ATP Dependence of Na$^+$/H$^+$ Exchange

Nucleotide Specificity and Assessment of the Role of Phospholipids

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ABSTRACT   We studied the ATP dependence of NHE-1, the ubiquitous isoform of the Na$^+$/H$^+$ antiporter, using the whole-cell configuration of the patch-clamp technique to apply nucleotides intracellularly while measuring cytosolic pH (pH$_i$) by microfluorimetry. Na$^+$/H$^+$ exchange activity was measured as the Na$^+$-driven pH$_i$ recovery from an acid load, which was imposed via the patch pipette. In Chinese hamster ovary (CHO) fibroblasts stably transfected with NHE-1, omission of ATP from the pipette solution inhibited Na$^+$/H$^+$ exchange. Conversely, ATP perfusion restored exchange activity in cells that had been metabolically depleted by 2-deoxy-D-glucose and oligomycin. In cells dialyzed in the presence of ATP, no "run-down" was observed even after extended periods, suggesting that the nucleotide is the only diffusible factor required for optimal NHE-1 activity. Half-maximal activation of the antiporter was obtained at ∼5 mM Mg-ATP. Submillimolar concentrations failed to sustain Na$^+$/H$^+$ exchange even when an ATP regenerating system was included in the pipette solution. High ATP concentrations are also known to be required for the optimal function of other cation exchangers. In the case of the Na/Ca^{2+} exchanger, this requirement has been attributed to an aminophospholipid translocase, or "flippase." The involvement of this enzyme in Na$^+$/H$^+$ exchange was examined using fluorescent phosphatidylserine, which is actively translocated by the flippase. ATP depletion decreased the transmembrane uptake of NBD-labeled phosphatidylserine (NBD-PS), indicating that the flippase was inhibited. Diamide, an agent reported to block the flippase, was as potent as ATP depletion in reducing NBD-PS uptake. However, diamide had no effect on Na$^+$/H$^+$ exchange, implying that the effect of ATP is not mediated by changes in lipid distribution across the plasma membrane. K-ATP and ATPγS were as efficient as Mg-ATP in sustaining NHE-1 activity, while AMP-PNP and AMP-PCP only partially substituted for ATP. In contrast, GTPγS was ineffective. We conclude that ATP is the only soluble factor necessary for optimal activity of the NHE-1 isoform of the antiporter. Mg^{2+} does not appear to be essential for the stimulatory effect of ATP. We propose that two mechanisms mediate the activation of the antiporter by ATP: one requires hydrolysis and is likely an energy-dependent event. The second process does not involve hydrolysis of the γ-phosphate, excluding mediation by protein or lipid kinases. We suggest that this effect is due to binding of ATP to an as yet unidentified, nondiffusible effector that activates the antiporter.

KEY WORDS: Na$^+$/H$^+$ antiporter • phospholipid translocase • intracellular pH

INTRODUCTION

Na$^+$/H$^+$ exchangers (NHEs),[1] or antiporters, are ubiquitous membrane transport proteins that play a major role in the regulation of intracellular pH (pH$_i$) and of cellular volume (Grinstein et al., 1985; Moolenaar, 1986; Demaurex and Grinstein, 1994). Under physiological conditions, NHEs catalyze the electroneutral exchange of extracellular Na$^+$ for intracellular H$^+$, a process which is competitively inhibited by amiloride and its analogues (see Grinstein et al., 1989; Wakabayashi et al., 1992b). The rate of exchange is dictated largely by the state of protonation of an allosteric "modifier" site on the cytosolic side of the antiporter. Protonation of the modifier stimulates Na$^+$/H$^+$ exchange, defending the cell from excessive acidification, while the occurrence of deprotonation near the physiological pH$_i$ deactivates the antiporter, precluding a potentially deleterious alkalinization of the cytosol (Aronson et al., 1982; Grinstein et al., 1984; Aronson, 1985).

Na$^+$/H$^+$ exchange activity has been observed in virtually all eukaryotic cells studied thus far. Yet, the kinetic and pharmacological properties of the exchange process vary widely between individual cell types and even between different domains of the plasma membrane in the case of asymmetric cells, e.g., the apical and basolateral membranes of epithelial cells (Haggerty et al., 1988). The structural basis of this functional diversity is attributed, at least in part, to the existence of distinct isoforms of the NHEs. In mammalian tissues, five separate isoforms have been identified to

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[1]*Abbreviations used in this paper:* CHO, Chinese hamster ovary; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; NBD-PS, 1-C$_6$-2-C$_{12}$-NBD-phosphatidylserine; NHE, Na/H exchanger; pH$_i$, intracellular pH; PS, phosphatidylserine.

date (Sardet et al., 1989; Orlowski et al., 1992; Tse et al., 1992; Wang et al., 1993; Klanke et al., 1995). All NHE isoforms are integral plasma membrane proteins of ~80–110 kD, with 10–12 predicted transmembrane domains and a long COOH-terminal cytosolic tail. The transmembrane-spanning segments are highly conserved between isoforms and contain the ion transport and inhibitor-binding sites. The cytosolic tail, which is more variable in sequence, is involved in the regulation of antiporter activity, as it contains numerous potential sites for phosphorylation by protein kinases and, in the case of NHE-1, a calcium/calmodulin-binding site. NHE-1, the "housekeeping" isoform, is present in most mammalian cells and is localized to the basolateral membrane of epithelial cells. The other isoforms have a more restricted tissue distribution: NHE-2, 3, and 4 are expressed predominantly in the kidney, intestine, and stomach, whereas NHE-5 is found mostly in the brain, spleen, and testis.

Fluxes through the antiporter are driven by the combined chemical gradients of Na^+ and H^+ and hence do not directly consume metabolic energy. The lack of requirement for ATP hydrolysis during ion transport is demonstrated by the ability to measure Na^+/H^+ exchange in plasma membrane vesicles prepared in the absence of ATP (Murer et al., 1976; Kinsella and Aronson, 1980). In intact cells, however, ATP appears to be required for optimal function of the antiporter. Procedures that reduce intracellular ATP levels drastically inhibit Na^+/H^+ exchange in a variety of native systems (Grinstein et al., 1985; Cassel et al., 1986; Little et al., 1988; Burns et al., 1991) and in antiport-deficient cells transfected with either NHE-1, 2, or 3, the only isoforms thus far expressed in heterologous systems (Wakabayashi et al., 1992a; Levine et al., 1993; Demaurex and Grinstein, 1994; Kapus et al., 1994). The effect of ATP depletion cannot be explained by dissipation of the Na^+ and H^+ gradients, as in most studies a defined intracellular pH was maintained and changes in the transmembrane Na^+ gradient were minimized. Thus, the effect of ATP is not attributable to changes in the concentration of the substrate or regulatory ions. Kinetic analysis showed that the predominant effect of ATP depletion is to alter the sensitivity of the antiporter to intracellular $[H^+]$; much more acidic levels must be reached to stimulate exchange, resulting in virtual inactivity of Na^+/H^+ exchange at pH_i levels higher than 6.5. In most studies, a parallel reduction in the maximal rate of transport (V_{max}) has also been observed, although estimates of V_{max} are limited by the range of pH_i values that can be imposed without affecting cellular integrity.

As the NHE-1 isoform is phosphorylated in unstimulated cells (Sardet et al., 1990, 1991), it was proposed that the inhibition caused by ATP depletion was due to the loss of constitutive phosphate groups. However, recent studies failed to demonstrate a difference in the phosphorylation state of control and ATP-depleted NHE-1 transformants. One-dimensional phosphopeptide maps of control and depleted cells were indistinguishable (Goss et al., 1994). Furthermore, normal exchange activity was observed in a deletion mutant lacking all major phosphorylation sites (Wakabayashi et al., 1994). Thus, changes in the phosphorylation state of the antiporter itself do not appear to mediate the effect of ATP. Since no consensus nucleotide-binding sequences such as Walker motifs are apparent in the primary structure of the NHE isoforms (Sardet et al., 1989; Orlowski et al., 1992; Wang et al., 1993), direct ATP binding to the antiporter appears equally unlikely. The effect of ATP thus appears to involve separate, as yet unidentified components.

Like the NHEs, the Na^+/Ca^{2+} antiporter is equally susceptible to depletion of ATP (Baker and McNaughton, 1976; Hilgemann, 1990; Collins et al., 1992; Hilgemann et al., 1992). Though the Na^+/Ca^{2+} exchanger bears no structural analogy to the NHE family, the two systems share some functional features. In particular, both transporters are activated allosterically by their intracellular substrate ion (Ca^{2+} and H^+, respectively) and depletion of ATP results in altered allosteric behavior in both systems. Thus, lessons learned from the Na^+/Ca^{2+} exchanger could help clarify the mode of action of ATP on the NHE. Specifically, the ATP sensitivity of the Na^+/Ca^{2+} exchanger has been reported to involve changes in the lipid distribution of the plasma membrane (Hilgemann and Collins, 1992). The asymmetric composition of the plasma membrane is actively maintained by phospholipid flippases, which preferentially distribute negatively charged lipids to the inner (cytosolic) leaflet (for review, see Zachowski, 1993). The negative surface charge, or a particular lipid composition of the membrane, has been postulated to stabilize the Na^+/Ca^{2+} protein in its functional state. Inhibition of the flippase upon ATP depletion is expected to cause a redistribution of the phospholipids across the bilayer (e.g., Dumaswala et al., 1996), resulting in an inhibition of exchange. Accordingly, in studies using excised macropatches, adding exogenous phosphatidylserine (PS) to the inner (cytosolic) leaflet increased Na^+/Ca^{2+} exchange, whereas transport was inhibited by screening PS head groups or by inhibiting the flippase (Hilgemann and Collins, 1992; Collins and Hilgemann, 1993). Given the similarities between the two cation exchangers, a similar mechanism might also underlie the ATP dependence of Na^+/H^+ exchange.

To gain insight into the mechanism of ATP dependence of Na^+/H^+ exchange, we used two approaches. Glass micropipettes were used to perfuse various nucleotides into cells while measuring NHE activity using a

pH-sensitive dye. In addition, the involvement of the flippase was assessed by measuring phospholipid translocation, using single cell fluorescence imaging. To simplify the analysis, cells expressing only a single, well defined isoform of the exchanger were employed. For this purpose, mutagenized Chinese hamster ovary (CHO) cells devoid of a functional antiporter (AP-1) were stably transfected with the rat NHE-1, the ubiquitous isoform of the exchanger.

METHODS

Materials and Solutions

Dioleoylphosphatidylcholine and 1-C_6-2-C_{12}-NBD-phosphatidylserine (NBD-PS) were from Avanti Polar Lipids (Alabaster, AL). Nigericin, $2'7'bis$-(2-carboxyethyl)-5-(and 6) carboxyfluorescein (BCECF) free acid and acetoxy methyl ester were purchased from Molecular Probes (Eugene, OR). Mes and medium RPMI-1640 were from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade and were obtained from Aldrich Chemical Co. (Milwaukee, WI). Bath solutions contained 135 mM aspartate and 100 mM Tris, titrated to pH = 8.0 with 85 mM of either NaOH (Na^+-medium) or CsOH (Na^+-free medium). Pipette solutions contained 150 mM Cs-aspartate and 5 mM Mes, pH 6.0–7.0, as indicated. Unless otherwise specified, all solutions contained 1 mM $MgCl_2$ and 1 mM ethylene glycol-bis(amino-ethyl ether)N,N,N',N'-tetra acetic acid (EGTA); pipette solutions also contained 200 μM BCECF (free acid). The osmolarity was set to 280 ± 5 mosM (pipette) and to 300 ± 5 mosM (bath).

Cells

AP-1 cells were stably transfected with the full-length cDNAs encoding the NHE-1 isoform of the rat antiporter, as previously described (Orlowski, 1993; Wang et al., 1993). The transfected cells were grown in α-minimal essential medium (Ontario Cancer Institute, Toronto, Canada) supplemented with 10% FCS and 1% antibiotic solution (penicillin and streptomycin, GIBCO BRL, Gaithersburg, MD) and passaged twice a week. Cultures were reestablished from frozen stocks, and cells from passages 3 to 12 were grown on glass coverslips for 24–48 h before the experiments.

Fluorescence Microscopy

Ratio fluorescence imaging was performed on a Zeiss Axiovert 100 TV inverted microscope (Zeiss, Obeskochen, Germany) equipped with a 75 W Xenon epifluorescence lamp (XB0 75; Zeiss), a shutter/filter-wheel assembly (Lambda 10; Sutter Instrument Co., Novato, CA), a NeoFluar 63×/1.25 objective and a cooled, digital CCD camera (TEA/CCD 1317; Princeton Instruments, Trenton, NJ) with a high resolution detector (1,317 × 1,025 pixels, KAF 1400; Eastman Kodak Co., Rochester, NY), interfaced to a Pentium 90 computer (Dell Inc., Canada) via a 12 bit, 1 MHz camera controller board (ST-38; Princeton Instruments). Image acquisition and excitation filter selection was controlled by the Metafluor software (Universal Imaging Corp.). Dynamic ratio fluorescence measurements were performed on a Nikon Diaphot TMD microscope (Nikon Canada, Toronto, Canada) equipped with a 100 W Xenon lamp, a shutter/rotating mirror/fiber optic assembly (RatioMaster; Photon Technologies Inc., South Brunswick, NJ), a Fluor 40×/1.3 oil-immersion objective, and a high-sensitivity photometer (D-104; Photo Technologies Inc.) interfaced to a 386 computer (NEC, Canada) via a 12 bit A/D board (Labmas-

ter; Scientific Solutions Inc., Solon, OH). Photometric data were acquired at 10 Hz using the Oscar software (PTI). Both the Nikon and Zeiss microscopes were equipped with a separate light source for long-wavelength transillumination (λ > 620 nm), a 580-nm emission dichroic mirror, and a separate video camera (MTI 72; Dage-MTI, Michigan City, IN) to allow continuous differential interference contrast (DIC) or Hoffmann-enhanced visualization of the cells during the fluorescence measurements.

Electrophysiology

Cells were voltage-clamped at −60 mV in the whole-cell configuration of the patch clamp technique using an Axopatch-1D amplifier (Axon Instruments Inc., Foster City, CA), as described (Demaurex et al., 1995). Electrodes were made from borosilicate glass (World Precision Instruments, Sarasota, FL) using a horizontal puller (P-87; Sutter Instrument Co., Novato, CA) and a microforge (MF-9; Narishige USA, Greenvale, NY). Pipette resistance ranged from 2 to 10 MΩ and seal resistance from 10 to 50 GΩ. Series resistance varied between 5 and 30 MΩ and cell capacitance between 12 and 34 pF.

Measurement of Phospholipid "Flipping"

The ability of cells to translocate lipids was assessed either directly by measuring the internalization of fluorescent phosphatidylserine (NBD-PS), or indirectly by quantifying the binding of extracellular rhodamine-heptalysine (the kind gift of Dr. D. Hilgemann, University of Texas Southwestern Medical Center, Dallas, TX) to cell membranes. Unilamellar vesicles containing NBD-PS/DOPC (1:2, mol/mol) were prepared by ethanol injection, according to Hanada and Pagano (1995). Briefly, aliquots of the lipids in solution were mixed, dried under N_2, redissolved in 50 μl ethanol, and injected into 5 ml of deionized water using a Hamilton syringe while vortex mixing. Then, 5 ml of twofold concentrated PBS was added, yielding a final concentration of 20 μM NBD-PS. To measure lipid uptake, cells adhered to coverslips were rinsed twice with PBS, incubated for 10 min in the experimental buffer and then for 10 min with PBS supplemented with 1 ml of the suspension of NBD-PS-containing donor vesicles. NBD-PS was removed from the cell surface by a 3 × 10 min incubation with 2 ml of a solution containing 2% defatted albumin at 4°C (back-exchange), and membrane-associated fluorescence was measured on the imaging system using 450-nm excitation and 542-nm emission filters. For Rhod-Lys7 binding measurements, cells were incubated for 10 min in the indicated buffer and subsequently for 10 min with PBS supplemented with 2 μM Rhod-Lys7, rinsed twice with PBS and fluorescence was measured on the imaging system using 535-nm excitation and 565-nm emission filters.

Measurement of Na^+/H^+ Exchange in Patch-clamped Cells

NHE activity in voltage-clamped cells was measured microfluorimetrically on the photometric system described above (see also Demaurex et al., 1995). Cells were incubated for 15 min with 2 μM BCECF-AM, then patch-clamped in the whole-cell configuration and used to measure BCECF fluorescence using alternate 495/440-nm illumination. The pipettes contained acidic solutions of low buffering power (5 mM Mes) to facilitate detection of NHE-induced changes of intracellular pH. When the cells were perfused in Na^+-free media, the cytosolic pH had equilibrated with the pipette pH after 5 min. NHE was initiated by switching the perfusate to a Na^+-containing medium. Cells were perfused at 0.5 ml/min and bath exchange was complete within 1 min. Calibration of fluorescence ratio vs. pH was performed on intact cells (not patched), using nigericin (5 μM final) according to Thomas et al. (1982). One calibration curve was obtained ev-

ery day by averaging data from three to six cells sequentially perfused with KCl media buffered at four different pH values ranging from 6.0 to 7.5. All measurements were carried out at 37°C.

Data Analysis and Statistics

Quantification of cell-associated fluorescence was performed using the Metamorph/Metafluor package (Universal Imaging, Inc.). Data were graphed using the Origin software (MicroCal Software Inc., Northampton, MA) and are shown as means ± one standard error (SE) of the number of experiments indicated.

R E S U L T S

To date, the ATP dependence of the NHE has been studied only in intact cells, which were subjected to metabolic depletion by the combined use of mitochondrial and glycolytic inhibitors. This approach precludes detailed study of the ATP concentration dependence and of the reversibility of the process. Moreover, the nucleotide specificity of the phenomenon cannot be explored in intact cells. To circumvent these limitations, we studied the activity of the antiport in cells where the cytosol could be perfused with the solution of choice via a patch pipette in the "whole-cell" configuration (i.e., after applying suction to rupture the membrane patch). Cytosolic pH was measured microfluorimetrically using the excitation ratio of BCECF. We found earlier that, when using cells with robust NHE activity and pipettes containing solutions of low buffering power, the extrusion of H^+ by the antiport can outstrip the diffusion of buffers to and from the pipette, resulting in measurable pH_i changes (Demaurex et al., 1995). In these conditions, the plateau pH_i reached after recovery is not necessarily the "set-point" of the antiporter, but an equilibrium between acid extrusion and acid diffusion from the pipette into the cell.

ATP perfusion restores Na^+/H^+ exchange in depleted cells. Intact, untreated NHE-1 transfectants displayed robust Na^+/H^+ exchange activity, readily detectable as a Na^+-dependent pH_i recovery from an acid load (Fig. 1 A, top trace). In agreement with earlier findings (Goss et al., 1994; Kapus et al., 1994), the antiport activity in these cells was markedly inhibited when intracellular ATP levels were reduced by a short (10 min) incubation with 5 mM 2-deoxy-D-glucose and 5 μg/ml oligomycin (Fig. 1 A, lower trace). To test whether the effect of metabolic inhibition was reversible, the nucleotide was re-introduced to depleted cells using a patch pipette. After metabolic depletion, the cells were patch-clamped in the whole-cell configuration using a pipette containing 10 mM ATP-Mg, and the cytosol was allowed to equilibrate with the perfusing solution for 5 min prior to recording. Acidic pipette solutions (pH = 6.0) of low buffering power (5 mM Mes) were used to maximize NHE activation and facilitate the detection of the pH_i changes. With ATP-Mg present in the patch pipette, Na^+/H^+ exchange could be readily restored in
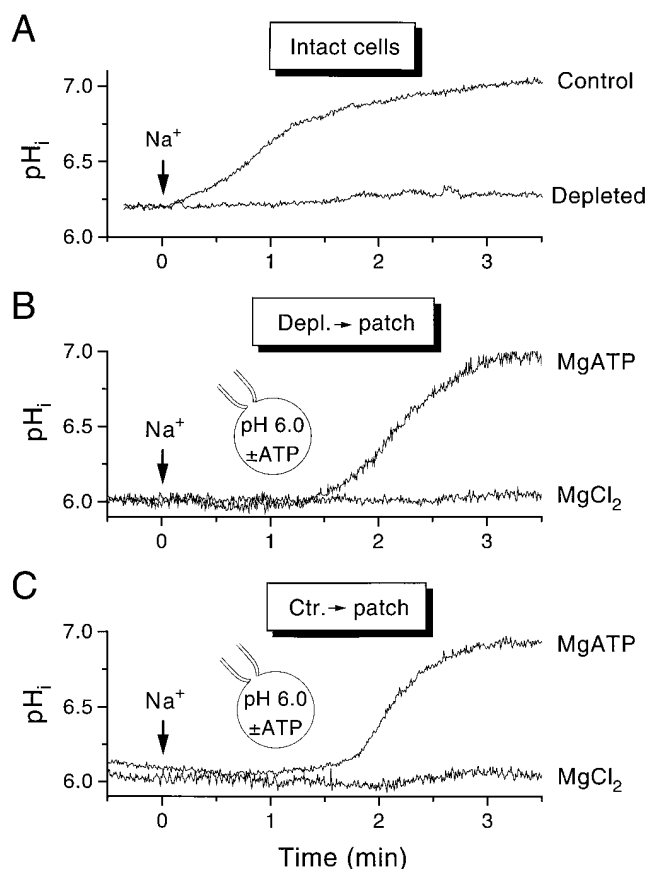

FIGURE 1. ATP perfusion restores functional Na^+/H^+ exchange. The cytosolic pH (pH_i) of single intact NHE-1-transfected cells (A) or of cells patch-clamped in the whole-cell configuration (B and C) was measured with BCECF. Intact cells were acid-loaded by an ammonium prepulse. Cytosolic acidification of patched cells was accomplished by equilibration with the pipette solution, which was titrated to pH 6.0. The cells were bathed in Na^+-free, Cs^+ medium at the start of the traces. The solution was replaced by Na^+-medium where indicated (time = 0). (A) Top trace: untreated cells (Control). Lower trace: cells subjected to ATP depletion by treatment with 5 mM 2-deoxy-D-glucose and 5 μg/ml oligomycin for 10 min before the onset of the experiment (Depleted). (B) Intact cells were ATP depleted as in A, prior to patch-clamping with pipettes containing either 10 mM Mg-ATP (top trace) or $MgCl_2$ without ATP (lower trace). The cells were allowed to equilibrate for 5 min with the pipette solutions before reintroduction of Na^+ to the medium. (C) Nondepleted (Ctr.) cells were patch-clamped with pipette solutions containing either 10 mM Mg-ATP (top trace) or 10 mM $MgCl_2$ (bottom trace). Traces in A–C are representative of at least six experiments of each kind.

cells that had been metabolically depleted (Fig. 1 B, top trace), although the lag time between Na^+ addition and the onset of the alkalinization was consistently prolonged (see below). In contrast, no exchange activity was observed when the depleted cells were perfused with a pipette solution devoid of ATP (Fig. 1 C, bottom trace). Thus, ATP-Mg is sufficient to restore Na^+/H^+ exchange in metabolically poisoned cells.

ATP Removal Inhibits NHE

In addition to depleting intracellular ATP, mitochondrial and/or glycolysis inhibitors increase the intracellular concentrations of ADP and AMP. As all previous studies of the ATP dependence of NHE have relied on metabolic inhibitors, increased ADP and AMP, instead of decreased ATP, could possibly account for the inhibition of Na^+/H^+ exchange. To test this possibility, we used the patch pipette to deplete the cells of all adenine nucleotides. A 5-min equilibration of otherwise untreated cells with a pipette devoid of nucleotides resulted in full inhibition of Na^+/H^+ exchange (Fig. 1 *C, bottom trace*), suggesting that this procedure lowered cytosolic ATP levels as efficiently as a 10-min incubation with metabolic inhibitors. Since intracellular ADP and AMP levels are expected to decrease in parallel with ATP, the inhibition observed under these conditions could not have been caused by an increase in the mono- or diphospho-nucleosides.

When 10 mM ATP was present in the pipette, the response observed in untreated cells was similar to that reported above for cells that had been metabolically depleted prior to patching (cf. Fig. 1, *B* and *C*). This implies that exposure to the metabolic inhibitors had no effects on NHE-1 activity other than those associated with ATP depletion. Thus, ATP removal mimicked the effects of metabolic inhibitors, whereas ATP re-perfusion restored Na^+/H^+ exchange, demonstrating that intracellular ATP-Mg is both necessary and sufficient for normal NHE-1 function.

Kinetics of the pH_i Changes Observed in Perfused Cells

The pH_i changes observed in patch-clamped NHE-1 transfectants differed somewhat from the response observed in intact cells, in that the lag-time between Na^+ re-addition and the onset of the alkalinization was markedly increased (Fig. 1). As the cytosol had been extensively dialyzed during the 5-min pre-equilibration with the pipette solution, this long lag-time could be due to the loss of a soluble component(s) required for optimal Na^+/H^+ exchange. However, analysis of the pH dependence of the effect suggested a simpler explanation. As shown in Fig. 2 *A*, the lag time was much reduced when the pH of the pipette was more alkaline (e.g., 6.5). Systematic study of this relationship in multiple experiments showed that the lag was progressively decreased as the pipette pH was elevated (Fig. 2 *B, inset*). We therefore believe that the lag period reflects, at least in part, diffusional delay of H^+ (equivalents) from the membrane to the area where fluorescence is recorded, which includes out-of-focus fluorescence from the pipette shank. Because patch-perfused cells are an open system, H^+ (equivalents) extruded from the cells by NHE can be replaced not only by cellular buffers,
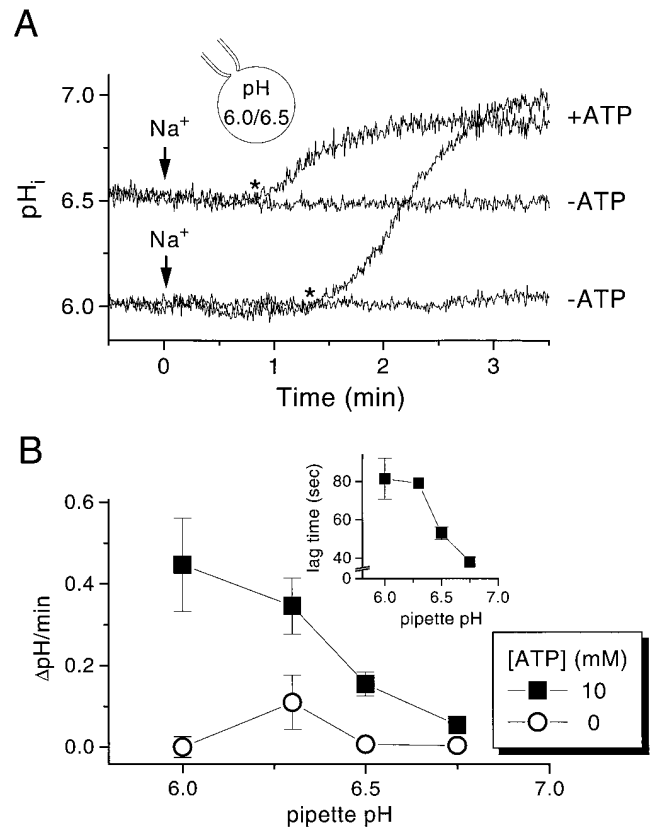

FIGURE 2. pH_i dependence of Na^+/H^+ exchange in the presence or absence of ATP. (*A*) Cells bathed in Cs^+ medium were patched in the whole-cell configuration and allowed to equilibrate with pipettes containing either 10 mM MgATP or $MgCl_2$, buffered to pH 6.0 or 6.5, as indicated. After 5 min, the bathing medium was replaced by Na^+ solution (time = 0). (*B*) Effect of pipette pH on the maximal rate of pH_i recovery, estimated from a linear fit of the steepest 30-s interval during the recovery phase. Because the buffering power and H^+ diffusion rates of the open cell/pipette system cannot be reliably estimated, alkalinization rates, rather than transmembrane H^+ fluxes, are graphed. *Inset*: relationship between the pipette pH and the lag time observed between introduction of Na^+ and detection of an alkalinization (e.g., asterisks in *A*). Results are means ± SEM of 3–15 cells.

but also by incoming pipette buffer. It therefore requires larger amounts of H^+ and longer times to detect pH_i changes in perfused cells than in intact ones. This delay is more noticeable at low pH because the buffering power is greater. pH-dependent changes in the diffusional properties of the cells may also contribute to this effect. For instance, the state of actin polymerization and cross-linking is known to increase at lower pH (Yuli and Oplatka, 1987; Edmonds et al., 1995), likely retarding diffusion across the cytosol.

pH Dependence of NHE in Perfused Cells

Despite the variable lag period, the effect of pH_i on the rate of NHE could be compared in ATP-depleted and ATP-replete cells. Consistent with the allosteric activa-

tion of the antiporter by intracellular H$^+$ ions, the pH recovery rates of ATP-containing cells were high at acidic pH$_i$ and were reduced at more alkaline levels, becoming virtually undetectable at values higher than pH 6.75 (Fig. 2 *B*). By comparison, NHE activity was insignificant in the depleted cells down to pH 6.0. If, as proposed earlier, the effect of ATP depletion is merely a shift in the pH dependence of the antiporter (Cassel et al., 1986; Wakabayashi et al., 1992a), the shift in the transfected cells is very large (>0.75 pH U), since the threshold of activation was not reached even at the lowest pH values tested in our experiments. It must be borne in mind, however, that diffusion of acidic solution from the pipette might blunt small NHE responses, shifting the apparent threshold of activation to lower values.

The Antiporter Requires Millimolar Intracellular ATP Concentrations

Since the patch pipette could both deplete and restore intracellular ATP levels without need for metabolic inhibitors, we used this approach to establish the ATP concentration dependence of Na$^+$/H$^+$ exchange. In cells that had not been exposed to the metabolic inhibitors, intracellular perfusion of submillimolar ATP concentrations failed to sustain Na$^+$/H$^+$ exchange, whereas a maximal response was obtained with doses above 10 mM (Fig. 3). A similar dose-response was observed in cells exposed to metabolic inhibitors (not shown).

As the perfused cells likely consume some ATP, conceivably approaching the rate of nucleotide diffusion from the pipette, our assay may overestimate the ATP concentration required for optimal antiport function. To ensure that the concentrations in the pipette truly reflected the prevailing concentration of the nucleotide in the cytosol, we compared the effects of perfusion in the presence and absence of an ATP-regenerating system. For these experiments, an ATP dose near the activation threshold was chosen, so that small increases in the effective concentration would result in sizable changes in activity. The presence of creatine-phosphate (10 mM) and creatine kinase (80 U/ml) did not enhance the response to 1 mM ATP (Fig. 3 *B, open circle*), suggesting that the rate of delivery of ATP through the pipette exceeded the intracellular consumption of the nucleotide. Thus, we believe that the dose-response shown in Fig. 3 *B* accurately reflects the ATP dependence of NHE-1.

ATP Depletion Inhibits the Phospholipid Flippase

Direct phosphorylation of the antiporter had been suggested to account for its ATP dependence (Wakabayashi et al., 1992b). However, most protein kinases have affinities for ATP in the micromolar range. The unusually high ATP dependence of the Na$^+$/H$^+$ exchanger is
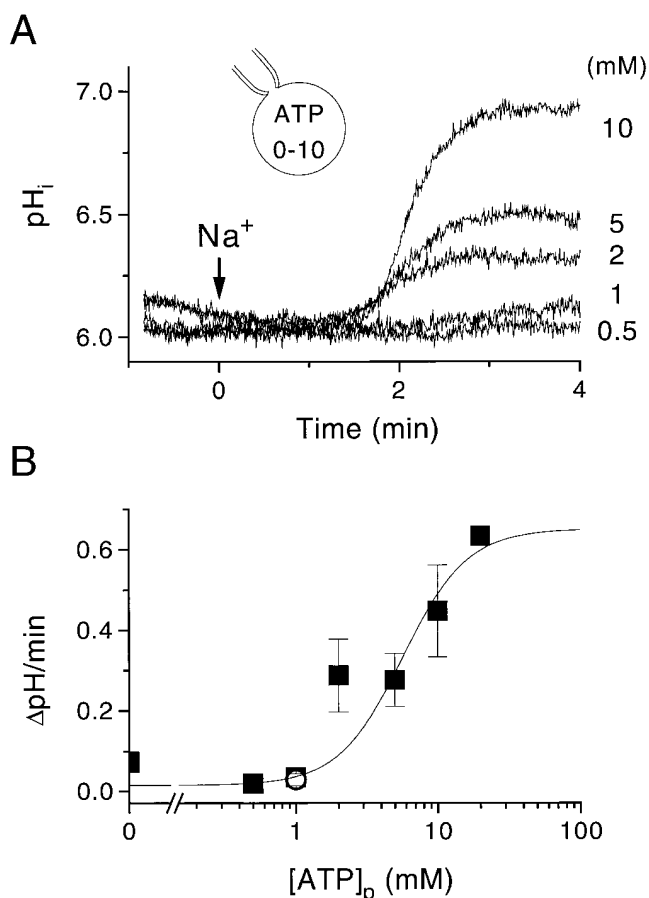

FIGURE 3. ATP concentration dependence. (*A*) Cells were ATP depleted as in Fig. 1 *A* and then patched with pipettes containing varying concentrations of ATP, pH = 6.0. Representative traces showing the effect of increasing pipette ATP concentrations on the Na$^+$-dependent cytosolic alkalinization. (*B*) pH$_i$ recovery rates measured at varying ATP concentrations in the absence (*solid squares*) or presence (*open circle*) of an ATP regenerating system (80 U/ml creatine-kinase + 10 mM creatine phosphate). Maximal rates of alkalinization were measured as in Fig. 2 and are plotted against the pipette ATP concentration. Results are mean ± SE of 3–24 cells. An equation of the form: $y = y_{min} - y_{max}/1 + e^{(x-KD)/dx} + y_{max}$ was fitted to the data (*solid line*), yielding an apparent dissociation constant for ATP, K_D, of 5 mM.

incompatible with this model and instead resembles that reported for the cardiac Na$^+$/Ca^{2+} exchanger (Collins et al., 1992). In the latter case, high concentrations of ATP are thought to be required to support "flipping" of membrane phospholipids (Hilgemann and Collins, 1992). The asymmetric distribution of membrane phospholipids, which is maintained actively by the ATP-dependent "flippase," has been shown to be essential for optimal Na$^+$/Ca^{2+} exchange activity (Hilgemann and Collins, 1992; Collins and Hilgemann, 1993; see INTRODUCTION).

To investigate whether phospholipid flipping underlies the ATP sensitivity of the antiporter, we estimated flippase activity in control and ATP-depleted cells. To

obtain a direct measure of the activity of the enzyme, which translocates lipids from the outer to the inner monolayer, we measured the incorporation of fluorescent PS into the cells. Liposomes containing NBD-labeled PS were added to adherent cells, which were allowed to incorporate the fluorescent lipid. A 10-min incubation at 37°C ensued, during which the lipid was allowed to translocate to the inner monolayer and subsequently to endomembrane compartments. Next, fluorescent lipid bound to the extracellular face of the plasma membrane was removed (back-exchange) by addition of defatted albumin, and the intracellular NBD-PS was finally measured by fluorescence imaging. After a 10-min incubation with 2 µM NBD-PS, bright fluorescence was observed in control cells (Fig. 4 A, left), whereas no signal was discernible in either ATP-depleted or in cells treated with diamide, a known inhibitor of the phospholipid flippase (Hilgemann and Collins, 1992) (Fig. 4 A, middle and right). Quantitation of the fluorescence images confirmed that ATP depletion and diamide inhibited the incorporation of fluorescent phosphatidylserine by 96 and 84%, respectively (Fig. 4 B). Thus, the ATP depletion protocol used for assessment of NHE activity was indeed sufficient to inhibit the plasma membrane phospholipid flippase.

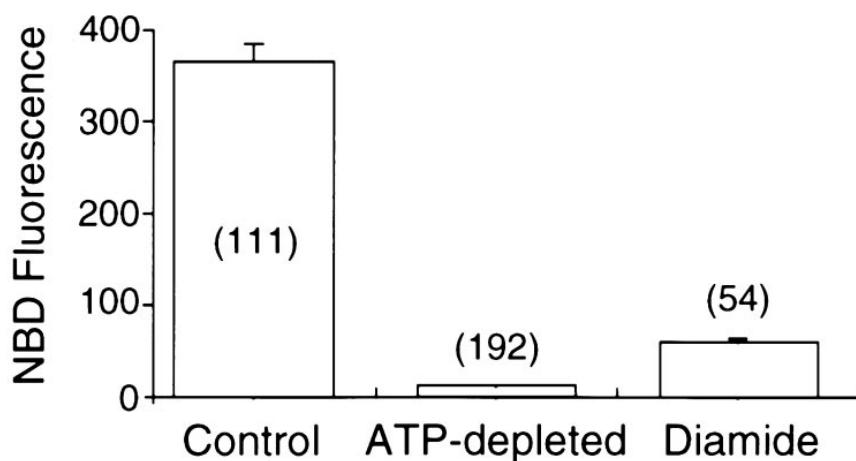If inhibition of the flippase is responsible for the ef-
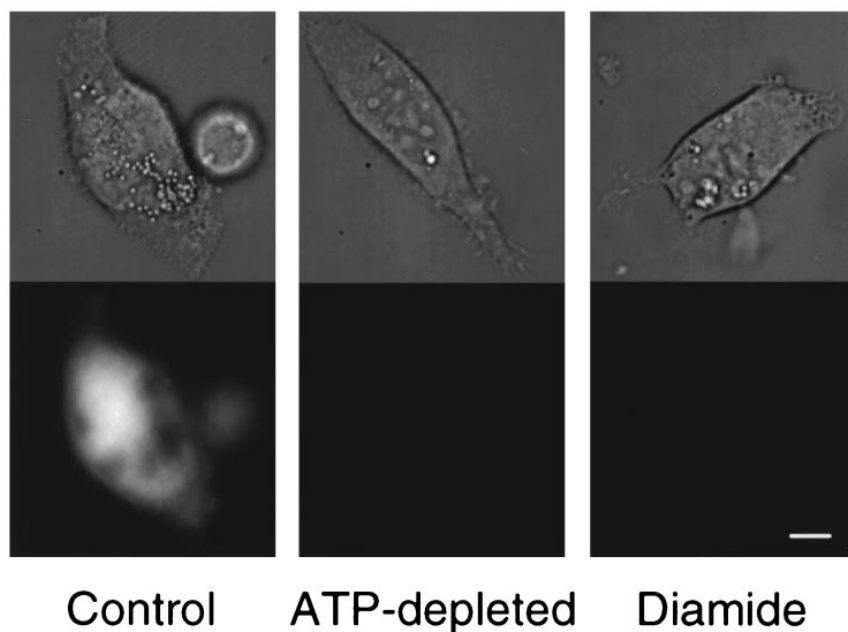
FIGURE 4. ATP depletion inhibits phospholipid "flipping." The internalization of exogenous phospholipids was measured using NBD-phosphatidylserine (NBD-PS). Cells were incubated for 10 min with unilamellar vesicles containing NBD-PS, washed three times at 4°C with defatted BSA to remove fluorescent lipids from the outer leaflet (back-exchange), and imaged at $450_{ex}/542_{em}$ nm. (A) Representative DIC (top row) and NDB-PS fluorescence (bottom row) of control (left), ATP-depleted (middle), and diamide-treated (right) cells. (B) Quantification of cell-associated NBD-PS fluorescence. Results are means ± SE of the fluorescence signal (in 12 bit AD units) of the indicated number of cells. Size bar: 10 µm.
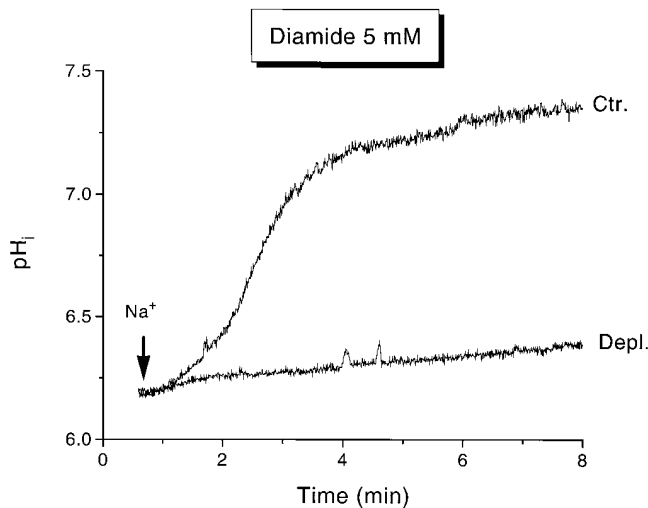
FIGURE 5. Inhibition of phospholipid flippase by diamide does not affect Na^+/H^+ exchange. NHE-1–transfected cells were first either ATP depleted (*Depl*) or left untreated (*Ctr*). Next, both groups of cells were incubated with 5 mM diamide for 10 min at 37°C. Finally, pH_i was measured in Na^+-rich medium after acid-loading by an ammonium pre-pulse. Results are representative of 3 experiments.

FIGURE 6. Na^+/H^+ exchange does not require cytosolic magnesium. Cells were ATP depleted and then dialyzed in the whole-cell configuration with Mg^{2+}-containing (10 mM) or Mg^{2+}-free pipettes (no Mg^{2+}, 2 mM EDTA) at pH 6.5. To ensure complete Mg^{2+} chelation, 5 mM EDTA was added to the external medium. (*A*) Representative pH traces obtained upon Na^+ readdition to Mg^{2+}-containing and Mg^{2+}-depleted cells. The pipette contained 0 or 10 mM ATP, Mg-ATP replaced by K-ATP. (*B*) pH_i recovery rates observed with 2 and 10 mM Mg-ATP or K-ATP, estimated as in Fig. 2, were normalized to the maximal response observed. Results are mean ± SE of the indicated number of cells.

fects of ATP depletion on NHE-1, diamide would be predicted to be an equally effective inhibitor of Na^+/H^+ exchange. In contradiction with this hypothesis, a 10-min incubation with 5 mM diamide, which inhibits the flippase by >80% (Fig. 4 *B*), did not alter Na^+/H^+ exchange (Fig. 5, *top trace*; compare with Fig. 1 *A*). Higher diamide concentrations and/or longer incubation times were similarly ineffective (not shown). More importantly, NHE-1 activity in diamide-treated cells was still sensitive to ATP depletion (Fig. 5, *lower trace*), implying that the step that confers ATP dependence to NHE-1 is insensitive to diamide. Thus, it appears most unlikely that the ATP dependence of NHE-1 is mediated by the phospholipid flippase.

Analysis of the divalent cation dependence of the exchanger also suggests that the flippase does not account for the effects of ATP on NHE. The lipid transferase is characterized by a strict requirement for magnesium (Auland et al., 1994). In contrast, in ATP-depleted cells Na^+/H^+ exchange could be restored if the potassium salt of ATP was used instead of Mg-ATP (Fig. 6, *A* and *B*). These experiments were performed adding 2 mM EDTA to the perfusion pipette, to ensure complete chelation of endogenous (cellular) Mg^{2+}, and 5 mM EDTA to the external medium, to exclude influx and the possibility of submembraneous Mg^{2+} microdomains. As shown in Fig. 6 *C*, the ATP dose-dependence obtained with K-ATP and Mg-ATP was indistinguishable. Together, these findings indicate that the biological process underlying the ATP-sensitivity of NHE-1 is Mg^{2+}-insensitive and is therefore unlikely to be the lipid flippase.
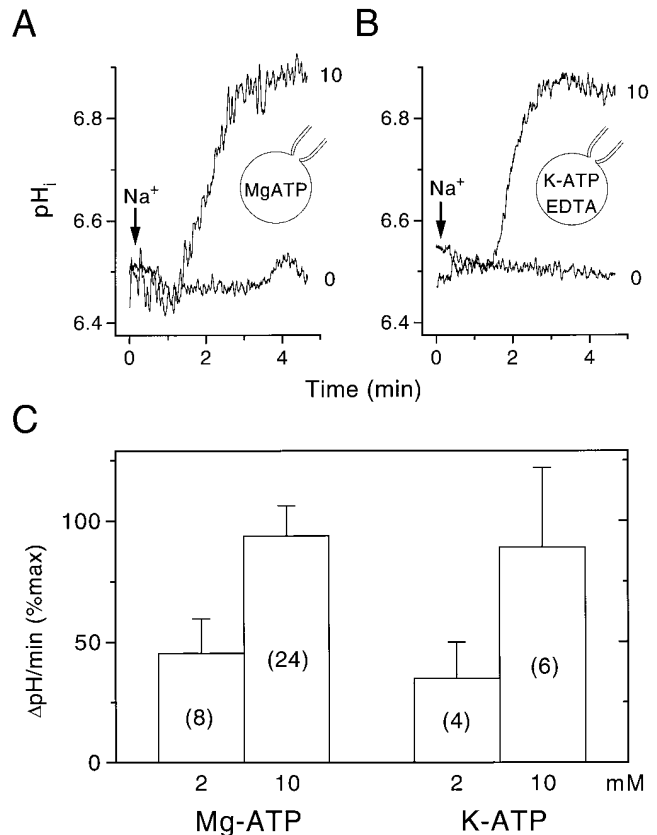
ATP Hydrolysis Is Not Absolutely Required for Stimulation of Na^+/H^+ Exchange

The complex of ATP with Mg^{2+} (or Ca^{2+}) is generally the substrate used by hydrolases. The finding that divalent cation chelation by EDTA did not impair the stimulatory effect of ATP on NHE suggested that hydrolysis of the nucleotide may not be required. This notion was tested by comparing the effects of ATP with those of poorly hydrolyzable or nonhydrolyzable analogues. Cells were ATP depleted, and the ability of different nucleotides to restore Na^+/H^+ exchange was evaluated in internally perfused cells. ATPγS, a poorly-hydrolyzable analogue, was nearly as effective as ATP in sustaining Na^+/H^+ exchange in NHE-1 transfectants at 2 and 10 mM (Fig. 7, *A* and *B*). Truly nonhydrolyzable analogues, such as AMP-PNP and AMP-PCP, partially substituted for ATP. The maximal rate of recovery ob-
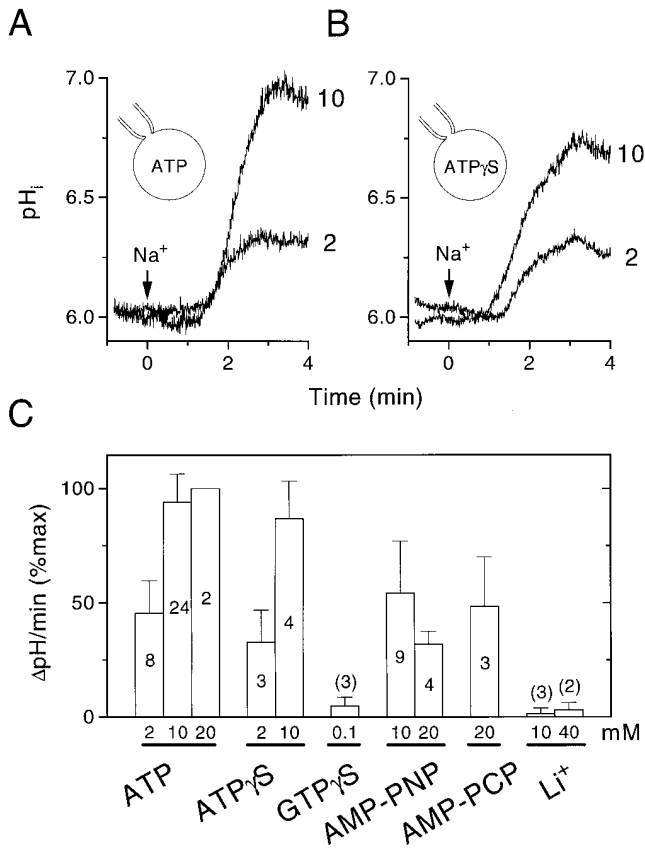
FIGURE 7. Nucleotide selectivity of Na$^+$/H$^+$ exchange. Cells were ATP depleted, perfused internally with various nonhydrolyzable ATP analogues, and used for measurement of pH$_i$ after an acid load, as in Fig. 1 B. (A) Representative traces recorded in cells perfused with 2 or 10 mM ATP (*left*) or ATPγS (*right*) in the patch pipette, pH = 6.0. (B) Summary of results obtained in experiments like those in A, using various nucleotides at the indicated concentrations (in mM). A control using Li$^+$, but without nucleotides, is also shown. Na$^+$-induced rates of pH$_i$ recovery, normalized to the response recorded with 20 mM Mg-ATP, were calculated as in Fig. 2. Results are means ± SE of the indicated number of cells.

tained with 10–20 mM of either AMP-PNP or AMP-PCP averaged ∼50% of the maximal response obtained with ATP (Fig. 7 C). Increasing the concentration of the nonhydrolyzable analogues up to 40 mM had no further effects (not shown). These nucleotides were added as the Li$^+$ salt. Because Li$^+$ has been reported to stimulate antiport activity (Aronson, 1985), we were concerned that the observed activation may be due to the cation, as opposed to the nucleotide. This possibility was ruled out by introduction of an equimolar concentration of Li$^+$ to depleted cells, in the absence of nucleotides. As shown by Fig. 7 C, Li$^+$ alone had not effect on NHE in ATP-depleted cells.

Finally, we considered that the effects of ATPγS or the nonhydrolyzable analogues might have been due to formation of poorly hydrolyzable analogues of GTP, via the nucleoside diphosphokinase (Vu and Wagner,

1993). GTP analogues are effective stimuli of monomeric and heterotrimeric G proteins, which have been in turn shown to alter NHE activity in some circumstances. This notion was tested by addition of 100 μM GTPγS to ATP-depleted cells. This concentration of the analogue effectively stimulates most G proteins, but failed to restore Na$^+$/H$^+$ exchange. These observations suggest that stimulation of G proteins by nonhydrolyzable analogues of GTP, generated by the diphosphokinase, are unlikely to explain the stimulatory effects of ATPγS and the other analogues of ATP. Thus, binding of ATP without hydrolysis may suffice for activation of NHE-1.

DISCUSSION

Physiological ATP levels are required in order for the antiporter to catalyze Na$^+$/H$^+$ exchange. This ATP dependence is shared by all the NHE isoforms identified to date and can be observed both in native tissues and in cells transfected with defined isoforms of the antiporter (for review, see Demaurex and Grinstein, 1994). Though the ATP dependence of NHE has been known for over ten years, the underlying mechanism remains to be defined. There are no conventional nucleotide-binding motifs identifiable in the sequence of any of the NHE isoforms. Structure-function analysis has thus far failed to clarify the mechanism, since results obtained with truncated mutants of NHE-1 and NHE-3 have yielded conflicting results. Progressive deletions of the cytosolic tail abolished the ATP dependence of NHE-1, but not of NHE-3 (Goss et al., 1994; Cabado et al., 1996). Thus, whereas the transmembrane domain is sufficient to confer ATP sensitivity to the NHE-3 isoform, the cytosolic tail appears to play a role in the ATP dependence of NHE-1.

Despite the fact that NHE-1 is constitutively phosphorylated, no changes in the phosphorylation state of this isoform were detected when cells were metabolically depleted (Goss et al., 1994). Based on these findings, it was suggested that direct phosphorylation of the antiporter is not likely to mediate the effects of ATP. In accordance with these findings, we found that analogues of ATP having either poorly hydrolyzable or nonhydrolyzable γ-phosphates substitute at least partially for the native nucleotide on NHE. The presence and contribution of nucleotide diphosphokinase(s) capable of generating small amounts of ATP from other nucleoside cannot be ruled out. However, metabolic depletion is expected to deplete GTP and other triphosphates pari passu with ATP. Moreover, in the patched cells, any remaining nucleoside triphosphates are lost from the cells as the cytosol is dialyzed against the pipette solution. These observations imply that ATP is virtually negligible and argue not only against direct phosphoryla-

tion of the antiporter, but also suggests that protein kinases may not be essential. This conclusion is supported by the finding that NHE was unaffected by complete removal of Mg^{2+}, an essential cofactor of most protein kinases.

The effect of nonhydrolyzable analogues and the independence of Mg^{2+} similarly suggest that lipid kinases or transferases are not responsible for the effects of ATP. Phospholipid transferases (flippases) presented an attractive alternative, inasmuch as the ATP dependence of the Na^+/Ca^{2+} exchanger has been attributed to these enzymes (Hilgemann and Collins, 1992). The possibility that phospholipid flipping accounted for the ATP dependence of NHE, however, was not borne out by our experimental data. Although ATP depletion did reduce flipping (Fig. 4), causing redistribution of membrane phospholipids (as measured by binding of rhodamine-heptalysine to the external face of the membrane; data not shown), extensive inhibition of the flippase with the oxidizing agent diamide did not alter Na^+/H^+ exchange (Fig. 5). These findings indicate that stringent regulation of the lipid composition of the plasma membrane is not essential for adequate NHE-1 function, as massive redistribution of membrane phospholipids occurred upon diamide treatment. More importantly, they rule out the notion that phospholipid flippases are solely responsible for the ATP dependence of Na^+/H^+ exchange.

GTP-binding proteins have also been invoked as possible regulators of antiporter activity (Davis et al., 1992). Depletion of GTP might in principle underlie the ATP dependence of Na^+/H^+ exchange, as the levels of GTP are expected to decline in parallel with ATP during metabolic inhibition. However, we found that GTPγS, when perfused at doses inducing maximal heterotrimeric G-protein activation, did not restore Na^+/H^+ exchange activity in nucleotide-depleted cells (Fig. 7). These findings, however, cannot rule out the participation of small, monomeric GTP-binding proteins, some of which fail to activate their effectors in the presence of GTPγS (Bokoch and Der, 1993).

Dialysis of otherwise untreated cells with solutions devoid of ATP produced inhibition of the exchanger (Figs.1, 2, and 3). Conversely, re-introduction of ATP to depleted cells sufficed to restore transport activity. Two conclusions can be derived from these findings. First, that the decreased transport rate caused by metabolic inhibition was reversible and due to the depletion of ATP, rather than to other, unrelated effects of oligomycin and/or deoxyglucose. Second, that intracellular ATP (or a metabolite thereof) is the only diffusible component required for optimal antiport activity. It is noteworthy that near normal Na^+/H^+ exchange activity was maintained in patch-perfused cells despite extensive dialysis of the cytosol, suggesting that the ATP sensitivity may be an intrinsic property of the antiporter. Certain ion transporters, such as the cystic fibrosis transmembrane regulator, are directly regulated by binding of ATP (Anderson et al., 1991; Welsh et al., 1992). Although consensus ATP-binding motifs such as Walker lysines are not present in the primary structure of NHE-1, the binding of the nucleotide to an as yet unidentified motif on the antiporter cannot be ruled out. Alternatively, the ATP sensitivity of NHE may be conferred by a nondialyzable ancillary component possessing a nucleotide-binding domain.

Whereas the poorly hydrolyzable analogue ATPγS could fully substitute for ATP, analogues where the γ-phosphate is truly nonhydrolyzable, such as AMP-PNP and AMP-PCP, restored exchange activity only partially, even when high concentrations were used (Fig. 7). Thus, although the direct binding of ATP to target effector proteins may be sufficient for detectable antiport function, optimal activity also requires the hydrolysis of ATP. This suggests that two distinct mechanisms may be involved in the activation of the antiporter: one that needs only binding of ATP and another that requires hydrolysis of the nucleotide. As both mechanisms are preserved in cells dialyzed in the whole-cell configuration, they appear to involve nondiffusible cellular structures. ATP may be required to preserve the membrane composition or to stabilize cytoskeletal elements needed to maintain the antiporter in an optimal configuration.

In conclusion, we demonstrated that ATP, at millimolar concentrations, is necessary for adequate activity of the NHE-1 isoform of the antiporter. The antiporter does not require Mg^{2+} as a cofactor and hydrolysis of ATP is not essential. We ruled out that the effect of ATP is mediated by the membrane phospholipid flippase and suggest instead that optimal antiport activation requires both hydrolysis of ATP and direct binding of the nucleotide to an as yet unidentified effector. Regardless of the detailed mechanism of action of the nucleotide, the fact that the antiporter requires millimolar ATP ensures that Na^+/H^+ exchange activity is tightly coupled to the metabolic state of the cell. Such coupling would prevent dissipation of the Na^+ gradient that would result from excess Na^+/H^+ exchange in the absence of functional Na^+/K^+ ATPase. Impairment of Na^+/H^+ exchange would also protect the cells from osmotic swelling, despite the acidification that accompanies metabolic inhibition. Indeed, it has been noted that cellular edema does not occur during ischemia, but only after reperfusion, when ATP levels are restored.

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