cells but not in rat kidney vesicles (Table 1), nor does gluconate or aspartate inhibit uncoupled Na^+ efflux in pig kidney vesicles (10). While these types of differences in anion dependence of uncoupled Na^+ efflux have been discussed (7), the issue of their similarity to red cells could be more decisively resolved by direct measurements of anion fluxes in the kidney vesicle preparations. The only direct measurements of this type that we are aware of have been carried out (at our request) by Bliss Forbush III (Yale University). These studies (personal communication) differ from those referred to before (Table 1) in that they involved the use of right-side-out membrane vesicles isolated from dog kidney (17). By use of previously described methods (18), Forbush loaded these vesicles (see ref. 18 for details) with caged ATP and Na_2SO_4, in a manner such that SO_4^{2-} was the only inorganic anion present. The vesicles were suspended in a $Tris_2SO_4$ medium. Because the vesicles also contained tracer quantities of $^{22}Na^+$ and $^{35}SO_4^{2-}$, the efflux of both ions could be measured simultaneously upon activation of uncoupled Na^+ efflux by the photorelease of ATP from caged ATP. Forbush found in a variety of experiments of this type that whereas a ouabain-sensitive efflux of $^{22}Na^+$ was easily measured, there was no detectable efflux of $^{35}SO_4^{2-}$ under the same conditions. Other types of information available that bear on the possible involvement of anions in uncoupled Na^+ efflux are studies by I. M. Glynn, Y. Hara, J.F.H., and D.E.R. (unpublished data) of the involvement and/or effects of anions in/on occlusion and Na^+-ATPase activity of purified pig kidney Na^+,K^+-ATPase described by Dissing and Hoffman (7). Thus, anions of different sizes (Cl^-, Hepes, glutamate, tartrate) were tested for their effects on Na^+ occlusion and on Na^+-ATPase activity on α-chymotrypsin-treated pig kidney Na^+,K^+-ATPase (cf. ref. 12). Experiments were also carried out to determine whether small anions, such as $^{36}Cl^-$ or $^{77}Br^-$, were occluded together with Na^+. No effects of anions were found on Na^+ occlusion or on Na^+-ATPase activity. Nor was there any evidence for anion occlusion under circumstances when Na^+ was occluded.

While uncoupled Na^+ efflux in intact red cells is evidently different from that displayed by kidney vesicle preparations, it is not known whether these differences obtain when the comparison is made to intact kidney cells. Thus, we need

information regarding the properties of uncoupled Na^+ efflux in renal tubular cells or in cell lines developed from them. We are not aware of any studies of uncoupled Na^+ efflux from renal tissue. Even so, it is not clear why the characteristics of uncoupled Na^+ efflux differ between red cells and vesicles containing reconstituted Na^+/K^+ pumps. Possible causes of the differences relative to the vesicle properties could be due to changes in the lipid composition, the effects of exposure to detergents, or that Na^+/K^+ pumps of kidney undergo different posttranslational modifications than those of red cells. It would also be of interest when purified Na^+,K^+-ATPase from red cells is available to reconstitute this enzyme into proteoliposomes for comparison with the kidney preparations. Obviously, further work is needed to determine the extent to which the Na^+/K^+ pumps from the two sources differ.

1. Glynn, I. M. (1985) in *The Enzymes of Biological Membranes*, ed. Martonosi, A. N. (Plenum, New York), pp. 35–114.
2. Garrahan, P. J. & Glynn, I. M. (1967) *J. Physiol. (London)* 192, 159–174.
3. Sachs, J. R. (1970) *J. Gen. Physiol.* 56, 322–341.
4. Eilam, Y. & Stein, W. D. (1973) *Biochim. Biophys. Acta* 323, 606–618.
5. Lew, V. L., Hardy, M. A. & Ellory, J. C. (1973) *Biochim. Biophys. Acta* 323, 251–266.
6. Glynn, I. M. & Karlish, S. J. D. (1976) *J. Physiol. (London)* 256, 465–496.
7. Dissing, S. & Hoffman, J. F. (1990) *J. Gen. Physiol.* 96, 167–193.
8. Marín, R. & Hoffman, J. F. (1994) *J. Gen. Physiol.* 104, 1–32.
9. Cornelius, F. (1989) *Biochem. Biophys. Res. Commun.* 160, 801–807.
10. Goldshleger, R., Shahak, Y. & Karlish, S. J. D. (1990) *J. Membr. Biol.* 113, 139–154.
11. Jørgensen, P. L. (1974) *Biochim. Biophys. Acta* 356, 36–52.
12. Glynn, I. M., Hara, Y. & Richards, D. E. (1984) *J. Physiol. (London)* 351, 531–547.
13. Peterson, G. L. (1977) *Anal. Biochem.* 83, 346–356.
14. Karlish, S. J. D. & Pick, U. (1981) *J. Physiol. (London)* 312, 505–529.
15. Hoffman, J. F. (1966) *Am. J. Med.* 41, 666–679.
16. Munzer, J. S., Silvius, J. R. & Blostein, R. (1992) *J. Biol. Chem.* 267, 1–9.
17. Forbush, B., III (1982) *J. Biol. Chem.* 257, 12678–12684.
18. Forbush, B., III (1984) *Proc. Natl. Acad. Sci. USA* 81, 5310–5314.