Regulatory Interaction of ATP Na^+ and Cl^- in the Turnover Cycle of the NaK2Cl Cotransporter

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ABSTRACT To probe the mechanism by which intracellular ATP, Na^+, and Cl^- influence the activity of the NaK2Cl cotransporter, we measured bumetanide-sensitive (BS) ^{86}Rb fluxes in the osteosarcoma cell line UMR-106-01. Under physiological gradients of Na^+, K^+, and Cl^-, depleting cellular ATP by incubation with deoxyglucose and antimycin A (DOG/AA) for 20 min at 37°C reduced BS ^{86}Rb uptake from 6 to 1 nmol/mg protein per min. Similar incubation with 0.5 mM ouabain to inhibit the Na^+ pump had no effect on the uptake, excluding the possibility that DOG/AA inhibited the uptake by modifying the cellular Na^+ and K^+ gradients. Loading the cells with Na^+ and depleting them of K^+ by a 2–3-h incubation with ouabain or DOG/AA increased the rate of BS ^{86}Rb uptake to ~12 nmol/mg protein per min. The unidirectional BS ^{86}Rb influx into control cells was ~10 times faster than the unidirectional BS ^{86}Rb efflux. On the other hand, at steady state the unidirectional BS ^{86}Rb influx and efflux in ouabain-treated cells were similar, suggesting that most of the BS ^{86}Rb uptake into the ouabain-treated cells is due to K^+/K^+ exchange. The entire BS ^{86}Rb uptake into ouabain-treated cells was insensitive to depletion of cellular ATP. However, the influx could be converted to ATP-sensitive influx by reducing cellular Cl^- and/or Na^+ in ouabain-treated cells to impose conditions for net uptake of the ions. The BS ^{86}Rb uptake in ouabain-treated cells required the presence of Na^+, K^+, and Cl^- in the extracellular medium. Thus, loading the cells with Na^+ induced rapid ^{86}Rb (K^+) influx and efflux which, unlike net uptake, were insensitive to cellular ATP. Therefore, we suggest that ATP regulates a step in the turnover cycle of the cotransporter that is required for net but not K^+/K^+ exchange fluxes. Depleting control cells of Cl^- increased BS ^{86}Rb uptake from medium-containing physiological Na^+ and K^+ concentrations from 6 to ~15 nmol/mg protein per min. The uptake was blocked by depletion of cellular ATP with DOG/AA and required the presence of all three ions in the external medium. Thus, intracellular Cl^- appears to influence net uptake by the cotransporter. Depletion of intracellular Na^+ was as effective as depletion of Cl^- in stimulating BS ^{86}Rb uptake. Clamping internal Na^+ and measuring BS ^{86}Rb uptake into ATP-containing and ATP-depleted cells at different Na^+ concentrations showed

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that increasing Na_i^+ from 1 up to 10 mM inhibited ATP-sensitive fluxes without affecting ATP-insensitive fluxes. Increasing Na_i^+ from 10 up to 40 mM had little effect on the fluxes. Further increase in Na_i^+ up to 140 mM inhibited ATP-sensitive fluxes and stimulated ATP-insensitive fluxes in a reciprocal manner. These findings suggest that the interaction of Na^+ with the internal and external sites of the cotransporter is asymmetrical, and that Na_i^+ can influence the activity of the cotransporter by affecting at least two steps in a possible turnover cycle of the cotransporter.

INTRODUCTION

The NaK2Cl cotransporter plays an important role in regulating intracellular concentrations of monovalent ions, in particular during cell volume perturbations (O'Grady, Palfrey, and Field, 1987; Hoffmann and Simonsen, 1989). The cotransporter was described in many cell types, including red blood cells (Canessa, Bize, Adragna, and Tosteson, 1982; Ellory and Stewart, 1982; Haas and McManus, 1983), Ehrlich ascites cells (Geck, Pietrzyk, Burckhardt, Pfeiffer, and Heinz, 1980), epithelial cells (Greger, 1985), and several cell lines (O'Grady et al., 1987; Hoffmann and Simonsen, 1989) including UMR-106-01 (Whisenant, Zhang, Khademazad, Loessberg, and Muallem, 1991). Common to NaK2Cl cotransport in all cells is that all three ions must be present in the external medium for influx via cotransport to occur (Brown and Murer, 1985; O'Grady et al., 1987; Kim, Tsai, Franklin, and Turner, 1988; Kort and Koch, 1989). The transport of the ions by the cotransporter is electroneutral (Haas, Schmidt, and McManus, 1982). Indeed, in most cells a stoichiometry of $1Na^+:1K^+:2Cl^-$ was found (Hoffmann and Simonsen, 1989), although in squid axons (Russell, 1983) and ferret erythrocytes (Hall and Ellory, 1985) a stoichiometry of $2Na^+:1K^+:3Cl^-$ was reported. The cotransporter is sensitive to the "loop" diuretics, bumetanide and furosemide (O'Grady et al., 1987).

The turnover cycle of the cotransporter is only partially understood. The classical approach of studying the characteristics of partial reactions has been used in various red blood cells in an effort to probe this important problem. In human and duck red cells, the cotransporter can mediate at least two partial reactions, K^+/K^+ and Na^+/Na^+ exchange (Schmidt and McManus, 1977; McManus and Haas, 1981; Haas, Starke, and McManus, 1984; Brugnara, Canessa, Cusi, and Tosteson, 1986; Canessa, Brugnara, Cusi, and Tosteson, 1986; Lytle, Haas, and McManus, 1986). K^+/K^+ exchange required the presence of all three ions in the intracellular compartment, whereas only the presence of K^+ and Cl^- was required in the medium (Haas et al., 1984; Canessa et al., 1986). Na^+/Na^+ exchange required Na^+, K^+, and Cl^- in the medium and only Na^+ inside the cells (Brugnara et al., 1986; Lytle et al., 1986). On the basis of such findings, two models for the turnover cycle were proposed (Canessa et al., 1986; Lytle et al., 1986). One model assumes that the only forms of the cotransporter that can cross the membrane are the one bound with the three ions and the ion-free form. The conformational transition of the ion-bound form is faster than that of the ion-free form. The rate-limiting step is thus assumed to be the conformational transition of the ion-free carrier (Lytle et al., 1986; Lauf et al., 1987). The second model assumes that the carrier with either Na^+ or K^+ bound to it can also cross the membrane to mediate net efflux of the respective ions (Canessa et al.,

1986). The additional modes were required to explain the furosemide-sensitive uncoupled fluxes of these ions in human red cells (Brugnara et al., 1986; Canessa et al., 1986). The extent to which the characteristics of the partial reactions and the above turnover cycles apply to other cell types is not known since similar information is not available.

The dependency of the cotransporter on cellular ATP was first reported in the squid axon (Russell, 1976, 1979) and later confirmed in duck red blood cells (Palfrey and Greengard, 1981) and other cell types (Rindler, McRoberts, and Saier, 1982; Owen, 1984; Kim et al., 1988). In many cells the cotransporter is regulated by different agonists acting through changes in cellular cAMP (Riddick, Kreegnow, and Orloff, 1971; Palfrey and Greengard, 1981), cGMP (O'Donnell and Owen, 1986), or $[Ca^{2+}]_i$ (Brock, Brugnara, Cassa, and Gimbrone, 1986). It is therefore commonly assumed that the dependency of cotransport on cellular ATP reflects the need to activate the relevant protein kinases (Saier and Boyden, 1984). However, on the basis of his studies in squid axons, Russell (1979, 1983) raised the possibility that ATP may directly regulate the cotransporter. In subsequent studies the effect of ATP in these cells was also attributed to protein phosphorylation (Altamirano, Breitwieser, and Russell, 1988).

In these studies, we used the osteosarcoma cell line UMR-106-01 and bumetanide-sensitive ^{86}Rb fluxes to study the characteristics of the net and K^+/K^+ exchange reactions by the cotransporter and the influence of intracellular ATP, Cl^-, and Na^+. The findings allowed us to propose a turnover cycle for the cotransporter in which the conformational transition of the ion-free form is regulated by internal ATP. Intracellular Cl^- interacts with a regulatory site to inhibit net cotransport. Asymmetry for the interaction of Na^+ with external and internal sites was found. Na^+ interacts at least twice with the internal face of the cotransporter in each turnover cycle.

MATERIALS AND METHODS

Solutions

The standard solution used in these studies is solution A (SA) of the following composition (mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES (pH 7.4 with NaOH), 10 glucose, 10 pyruvate, and 0.1% BSA. The Cl^--free solution B (SB) contained 140 Na gluconate, 5 K gluconate, 1 $MgSO_4$, 1.2 Ca cyclamate, 10 HEPES (pH 7.4 with NaOH), 10 glucose, 10 pyruvate, and 0.1% BSA. Ca^{2+} concentration was increased to compensate for binding to gluconate and to keep free Ca^{2+} concentration at 1 mM. The Na^+-free solution C (SC) contained 140 *N*-methyl-D-glucamine (NMG) Cl^-, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES (pH 7.4 with Tris), 10 glucose, 10 pyruvic acid, and 0.1% BSA. A mixture of high Na^+ and high K^+ solutions was used to clamp intracellular Na^+ and K^+ concentration with ionophores. The high Na^+ solution, solution D (SD), was identical to the standard SA except that $CaCl_2$ was reduced to 0.2 mM, the pH was 7.2, and BSA was omitted to prevent binding of ionophores to BSA. The high K^+ solution, solution E (SE), contained 145 KCl, 1 $MgCl_2$, 0.2 $CaCl_2$, 10 HEPES (pH 7.2 with KOH), 10 glucose, and 10 pyruvic acid. The pH of SD and SE was set at 7.2, the measured internal pH of UMR-106-01 cells (Green, Yamaguchi, Kleeman, and Muallem, 1988). To test the effect of intracellular ATP on cotransport activity, the cells were incubated with solutions similar to those specified above except that glucose and pyruvate were omitted and the solutions were supplemented with 10 mM deoxyglucose (DOG) and 2 μM antimycin A (AA).

Cell Culture

UMR-106-01 cells were grown in 75-cm^2 culture plates in a 1:1 mixture of Ham's F-12–Dulbecco's modified Eagle's medium supplemented with 1.2 mM L-glutamate, 15 mM HEPES, 14.3 mM $NaHCO_3$, 2.5% fetal calf serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Cultures were kept in a humidified atmosphere of 95% air-5% CO_2 at 37°C. The cells were subcultured by seeding at a density of 25,000 cells/cm^2 in a 24-well culture plate. The cells were fed twice weekly with the above medium. After 4–5 d in culture, the cells reached confluency, and they were used for ^{86}Rb uptake between days 6 and 10 in culture.

Clamping Intracellular Na^+ and K^+ Concentrations

SD and SE were mixed to yield solutions of different Na^+ concentrations between 1 and 140 mM. A portion of each of these solutions was without BSA and was supplemented with 2 μM monensin and 1 μM nigericin. Another portion of the same solutions was with 10 mg/ml BSA. Cells grown to confluency in 24-well plates were washed twice with BSA- and ionophore-free solution of the desired Na^+ (1–140 mM) and K^+ (5–144 mM) concentrations and then incubated in the same solutions containing the ionophores for 5 min at 37°C. Finally, the cells were washed twice with ionophore-free solutions containing 10 mg/ml BSA and 0.5 mM ouabain. ^{86}Rb uptake into these cells was initiated within 2 min of the removal of the ionophores with the high BSA washes and was measured during a 2-min incubation at 37°C. We have shown previously (Green et al., 1988) that this technique clamps the intracellular Na^+ and K^+ at the desired concentrations and these concentrations remain stable for at least 15 min if the gradients are not interrupted and ouabain is included in the incubation medium.

Measurement of ^{86}Rb (K^+) Fluxes

The cells were routinely washed twice with SA to replace the tissue culture medium and remove the serum and then allowed to recover normal ionic gradients during a 30-min incubation at 37°C before any treatment. For measurement of ^{86}Rb uptake, the cells were treated with SA, ouabain, DOG/AA, Cl^--free SB, or Na^+-free SC for the indicated times. At the end of the pretreatment period, the cells were exposed to 0.2 ml of uptake medium, which was usually SA containing ^{86}Rb ($\sim 10^6$ cpm/ml) and 0.5 mM ouabain with or without 0.1 mM bumetanide. After the indicated incubation times, ^{86}Rb uptake was terminated by aspiration of the uptake medium and three rapid washes of the cell layer with a cold stop solution containing 100 mM $MgCl_2$ and 10 mM HEPES (pH 7.4 with Tris). The cells were dissolved in 0.5 ml of 0.1 M NaOH by a 30-min incubation at 60°C. The lysates were transferred to scintillation vials and counted for ^{86}Rb. Samples of lysates were removed for measurement of protein (Bradford, 1976). The linear portion of the uptake was determined for each experimental condition. Then the rates of uptake were determined from a 1–3-min uptake period (depending on the conditions). In this case, duplicate determinations were made and results from at least three separate experiments were used to calculate the mean ± SEM.

Measurement of ^{86}Rb (K^+) efflux was initiated after labeling the cells with ^{86}Rb. Cells were incubated in SA (control) or SA containing 0.5 mM ouabain (ouabain treated) for 2 h at 37°C. These cells were exposed to ^{86}Rb during the 2-h incubation (not shown) or an 8-min period (Fig. 4). Then the medium containing ^{86}Rb was carefully aspirated and the cells were exposed to 1 ml SA containing 0.5 mM ouabain with or without 0.1 mM bumetanide. At the indicated time, the efflux medium was removed and the cell layer was washed twice with the stop solution. Processing of the cells to determine the cellular content of ^{86}Rb was as described above for measurement of ^{86}Rb uptake. To calculate ^{86}Rb efflux in nanomoles, the specific activity of the internal K^+ was determined for control and ouabain-treated cells. After a 2-h incubation at 37°C, isotopic equilibrium was achieved in both cell types and the specific activity of the

internal K^+ was taken as that of the loading medium. When uptake was for 8 min, some cells were labeled to isotopic equilibrium (2 h, 37°C) with a sample of the same uptake medium. This value was used to determine the level of K^+ in ouabain-treated cells (37 ± 1.8 mM) relative to control (145 mM K^+; see Green et al., 1988) and thus the specific activity of the K^+ before the initiation of efflux.

RESULTS

Effect of ATP Depletion

In previous studies we reported that ~35% of ^{86}Rb uptake into UMR-106-01 cells was inhibited by ouabain and ~60% by bumetanide. We also showed that bumetanide-sensitive (BS) ^{86}Rb uptake is mediated by the Na/K/2Cl cotransporter (Whisenant et al., 1991). In the present studies, the same methodology was used to follow cotransporter activity. In the first set of experiments we attempted to understand if and how the cotransporter is regulated by ATP. To reduce cellular ATP concentration, the cells were incubated in a glucose-free medium containing 10 mM DOG and 2 μM AA. The effect of a 20-min incubation in this medium is shown in Fig. 1 (*left*). Incubation with DOG/AA inhibited the BS K^+ (^{86}Rb) uptake by 77%. K^+ uptake was linear for at least 10 min. Fig. 1 (*right*) shows that the inhibition due to incubation with DOG/AA was time dependent and near maximal after a ~20-min incubation at 37°C.

Inhibition of cotransporter by reduction in cellular ATP can occur on several levels. Among the possibilities considered were the requirement for phosphorylation of the cotransporter, the collapse of cellular Na^+ and K^+ gradients, and the requirement for ATP interacting at a regulatory site. We were able to rule out the collapse of ionic gradients by estimating the concentrations of intracellular ions after DOG/AA treatment and by comparing the effect of incubations with DOG/AA and ouabain. Using a null point titration (Green et al., 1988) we estimated that after a 20-min incubation with DOG/AA, internal K^+ was 115 ± 8 mM and internal Na^+ was 40 ± 6 mM ($n = 4$). Fig. 1 (*right*) shows that incubating the cells with 0.5 mM ouabain for up to 20 min at 37°C had no apparent effect on the subsequent rate of K^+ uptake measured during a 2-min incubation with ^{86}Rb. The effect of long incubation with DOG/AA or ouabain on BS ^{86}Rb uptake is shown in Fig. 2. Surprisingly, under both conditions the rate of ^{86}Rb uptake increased approximately twofold relative to uptake into untreated cells. In the case of DOG/AA treatment, the cells are expected to lose the Na^+ and K^+ gradients due to complete depletion of cellular ATP and inhibition of the Na^+ pump. Indeed, ouabain-sensitive ^{86}Rb uptake was inhibited by 100% after a 15-min incubation with DOG/AA and remained inhibited for the duration of the experiment (not shown). On the other hand, inhibition of the Na^+/K^+ pump by ouabain dissipates the Na^+ and K^+ gradients while sparing cellular ATP. If the same mode of transport by the NaK2Cl cotransporter mediates ^{86}Rb uptake in cells treated for a long period of time with DOG/AA or ouabain, then an important prediction is that the uptake in ouabain-treated cells is insensitive to depletion of cellular ATP. That this is indeed the case is shown in Fig. 3. Incubating the cells with ouabain for 2 h doubled the rate of BS ^{86}Rb uptake. When these cells were subsequently incubated in medium containing ouabain, DOG, and AA up to an additional 60 min, there was

no effect on the rate of ^{86}Rb uptake. In control cells incubated in the same manner, but in the absence of ouabain, BS ^{86}Rb uptake was inhibited as expected.

Unidirectional Influx and Efflux

The findings in Figs. 2 and 3 strongly suggest that different modes of transport mediate ^{86}Rb uptake in control cells and cells treated with ouabain. Since long incubation with ouabain is expected to set the Na^+ and K^+ gradients across the plasma membrane at equilibrium, it is possible that ^{86}Rb uptake into control cells

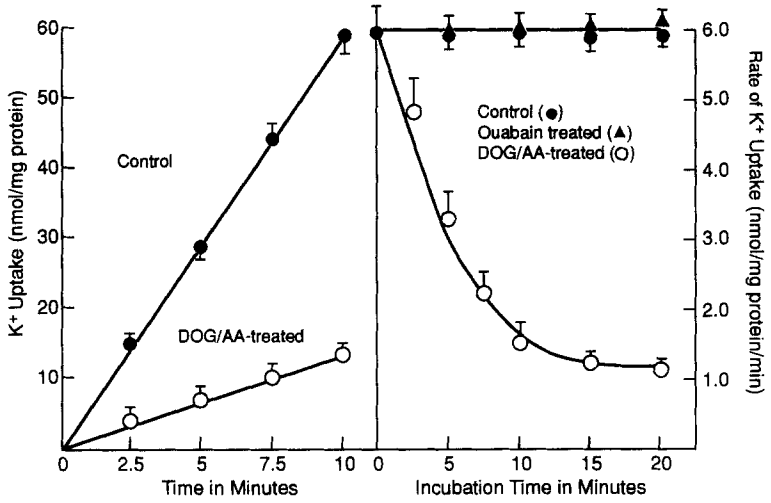


FIGURE 1. Effect of intracellular ATP on bumetanide-sensitive K^+ uptake. (*Left*) UMR 106-01 cells bathed in SA were incubated in glucose- and pyruvate-free SA containing 10 mM DOG and 2 μM antimycin A (DOG/AA) for 20 min at 37°C and then exposed to uptake medium of the same composition, also containing ^{86}Rb and 0.5 mM ouabain, with or without 0.1 mM bumetanide (○). In parallel, cells incubated in SA were exposed to SA containing ^{86}Rb and ouabain, with or without bumetanide (●). The fraction of BS ^{86}Rb uptake is plotted as a function of incubation time. (*Right*) Experimental protocol was as in the left panel except that the cells were incubated with DOG/AA for the indicated times before measurement of BS ^{86}Rb uptake during a 1-min incubation of 37°C (○). Cells were also incubated in SA containing 0.5 mM ouabain for the indicated time before exposure to SA containing ^{86}Rb and 0.5 mM ouabain, with or without 0.1 mM bumetanide for 1 min (▲). Control cells were incubated in SA before measurement of ^{86}Rb uptake (●).

represents net uptake of K^+, whereas in ouabain-treated cells it represents K^+/K^+ exchange by the cotransporter. This can be tested by comparing the unidirectional fluxes of K^+ in control and ouabain-treated cells. The results of such measurements are given in Fig. 4. In control cells, the rate of BS ^{86}Rb efflux was ~10 times slower than the rate of the influx under identical conditions. Similar differences in the rates of influx and efflux was reported in BALB/c3T3 cells (O'Brien and Krzeminski, 1983). In ouabain-treated cells, the rates of influx and efflux were similar. No difference between the rates could be obtained whether ^{86}Rb efflux was measured

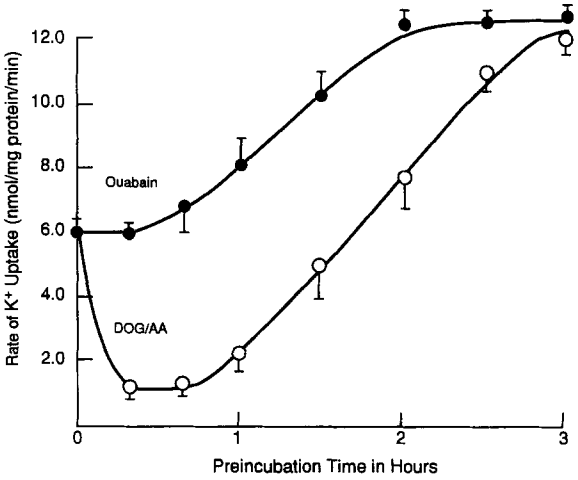


FIGURE 2. Effect of long incubation with ouabain or DOG/AA on K^+ uptake. Cells were incubated in SA containing 0.5 mM ouabain (●) or DOG/AA (○) for the indicated times before exposure to uptake medium with or without bumetanide for 4 min at 37°C. The figure shows the rates of BS K^+ (^{86}Rb) uptake.

after short (Fig. 4) or long equilibration of the cells with ^{86}Rb (not shown). Since Na^+ and K^+ gradients in control and ouabain-treated cells are at steady state, the results in Fig. 4 demonstrate the different kinetic properties of the transport mediating the BS ^{86}Rb uptake in control and ouabain-treated cells.

Considering the differences in ^{86}Rb uptake demonstrated in Fig. 4, it was important to establish that they were mediated by the NaK2Cl cotransporter. In addition, it was of interest to test whether the two types of transport show the same extracellular ionic requirements. It was previously shown in many cells (Hoffmann and Simonsen, 1989), including UMR-106-01 cells (Whisenant et al., 1991), that all three ions, Na^+, K^+, and Cl^-, must be present for ^{86}Rb uptake by the cotransport in control cells. Fig. 5 shows the ionic dependency of ^{86}Rb (K^+) uptake into ouabain-

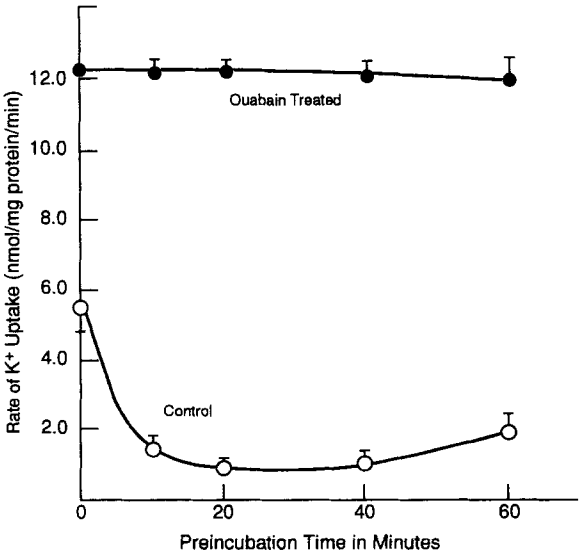


FIGURE 3. Effect of intracellular ATP on K^+ uptake in ouabain-treated cells. Cells were first incubated in SA (○) or SA containing 0.5 mM ouabain (●) for 2 h at 37°C. The cells in SA were then exposed to the DOG/AA medium (○) and cells treated with ouabain were exposed to DOG/AA medium also containing 0.5 mM ouabain (●) for the indicated times at 37°C. At the end of each incubation time, the rate of BS ^{86}Rb uptake was measured during a 3-min incubation at 37°C.

treated cells. It is clear that the influx required the presence of all ions, including Na^+, in the external medium.

Effect of Intracellular Cl^-

The unidirectional fluxes suggest that after ouabain treatment the cotransporter is performing K^+/K^+ exchange. This mode of transport is independent of cellular ATP. A simple test of such an interpretation is to impose a non–steady-state condition and

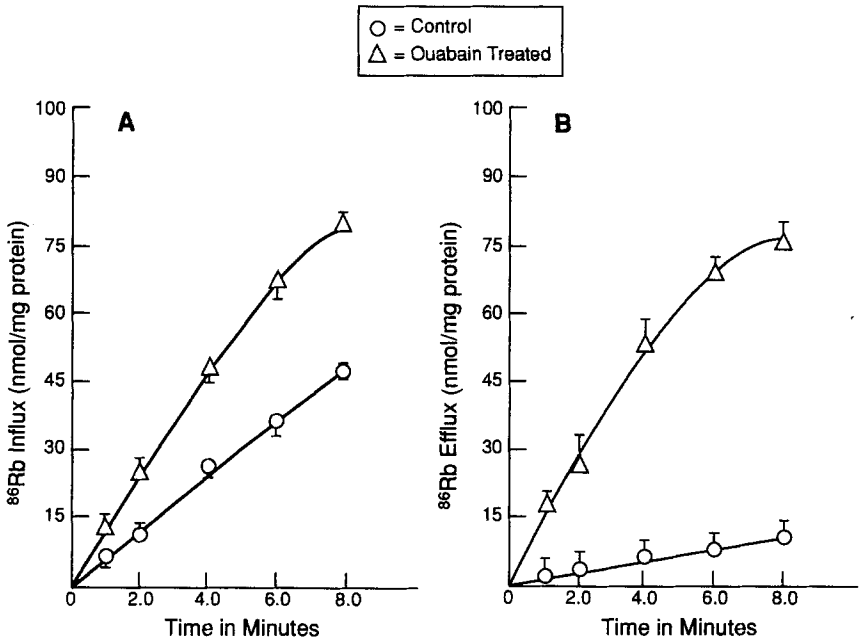


FIGURE 4. Effect of ouabain treatment on K^+ influx and efflux. Cells were incubated for 2 h at 37°C in SA (○) or SA containing 0.5 mM ouabain (△). (*A*) BS ^{86}Rb uptake was measured during an 8-min incubation at 37°C. (*B*) For measurement of ^{86}Rb efflux, control cells were exposed to SA containing ^{86}Rb (○) and ouabain-treated cells were exposed to SA containing ^{86}Rb and 0.5 mM ouabain (△) for 8 min at 37°C. The radioactive medium was then removed and replaced with 1 ml of SA containing 0.5 mM ouabain, with or without 0.1 mM bumetanide. At the indicated times, the cell layer was washed twice with the stop solution and the ^{86}Rb content of cells was determined. The specific activity of ^{86}Rb(K) at the beginning of efflux was determined from samples incubated for 2 h at 37°C (see Materials and Methods) and used to calculate the fraction of BS efflux at each time.

test its effect on the rate of transport and sensitivity to ATP. The first protocol we used was depletion of cellular Cl^-. Fig. 6 shows the results of such experiments in control and ouabain-treated cells. Incubating control, glucose-fed cells in Cl^--free medium for 30 min and then transferring them back to Cl^--containing medium (SA) resulted in a ~2.5-fold increase in BS K^+ uptake (Fig. 6*A*). The stimulated uptake required the presence of Na^+, K^+, and Cl^- in the uptake medium (Fig. 6*A*). The augmented rate of uptake persisted for ~6 min, after which the rate gradually

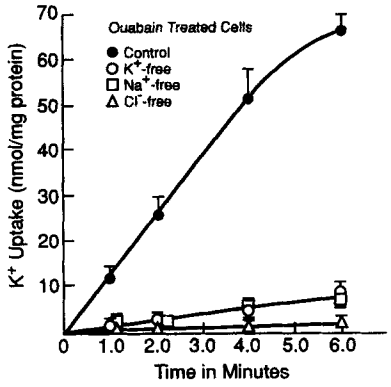


FIGURE 5. Effect of extracellular ions on K^+ uptake into ouabain-treated cells. Cells were incubated for 2 h at 37°C in SA containing 0.5 mM ouabain. ^{86}Rb uptake was then measured by exposing the cells to SA (●; control), SB (△; Cl^- free), SC (□; Na^+ free), or SA in which 5 mM NaCl replaced the 5 mM KCl (○; K^+ free). All uptake solutions contained 0.5 mM ouabain and were with or without 0.1 mM bumetanide. The fraction of BS uptake is plotted as a function of time.

declined to that measured in control cells. Chemical measurement showed that a 30-min preincubation in Cl^--free medium depleted cellular Cl^- in UMR-106-01 cells (Green, Yamaguchi, Kleeman, and Muallem, 1990). In these studies we confirmed this finding using cells loaded with the Cl^--sensitive dye, SPQ (not shown). Thus, the stimulated uptake appears to be the result of low initial intracellular Cl^-. To determine which mode of uptake was augmented, we tested the effect of treatment with DOG and AA. Fig. 6 *A* shows that the augmented uptake was completely inhibited by ATP depletion.

In the next stage, we used the Cl^--free protocol to test whether BS K^+ uptake into ouabain-treated cells could be converted to ATP-sensitive uptake simply by incubating ouabain-treated cells in Cl^--free, ouabain-containing medium. Fig. 6 *B* shows

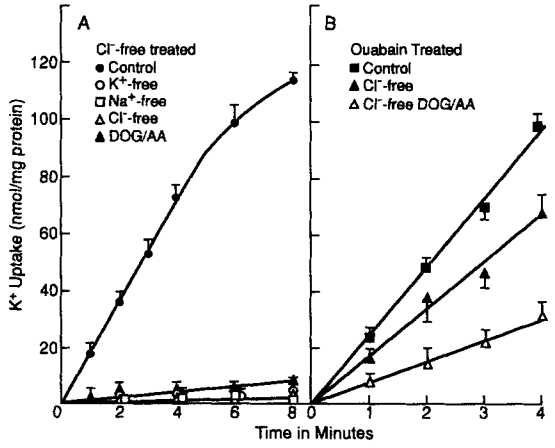


FIGURE 6. Effect of incubation in Cl^--free medium on K^+ uptake. (*A*) Cells were washed twice and then incubated in SB (Cl^- free) for 30 min at 37°C. BS ^{86}Rb uptake was then measured by adding uptake media composed of 0.5 mM ouabain and either SA (●; control), SB (△; Cl^- free), SC (□; Na^+ free), or K^+-free SA (○). Some cells were washed and incubated in glucose-free SB containing DOG/AA for 30 mn at 37°C before measurement of uptake in glucose-free SA containing DOG/AA (▲). (*B*) Cells were incubated for 2 h at 37°C in SA containing 0.5 mM ouabain. The cells were then incubated in SA and 0.5 mM ouabain (■; control) or SB and 0.5 mM ouabain (▲, △) for 30 min at 37°C. During the last 20 min of the second incubation, some cells were incubated in glucose-free SB containing 0.5 mM ouabain and DOG/AA (△). At the end of the preincubations, BS ^{86}Rb was measured by exposing all cells to SA containing ouabain and ^{86}Rb, with or without 0.1 mM bumetanide.

that this was partially achieved. Incubating ouabain-treated cells in Cl^--free medium before measurement of ^{86}Rb uptake in the presence of Cl^- resulted in significant (30 ± 6%, n = 5) inhibition, rather than augmentation, of ^{86}Rb uptake. This is probably the result of the high internal Na^+ in these cells (see below). The remaining uptake could be partially (46 ± 8%, n = 5) inhibited by depleting the cells of ATP. Hence, incubating ouabain-treated cells in Cl^--free medium converted some of the exchange flux to net K^+ influx.

Effect of Intracellular Na^+

Another protocol we used to modify the ionic gradients across the plasma membrane was depletion of intracellular Na^+ (Na_i^+) by incubating the cells in Na^+-free medium. Fig. 7 shows the results of such an experiment in control cells. Incubating the cells in Na^+-free medium for 10 min before uptake in the presence of Na^+ augmented the

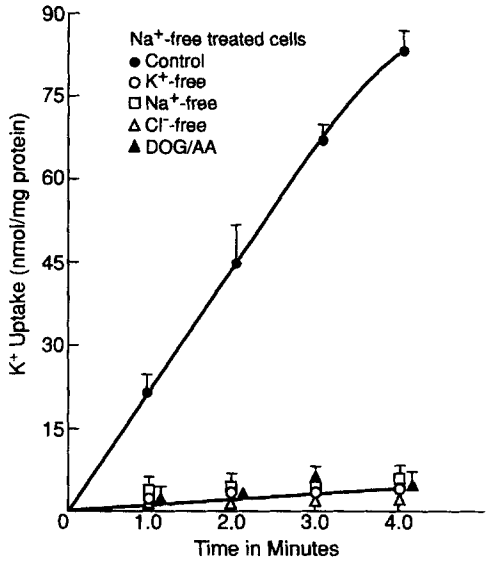


FIGURE 7. Effect of Na^+-free treatment on K^+ uptake. Cells in SA were washed twice and then incubated in SC (●, ○, □, △) or glucose-free SC containing DOG/AA (▲) for 20 min at 37°C. At the end of the preincubations, BS ^{86}Rb uptake was measured by exposing the cells to SA (●, ▲), SB (△), SC (□), or K^+-free SA (○) containing 0.5 mM ouabain and ^{86}Rb, with or without 0.1 mM bumetanide.

rate of BS ^{86}Rb uptake threefold. The uptake required extracellular Na^+, K^+, and Cl^- and was inhibited upon depleting the cells from ATP. Hence, the augmented uptake is mediated by the NaK2Cl cotransporter and represents net uptake of the ion into the cells. Interestingly, the rate of ^{86}Rb uptake by the cotransporter in Cl^-- or Na^+-free treated cells was similar (~ 18 nmol/mg protein per min) and the highest rate measured, suggesting that under this condition the cotransporter operates at its maximal possible rate.

The effect of incubation in Na^+-free medium on BS ^{86}Rb uptake into ouabain-treated cells is demonstrated in Fig. 8. Fig. 8 *A* shows that incubation in Na^+-free medium strongly (65 ± 7%, n = 4) inhibited the uptake. Furthermore, the remaining uptake was mostly resistant to depletion of cellular ATP. One possible explanation for the inhibition is that incubating the ouabain-treated (and therefore Na^+-loaded) cells in Na^+-free medium resulted in cytosolic acidification. The H^+ might have

interfered with transport by the cotransporter either by protonating negatively charged sites on the protein or by H^+ interacting with an internal Na^+site or sites. To test such a possibility, ouabain-treated cells were incubated in Na^+-free medium containing 0.5 mM amiloride to block the Na^+/H^+ exchanger, which dominates pH_i regulation in these cells under the conditions used for cotransport measurements (Green et al., 1988). Fig. 8 *B* shows that amiloride had no effect on BS ^{86}Rb uptake in ouabain-treated cells. Depletion of ATP had no effect on the cotransport and amiloride did not interfere with this activity. Now incubating the cells in Na^+-free medium resulted in a small inhibition of the uptake. Thus, amiloride reduced the inhibition of the uptake due to the incubation in Na^+-free medium. In the presence

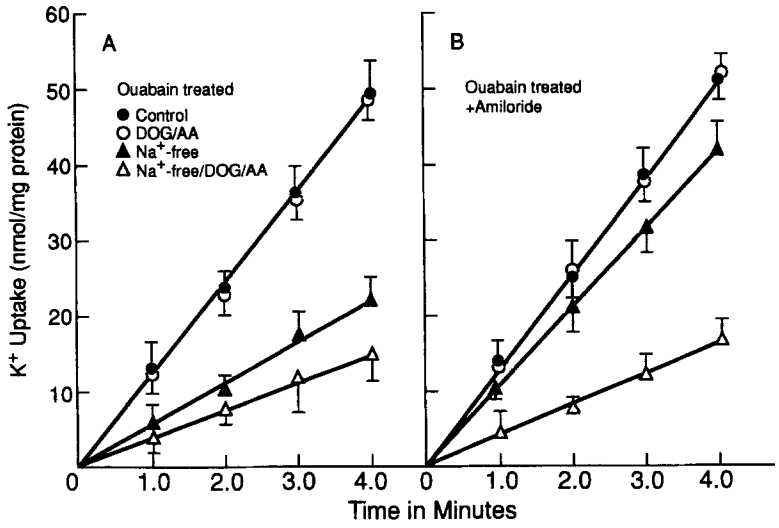


FIGURE 8. Effect of Na^+-free treatment on K^+ uptake into ouabain-treated cells. For the experiments in both panels, the cells were incubated in SA containing 0.5 mM ouabain for 2 h at 37°C before any further manipulations. (*A*) The ouabain-treated cells were further incubated in SA containing 0.5 mM ouabain (●), SA containing ouabain and DOG/AA (○), SC containing ouabain (▲), or SC containing ouabain and DOG/AA (△). After a 20-min incubation at 37°C, BS ^{86}Rb uptake was measured. (*B*) The protocols described for *A* were used except that all preincubation and uptake solutions also contained 0.5 mM amiloride.

of amiloride the uptake after treatment with DOG/AA was inhibited by 65 ± 9% (n = 3).

The results with Cl^-- and Na^+-free incubations suggest that ATP-sensitive transport by the cotransporter in ouabain-treated cells can be imposed by changing the ionic gradients across the plasma membrane. We have previously shown that the Na^+ and K^+ content of these cells can be reliably set at any desired level by treatment with the Na^+/H^+ and K^+/H^+ exchange ionophores monensin and nigericin, respectively (Green et al., 1988). Here we used this technique to measure the exact dependency of cotransport on intracellular Na^+ and K^+ concentrations. Fig. 9 (open circles) shows the results of these experiments. At very low Na^+ the rate of BS ^{86}Rb uptake was very

high. It declined sharply as Na_i^+ concentration was increased up to 10 mM. Then the uptake remained nearly unchanged upon increasing Na_i^+ up to 40 mM, after which the rate increased almost exponentially as Na_i^+ was increased. At the highest Na_i^+ concentration tested (140 mM) there was no apparent saturation of this effect.

In an attempt to distinguish between the uptake components due to net and exchange, parallel experiments were performed, but with the cells depleted of ATP by incubation with DOG/AA (Fig. 9, filled circles). These experiments show that depletion of ATP almost completely inhibited BS ^{86}Rb uptake measured in cells containing up to 40 mM Na^+. Subsequently, with increasing Na_i^+ the rate increased

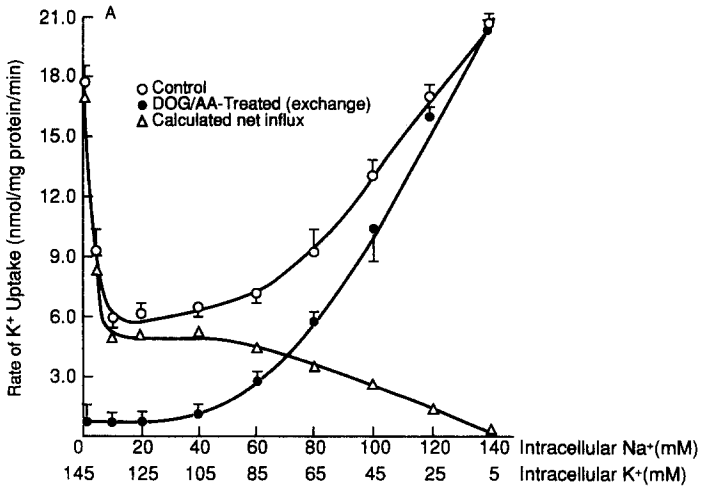
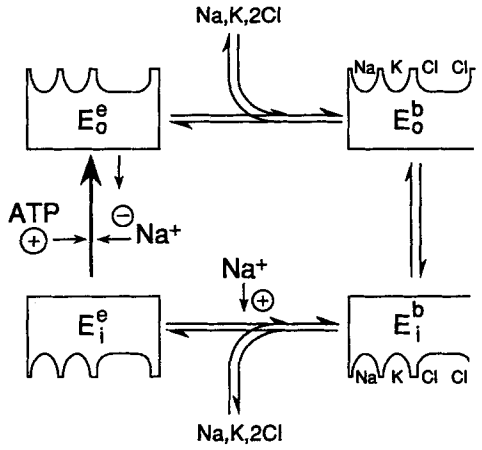


FIGURE 9. Effect of intracellular Na^+ concentration on net and exchange K^+ uptake. The cells were loaded with the indicated Na^+ and K^+ concentrations with the use of ionophores as described in Materials and Methods. One set of solutions contained 10 mM glucose and 10 mM pyruvate (○). The second set of solutions contained DOG/AA (●). The total incubation time with DOG/AA was kept constant at 20 min before initiation of uptake. Uptake medium was always SA containing 0.5 mM ouabain with or without 0.1 mM bumetanide. The medium was supplemented with either glucose/pyruvate or DOG/AA. Uptake was measured for 2 min at 37°C. The BS portion of the uptake was measured and then the difference between the uptake in the presence of glucose/pyruvate (○) and DOG/AA (●) was calculated to yield the fraction on net uptake (△) under all internal Na^+ concentrations.

until at 120 mM it was the same as that measured in fed, ATP-containing cells. The difference between the two experimental curves yield the calculated component of ATP-dependent influx due to the cotransporter (open triangles). This component shows two phases of inhibition by Na^+. A very sensitive phase in which the transport is reduced by 70% due to an increase in Na_i^+ up to 10 mM, and a shallow phase in which the remaining transport is gradually inhibited by increasing Na_i^+ up to 140 mM.

DISCUSSION

Most studies of the turnover cycle of the NaK2Cl cotransporter have been described in various red blood cells (Lauf, McManus, Haas, Forbush, Duhm, Flatman, Saier, and Russell, 1987). Measurement of the internal and external ionic dependencies of net transport, K^+/K^+ exchange, and Na^+/Na^+ exchange were performed. On the basis of these findings, a model for the turnover cycle of the cotransporter has been proposed (Lauf et al., 1987). Important features of the model include the assumption that only the fully loaded or the empty carrier can cross the membrane and that the mobility of the fully loaded carrier is greater than that of the empty carrier. The binding and dissociation of the ions are ordered, which explains the co-ion dependency of the exchange reactions. Detailed studies of the turnover cycle of the cotransporter in other cell types are not available. The findings in the present studies point to two regulatory features in the turnover cycle of the NaK2Cl cotransporter in UMR-106-01 cells. One is regulation by ATP and the other is regulation by intracellular Na^+ and Cl^-. To facilitate the identification of these sites and simplify the arguments in their favor, we describe below a minimal turnover cycle for the cotransporter in UMR-106-01 cells.



(Scheme 1)

The carrier can exist in two forms: E_o where the ion binding sites face the external medium and E_i where the ion binding sites face the internal medium. The two forms can be fully loaded with the ions (E_o^b and E_i^b) or empty (E_o^e and E_i^e). The two major conformational transitions move the ions into the cells ($E_o^b \rightarrow E_i^b$) and the binding sites of the empty carrier out of the cells ($E_i^e \rightarrow E_o^e$). All steps are assumed to be equally reversible except the $E_i^e \rightarrow E_o^e$ in the presence of high ATP. $E_i^e \rightarrow E_o^e$ is slow in the absence of ATP and $E_o^e \rightarrow E_i^e$ is slow in the presence or absence of ATP. Thus slippage is small compared with all other steps in the cycle. The notable differences between this model and that proposed previously are that binding and debinding of all ions are required for both the net and exchange reactions by the cotransporter,

and that high ATP accelerates the forward movement of the empty carrier. At physiological or high internal Cl^- and Na^+ concentrations, the movement of the loaded carrier is the rate-limiting step in the turnover cycle. At low internal ion concentrations, the rate-limiting step is the forward movement of the empty carrier.

It is important to note at this stage that this model may not be sufficient to account for all possible partial reactions by the cotransporter, as described for the Na^+/Na^+ and K^+/K^+ exchange in high Na^+ or high K^+ red blood cells, respectively. In support of this model, it is useful to note that all modes of transport reported here required the presence of Na^+ and K^+ in the external medium. In contrast, K^+/K^+ exchange in red blood cells was reported to be independent of external Na^+ (Haas et al., 1984; Canessa et al., 1986). However, this reaction shows the opposite dependence on external pH as compared with net transport. Thus, increasing pH from 6.6 to 8.0 strongly inhibited the exchange while it stimulated net uptake (Duhm, 1987). This would suggest that in red cells external H^+ is able to substitute for Na^+ interacting with the external site. Similarly, Fig. 8 argues that H^+ can interact with the internal Na^+ site(s) in UMR-106-01 cells. Hence, it is possible that the external Na^+ site must be occupied by either Na^+ or H^+ for K^+/K^+ exchange to occur in all cell types. Differences in the apparent affinity for external Na^+ and H^+ can account for the apparent differences in the external Na^+ requirement of K^+/K^+ exchange. Therefore, we believe that the model proposed here is the minimal model necessary and is adequate to account for the modes of transport measured in these studies.

Mode of Cotransport

BS ^{86}Rb uptake in control and ouabain-treated cells reflect different modes of cotransport. Most of the transport in control cells appears to be due to net K^+ uptake since the rate of ^{86}Rb influx far exceeds the rate of ^{86}Rb efflux under the same conditions. Furthermore, under control conditions, but not in ouabain-treated cells, the cotransporter increased cell volume (Khademazad, Zhang, Loessberg, and Muallem, 1991). Ouabain treatment more than doubled the rate of ^{86}Rb influx. In ouabain-treated cells the rates of ^{86}Rb influx and efflux were similar and about two-thirds of the maximal possible unidirectional influx. Thus, the same BS cotransporters must have mediated the influx and efflux reactions. The effect of ouabain can also be seen after a long incubation with DOG/AA. Loading the cells with Na^+ and depleting K^+ with ionophores had the same effect as long treatment with ouabain. Incubation of ouabain-treated cells in Na^+- or Cl^--free medium to impose an inwardly directed driving force was sufficient to modify the behavior of the cotransporter. These findings suggest that in control cells, BS ^{86}Rb uptake represents net K^+ uptake, whereas after ouabain treatment most of the BS ^{86}Rb uptake is due to K^+/K^+ exchange.

Although the evidence above strongly supports K^+/K^+ exchange as the mode of transport in ouabain-treated cells, we were unable to obtain evidence for obligate K^+/K^+ exchange as was shown for red blood cells. Hence, in UMR-106-01 cells net and exchange fluxes required all three ions, including Na^+ in the medium, whereas in red cells the exchange, but not net uptake, was independent of Na_o^+ (see below). When Na^+ and/or K^+ were removed from the efflux medium of ouabain-treated cells,

the efflux became dependent on cellular ATP (net efflux) in a manner similar to that shown in Figs. 6 and 8. Thus, the requirement for all ions during exchange flux precluded demonstrating an obligate K_o^+/K_i^+ exchange. Nonetheless, the overall properties of K^+ fluxes in ouabain-treated cells seem to be sufficient to conclude that they are mediated by a K^+/K^+ exchange reaction.

Several properties of net and exchange flux in red blood cells are quite different from those measured in UMR-106-01 cells. K^+/K^+ exchange in erythroid cells is usually measured as the Na_o^+-independent ^{86}Rb or K^+ influx (Haas et al., 1984; Canessa et al., 1986; Duhm, 1987). In human erythrocytes K^+/K^+ exchange was inhibited by Na_i^+ above 60 mM (Canessa et al., 1986; Duhm, 1987), whereas in UMR-106-01 the exchange became more prominent as Na_i^+ was increased beyond 60 mM (Fig. 9). In addition, net Na_o^+-dependent ^{86}Rb influx into red blood cells in medium containing 140 mM Na^+ was not affected by internal Na^+ of up to about 100 mM, while in UMR-106-01 cells the uptake declined on increasing Na_i^+ (Canessa et al., 1986; Duhm, 1987). Under physiological Na^+ and K^+ gradients, ~25–50% of furosemide-sensitive ^{86}Rb uptake into red blood cells is independent of extracellular Na^+ (Haas et al., 1984; Duhm, 1987). In contrast, under similar conditions, UMR-106-01 (Whisenant et al., 1991) and many other nonerythroid cell types (Rindler et al., 1982; Owen, 1984; Brown and Murer, 1985; O'Grady et al., 1987; Kim et al., 1988; Kort and Koch, 1989) show almost no Na_o^+-independent, furosemide-sensitive ^{86}Rb uptake. These differences suggest that the turnover cycle of the cotransporter is not necessarily identical in all cells. This is also evident from the stoichiometry of the transport in squid axons and ferret erythrocytes (Russell, 1983; Hall and Ellory, 1985). Because of the absolute requirement for external Na^+ (as well as external K^+ and Cl^-) for ^{86}Rb uptake into nonerythroid cells under various experimental conditions, we assume that binding and debinding of all ions in the external face of the carrier are required for net and exchange fluxes.

Site of ATP Interaction

Several studies have shown the dependence of net ion transport on cellular concentration of ATP (Russell, 1976; Palfrey and Greengard, 1981; Rindler et al., 1982; Kim et al., 1988). The cotransporter is also regulated by protein kinases (for review see Hoffmann and Simonsen, 1989). Hence, it is generally assumed that the dependency of the cotransport on cellular ATP reflects regulation by phosphorylation. The results presented here suggest another mode of regulation. The finding that the exchange is insensitive to cellular ATP levels, and that the rate of exchange is about two-thirds of the maximal overall net transported at very low Na_i^+ and Cl_i^- indicates that the presence of ATP or phosphorylation is not a primary requirement for cotransport to occur. However, under all conditions, net transport was sensitive to ATP depletion. Hence, the effect of ATP described here is probably regulatory in nature. The insensitivity of the exchange to ATP with the complete inhibition of net cotransport at relatively low ATP places the regulatory site for ATP in a step not common to the two modes of transport. The only such step is the conformational transition of the empty carrier. This mode of regulation is similar to that described for the Na^+/K^+ (Glynn, 1985) and Ca^{2+} pumps (Inesi, Sumibilla, and Kirtley, 1990; Carafoli, 1991).

The suggested regulation of the turnover cycle by ATP is, of course, not the only mode of regulation. As noted above, the cotransporter is regulated by many protein kinases which probably phosphorylate the cotransporter (O'Grady et al., 1987; Pewitt, Hegde, Haas, and Palfrey, 1990). Regulation of the cotransporter protein by direct phosphorylation was reported recently in shark rectal (Lytle and Forbush, 1992) and avian salt glands (Torchia, Lytle, Pon, Forbush, and Sen, 1992). Regulation by protein kinases may change the number of active cotransporters in the plasma membrane (Haas and Forbush, 1986; Franklin, Turner, and Kim, 1989; O'Donnell, 1989; Haas, Johnson, and Boucher, 1990) or any of the rate-limiting steps in the turnover cycle of the cotransporter. These can represent secondary regulatory modes that serve to stimulate or inhibit cotransport beyond the regulation by ATP interacting at a regulatory site.

Effect of Intracellular Cl^-

Incubating cells in Cl^--free medium greatly stimulated K^+ uptake from a medium containing Na^+, K^+, and Cl^-. By virtue of the sensitivity to ATP depletion, the augmented influx represents net uptake of the ions. It is important to note that in control and Cl^--free treated cells the driving force was always inwardly directed. Thus, the removal of Cl^- or Na^+ from the cytosol must have stimulated the uptake due to removal of an inhibitory effect by the ions.

The stimulatory effects due to removal of internal Cl^- have been described before in squid axons (Russell, 1976, 1979, 1983). Furthermore, it appears that the interaction of Cl^- with the internal site(s) is modified when the cotransporter is stimulated by cell shrinkage (Breitwieser, Altamirano, and Russell, 1990). It is possible that the stimulation observed here represents similar phenomena. Since we were unable to clamp intracellular Cl^- at various concentrations, we could not determine the step(s) in the turnover cycle affected by Cl^-. However, it is unlikely that internal Cl^- inhibited the transfer of the loaded carrier from out to in or stimulated the reverse mode, since such an effect should stimulate K^+/K^+ exchange. This was not the case, as increasing internal Cl^- during NaK2Cl uptake into Cl^--depleted cells inhibited net uptake by ~70% without inducing large K^+ efflux. It is therefore likely that Cl^- acts on one of the cytosolic-facing forms of the cotransporter.

Regulation by Intracellular Na^+

The results presented in Fig. 9 suggest quite complicated interactions of the cotransporter with internal Na^+. Our ability to clamp internal Na^+ and K^+ at any given concentration allowed reproducible measurements, and thus detailed description of this interaction. The first observation was the asymmetry of Na^+ interaction with the internal and external sites. While on the external face Na^+ appears to interact with only one site (Whisenant et al., 1991), the internal face appears to interact with Na^+ twice in each turnover. Thus, increasing Na^+ from ~1 to 10 mM strongly inhibited (~65%) net forward uptake while not inducing any exchange flux. Under these Na^+ concentrations, the driving force is inwardly directed, and therefore internal Na^+ must inhibit net cotransport by interacting with an inhibitory regulatory

site. In principle, intracellular Na^+ can inhibit any of the steps on the cytosolic face. Our results are not sufficient to conclusively identify this step. However, since increasing Na_i^+ up to 10 mM affected only net uptake, this step may be the conformational transition of the empty carrier. Through this regulation, cotransport activity can increase or decrease almost threefold. This could be an excellent $[Na^+]_i$-sensing mechanism, as a small reduction in Na_i^+ will result in activation of a large influx by the cotransporter. Once Na_i^+ is increased to ~10 mM, the rate of the cotransporter will return to normal.

Upon further increase in internal Na^+, net uptake was further inhibited and in parallel exchange flux was activated. This effect of internal Na^+ was not a consequence of reversing the driving force. Internal Cl^- concentration in control and ionophore-treated UMR-106-01 cells is ~30 mM (Green et al., 1990). The increase in internal Na^+ was accompanied by a decrease in internal K^+ so that under all conditions the driving force for cotransport was inwardly directed. The reciprocal nature of the inhibition of net and stimulation of exchange suggests that the interaction of Na^+ with the same site was responsible for the two effects. Furthermore, both activities (DOG-sensitive and DOG-insensitive fractions) show similar dependency on intracellular Na^+ concentrations. The reciprocity of the effect of Na_i^+ on the net and exchange fluxes also points to the possible site of interaction of Na_i^+. The inhibition of net flux cannot be due to inhibition of any of the forward reactions since such an effect will result in a parallel inhibition of net and exchange flux. Stimulation of any of the forward reactions by high Na_i^+ is also not required since the overall rate of these reactions is sufficiently fast (net uptake at very low Na_i^+). Thus, high Na_i^+ should stimulate one of the reactions in the reverse direction. To completely inhibit net uptake, high Na_i^+ should stabilize E_i^b. Such an effect can account for both maximal inhibition of net uptake and stimulation of exchange to a rate beyond that of net uptake at 10–40 mM Na_i^+.

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