DIDS inhibits Na-K-ATPase activity in porcine nonpigmented ciliary epithelial cells by a Src family kinase-dependent mechanism

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Shahidullah M, Wei G, Delamere NA. DIDS inhibits Na-K-ATPase activity in porcine nonpigmented ciliary epithelial cells by a Src family kinase-dependent mechanism. Am J Physiol Cell Physiol 305: C492–C501, 2013. First published May 15, 2013; doi:10.1152/ajpcell.00057.2013.—The anion transport inhibitor DIDS is known to reduce aqueous humor secretion but questions remain about anion dependence of the effect. In some tissues, DIDS is reported to cause Na-K-ATPase inhibition. Here, we report on the ability of DIDS to inhibit Na-K-ATPase activity in nonpigmented ciliary epithelium (NPE) and investigate the underlying mechanism. Porcine NPE cells were cultured to confluence on permeable supports, treated with drugs added to both sides of the membrane, and then used for 86Rb uptake measurements or homogenized to measure Na-K-ATPase activity or to detect protein phosphorylation. DIDS inhibited ouabain-sensitive 86Rb uptake, activated Src family kinase (SFK), and caused a reduction of Na-K-ATPase activity. PP2, an SFK inhibitor, prevented the DIDS responses. In BCECF-loaded NPE, DIDS was found to reduce cytoplasmic pH (pHc), PP2-sensitive Na-K-ATPase activity inhibition, 86Rb uptake suppression, and SFK activation were observed when a similar reduction of pH was imposed by low-pH medium or an ammonium chloride withdrawal maneuver. PP2 and the ERK inhibitor U0126 prevented robust ERK1/2 activation in cells exposed to DIDS or subjected to pH reduction, but U0126 did not prevent SFK activation or the Na-K-ATPase activity response. The evidence points to an inhibitory influence of DIDS on NPE Na-K-ATPase activity by a mechanism that hinges on SFK activation associated with a reduction of cytoplasmic pH.

CONTROLLING ELEVATED INTRAOCULAR PRESSURE (IOP) is currently the only remedy available to prevent or delay vision loss and retinal ganglion cell death in persons with glaucoma. Because reduction of aqueous humor (AH) secretion is an effective strategy used to lower IOP, there is interest in the process of AH formation. AH is secreted through translocation of solutes and water across the ciliary body epithelium (CE), a bilayer formed by two cells, the pigmented (PE) and nonpigmented (NPE) epithelium. The two epithelial layers contact each other at their apical surfaces where there are numerous gap junctions. The PE basolateral surface contacts the stroma of the ciliary process and the basolateral surface of the NPE contacts the AH that fills the posterior chamber of the eye. Solutes and water are taken up by the PE from the stromal fluid, pass through the gap junction to NPE, and then enter the posterior chamber. Anion transport, particularly Cl− and HCO3−, across the ciliary epithelium to the posterior chamber plays a major role in AH secretion (4, 7–9, 34, 40). The NPE constitutes the exit point for solute to enter the posterior chamber, and anion efflux from NPE appears crucial for AH secretion in many species including rabbit (8), bovine (10, 11), and porcine (4). Furthermore, in the monkey ciliary epithelium, the anion transport inhibitor 4,4-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) affects the short-circuit current (Isc) only when it is applied to the aqueous/NPE side but not the stromal/PE side (5).

DIDS, a stilbene compound, has been used as an anion-transport inhibitor for decades. Previously, we reported that DIDS reduced AH secretion in the isolated arterially perfused bovine (34) and in the porcine eye (31). DIDS is a nonselective anion transport inhibitor and has been shown to inhibit several transporters and channels in the CE, such as the Cl−/HCO3− exchanger (40), Na-HCO3 cotransporter (NBC) (6), and chloride channels (26). However, some aspects of the DIDS effect on the CE are puzzling. For example, although it has been postulated to act on the Cl−/HCO3− exchanger at the PE cells (25), it affects the Isc of monkey CE only when it is applied to the aqueous/NPE side but not the stromal/PE side (5). DIDS decreases the Isc in isolated CE preparations of both rabbit (8) and ox (12), but it has no inhibitory effect on the chloride secretion across the isolated bovine CE preparation (12). Despite this, stromal perfusion of DIDS inhibited AH secretion in the bovine eye by 56% (34). Thus, the mechanism of action of DIDS on CE may involve a nonchloride pathway that contributes to AH secretion. Interestingly, DIDS and another stilbene derivative, 4-acetamido-4'-isothiocyanato-2,2'-stilbenedisulfonic acid disodium salt hydrate (SITS), are reported to have a potent inhibitory effect on Na-K-ATPase activity (14, 38). Na-K-ATPase is the primary active transporter that establishes the ion gradients that drive AH formation. In the intact eye, Na-K-ATPase inhibition by ouabain reduces AH secretion by ~62% (34). Na-K-ATPase is localized to the basolateral surface of both layers, but expression is considerably more abundant in the NPE than the PE (15). Recently, we have shown rich expression of all three α-isoforms of Na-K-ATPase (32), as well as anion transporters NBC and Cl−/HCO3− exchanger (33) in the porcine NPE. Here we report evidence that suggests an inhibitory influence of DIDS on NPE Na-K-ATPase activity by a mechanism that hinges upon Src family kinase (SFK) activation associated with a reduction of cytoplasmic pH.1

MATERIALS AND METHODS

Cells and reagents. Porcine eyes purchased from the University of Arizona Meat Science Laboratory and Hatfield Quality Meats (Philadelphia, PA) were delivered overnight on ice. The use of porcine tissue was approved by the University of Arizona Institutional Animal Care and Use Committee and conformed to the ARVO Resolution for the Use of Animals in Ophthalmic and Vision Research. Porcine NPE

1 This article is the topic of an Editorial Focus by Mortimer M. Civan (7a).
was established in primary culture as described earlier (32) and grown in HEPES-buffered DMEM containing 10% fetal bovine serum. Before use, the cell monolayers were serum starved for 3 h and the medium was then replaced with Krebs solution that contained (in mM) 119 NaCl, 4.7 KCl, 1.2 KH2PO4, 25 NaHCO3, 2.5 CaCl2, 1 MgCl2, and 5.5 glucose, equilibrated with 5% CO2 and adjusted to pH 7.4. Experiments were carried out in Krebs solution at 37°C in a humidified incubator, saturated with 95% air and 5% CO2.

HEPES-buffered DMEM, fetal bovine serum, and newborn calf serum were purchased from Invitrogen (Carlsbad, CA). 1,4-Diaminobenzene-2,3-dicyano-1,4-bis-[2-aminophenylthio]butadiene (U0126), dimethyl sulfoxide (DMSO), DIDS, SITS, and other chemicals used to prepare the Krebs solution were purchased from Sigma (St. Louis, MO). Alamethicin and 4-amino-3-(4-chlorophenyl)-1-(t-butyl)-1H-pyrazolo[3,4-d]pyrimidine (Minneapolis, MN). Rabbit polyclonal anti-Na-K-ATPase α1-antibody was purchased from Sigma. Rabbit monoclonal anti p44/42 MAPK (Thr202/Tyr204) antibody, rabbit polyclonal anti-phospho-ERK1/2 (Ser16)-antibody, rabbit polyclonal anti-phospho-Src-family (Tyr416) antibody, and rabbit polyclonal anti-β-actin antibody was obtained from Cell Signaling Technology (Danvers, MA). Mouse monoclonal anti-β-actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit or anti-mouse IRDye 800 conjugated and goat anti-mouse or anti-rabbit IRDye 680 conjugated secondary antibodies were purchased from Rockland Immunochemicals (Gilbertsville, PA).

Sample preparation and Na-K-ATPase activity assay. NPE cells were grown to confluence on 24-mm polyester-permeable culture inserts with a 0.4-µm pore size (Corning). The cells were preincubated with Krebs solution for 1 h and the Krebs solution was then changed, exposing both surfaces of the culture insert to test compounds. Following treatment, the membrane with the cell monolayer was removed from each culture insert using a custom-made stainless steel cutter. Cells from two inserts were pooled as one sample for the Na-K-ATPase assay. The two inserts were arranged into a sandwich with the cells facing inside, and the membrane sandwich was then cut into small pieces, placed in a 2.0-ml Eppendorf tube, frozen in liquid nitrogen, and stored at −80°C until the Na-K-ATPase assay.

The culture insert membranes, which are brittle when frozen, were pulverized using a glass pestle that fits snugly in the 2.0 ml Eppendorf tube. The samples were subjected to two cycles of freezing in liquid nitrogen and pulverization, then 300 µl of ice cold 2× strength ATPase assay buffer was added. Assay buffer composition was (in mM) 80 l-histidine, 200 NaCl, 10 KCl, 6.0 MgCl2, 2.0 EGTA (pH 7.4) and a protease inhibitor mixture (1 tablet for each 7 ml of the ATPase buffer, Roche Applied Science, Mannheim, Germany). The mixture was homogenized for 1 min (4 strokes of 15 s at 5-s intervals) using Misonix S3000 sonicator at a 6W power setting (Misonix). The homogenate was subjected to centrifugation at 13,000 x g for 30 min at 4°C to remove the polyester membrane fragments, cell nuclei, and larger mitochondria. Protein in the supernatant was measured by bicinchoninic acid (BCA) assay (35) (Pierce Biotechnology, Rockford, IL), using bovine serum albumin as a standard. The supernatant was used to measure Na-K-ATPase activity.

Na-K-ATPase activity was measured according to our previously described method (30). Samples obtained from treated or control cells (80 µl) were placed in glass assay tubes, and an additional 120 µl of 2× strength assay buffer was added to each tube. To improve access of ions and ATP to membrane vesicles, alamethicin solution in ethanol (5 µl) was added to give a final approximate concentration of 0.1 µg of alamethicin per mg protein (41). Half the tubes received ouabain, a highly specific Na-K-ATPase inhibitor (3) (final concentration 300 µM), and the remaining tubes received an equivalent volume (5 µl) of distilled water. An additional 150 µl of distilled water was added to each tube. The tubes were preincubated at 37°C for 5 min, and ATP stock solution (40 µl) was then added to each tube (final ATP concentration 2 mM), bringing the total assay mixture volume to 400 µl, diluting the 2× strength Na-K-ATPase buffer to single strength. After 30 min of incubation at 37°C in the dark, the ATP hydrolysis reaction was stopped by the addition of 150 µl of 15% ice-cold trichloroacetic acid (TCA) and placing the tubes on ice for 20 min with occasional shaking.

ATP hydrolysis was determined by measuring the amount of inorganic phosphate released in each reaction tube. To detect inorganic phosphate, each tube was placed in a centrifuge at 3,000 rpm (2,680 g) for 15 min at 4°C, then 400 µl of the supernatant was removed, placed in premarked glass tubes, and mixed with 400 µl of 4.0% FeSO4 solution in ammonium molybdate (1.25 g of ammonium molybdate in 100 ml of 2.5 N sulfuric acid). Standard solutions containing NaH2PO4 equivalent to 0, 10, 62.5, 125, 250, and 500 nmol PO4 were treated similarly. After 5 min at room temperature the tubes with the samples (not the standards) were placed in a centrifuge at 3,000 rpm (2,680 g) for 10 min to pellet additional precipitates. A 250-µl aliquot of each standard or sample was then transferred to each well of a 96-well plate, and the absorbance was measured at 750 nm in a Perkin Elmer plate reader (Victor V3, Perkin Elmer). Na-K-ATPase activity was calculated as the difference between ATP hydrolysis in the presence and in the absence of ouabain. Values are presented as nmol ATP hydrolyzed per milligram protein per 30 min. Because Na-K-ATPase activity was variable between batches of cells, data for different experiments were not pooled.

Measurement of 86Rb uptake. NPE monolayers grown to confluence on 24-mm permeable culture inserts were preincubated in Krebs solution for 1 h at 37°C. Then the Krebs solution was changed and the cells were incubated in the presence or absence of test compounds added to both sides of the culture insert for a specified time (10 min in the case of DIDS) before the addition of 86RbCl (1 µCi/ml) for 5 min. The total concentration of radioactive and nonradioactive Rb was 0.01 mM in the 86RbCl-containing Krebs solution. Half the samples received ouabain (500 µM) along with the 86RbCl. Uptake of 86Rb was stopped by removing the culture inserts and washing them three times by quick submersion in ice-cold Krebs solution. The cell
monolayer and permeable membrane were cut from each insert and placed in 7 ml of scintillation fluid in a scintillation vial. Radioactivity was measured in a scintillation counter. Results are expressed as cpm/insert and Na-K-ATPase-mediated 86Rb is the difference between uptake in the presence and absence of ouabain.

**Western blot analysis.**

NPE monolayers cultured to confluence on 60-mm dishes were preincubated in control Krebs solution and were then exposed to Krebs solution containing specified test compounds. After a specified time the incubation medium was removed and the cells were lysed in 200 μl RIPA buffer containing (in mM) 50 HEPES, 150 NaCl, 1.0 EDTA, 10 sodium pyrophosphate, 2.0 sodium orthovanadate, 10 sodium fluoride, and 1 phenylmethylsulfonyl fluoride (PMSF) with 10% glycerol, 1.0% Triton X-100, 1.0% sodium deoxycholate, and Complete Mini Protease Inhibitor Cocktail tablets (3 tabs/20 ml; Roche Diagnostics, Indianapolis, IN). Cell lysate from each dish was collected in premarked Eppendorf tubes and homogenized as described above. The homogenate was centrifuged at 14,000 g for 30 min, the supernatant was collected, and protein concentration was measured with a BCA protein assay kit (Pierce). The supernatant was mixed with Laemmli buffer, and the proteins were separated by electrophoresis on a 7.5% SDS-polyacrylamide minigel. Proteins were then transferred by electrophoresis to nitrocellulose membrane which was blocked overnight with a blocking buffer (East Coast Bio). The nitrocellulose membranes were incubated overnight with a blocking buffer (East Coast Bio).

**Fig. 2.** The influence of PP2 on Na-K-ATPase activity in cells exposed to DIDS. Na-K-ATPase activity (ouabain-sensitive ATP hydrolysis) was measured in samples obtained by homogenizing cells that had been preincubated with PP2 (10 μM) for 40 min before being exposed to DIDS (100 μM) for 10 min in the continued presence of PP2. Values are means ± SE of results from 4–6 samples, each pooled from 2 cell monolayers. *****P < 0.001, significant difference from control.**

**Fig. 3.** Comparison of Na-K-ATPase activity inhibition elicited by DIDS and SITS. Na-K-ATPase activity (ouabain-sensitive ATP hydrolysis) was measured in samples obtained by homogenizing cells that had been exposed to DIDS (100 μM) or SITS (100 μM) for 10 min. Values are means ± SE of results from 4–6 samples, each pooled from 2 cell monolayers. *P < 0.05, significant difference from control.

**Fig. 4.** Src family kinase (SFK) phosphorylation detected by Western blot analysis in nonpigmented ciliary epithelial (NPE) cells exposed to DIDS (100 μM). A: a typical time course study. B: effect of PP2 on DIDS-induced SFK phosphorylation. Cells were preincubated with PP2 (10 μM) for 40 min before being exposed to DIDS (100 μM) for 10 min in the continued presence of PP2. In each panel, a typical Western blot is presented together with a bar graph that shows band density results pooled from 3 independent experiments (means ± SE). *P < 0.05, significant difference from control.
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the following primary antibodies: rabbit polyclonal anti-Na-K-ATPase ε4 (1:450), rabbit monoclonal anti-p44/42 MAP kinase (1:1,000), mouse monoclonal anti-phospho-p44/42 MAPK (Thr202/Tyr204) (1:2,000), rabbit polyclonal anti-phospho Tyr416 Src family kinase (1:1,000), rabbit polyclonal anti-β-actin (1:3,000), mouse monoclonal anti-β-actin (1:10,000), and mouse monoclonal anti-p38 MAPK (1:1,000). All antibodies were diluted in the blocking buffer. After three washes in 30 mM Tris, 150 mM NaCl, and 0.5% (vol/vol) Tween 20 (TTBS) at pH 7.4, each nitrocellulose membrane was incubated for 1 h with an appropriate secondary antibody conjugated with IRDye 800 or 680; IRDye goat anti-mouse or anti-rabbit secondary antibody (1:20,000) or IRDye 680 goat anti-rabbit or goat anti-mouse secondary antibody (1:20,000) (Licor Biosciences). Protein bands were visualized and band density was quantified by infrared laser scan detection (LI-COR Odyssey). By using secondary antibodies at two different infrared wavelengths, two proteins were quantified simultaneously, permitting calculation of a band density ratio.

**Measurement of cytoplasmic pH.** Cytoplasmic pH was measured by imaging microscopy using the pH-sensitive dye 2.7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF). Cells were loaded with the acetoxymethylester (BCECF-AM). NPE cells grown to semiconfluence on a 35-mm plastic dish (Corning) were incubated at 37°C for 10 min with BCECF-AM (5.0 µM) as described earlier (31). Then the cells were washed five times with Krebs solution and incubated for another 10 min in Krebs solution to allow deesterification of the dye. Deesterification transforms BCECF-AM (ester form) to membrane-impermeable BCECF (acid form), which is trapped in the cytoplasm. The cells were then washed again several times to remove any traces of external dye. The dish containing the cells was then placed in a temperature-controlled perfusion microincubator (PDMI-2, Harvard Biosciences, Holliston, MA) on the stage of an upright epifluorescence microscope (Nikon Eclipse) where the preparation was superfused (2.0 ml/min) with a Krebs buffer. The inflow and outflow rate was controlled using peristaltic pumps (Watson-Marlow, 520S, Cornwall, TR11 4RU, UK). The microscope was fitted with a high-resolution video camera (DVC 340M-00-CL) to continuously monitor and record the BCECF fluorescence intensity in the cells. Fluorescence intensity was measured at an emission wavelength of 535 nm using alternating excitation wavelengths of 488 nm and 460 nm programmed by an InCyt Im2 imaging system (Intracellular Imaging, Cincinnati, OH). The fluorescence intensity ratio I488/I460 was calibrated by titrating BCECF-free acid with a range of buffers with defined pH values (5.49 to 8.5). Calibration was done in vitro using the calibration chamber supplied by the manufacturer (Intracellular Imaging). The camera and the microscope settings for the calibration and for conducting experiments were the same.

**Fig. 5.** The effect of DIDS and low-pH medium on cytoplasmic pH (pHi). Cells were loaded with the pH-sensitive dye BCECF, washed, and continuously superfused with normal medium (pH 7.4) while baseline intracellular pH was recorded for 3 min. Then, the superfusate was replaced with one containing 100 µM DIDS or with low-pH medium (pH 6.5 and 6.0) and recording was continued for 20 min. A–C: typical pH responses to DIDS (A), low-pH medium 6.5 (B), and low-pH medium 6.0 (C). D and E: bar graphs of cytoplasmic pH at 5, 10, 15, and 20 min after exposure to DIDS (D) and low-pH medium (E). Results are shown as means ± SE of 5–7 individual experiments. *P < 0.05, **P < 0.01, and ***P < 0.001, significant difference from control for DIDS and low-pH medium 6.5; ###P < 0.01 and ####P < 0.001, significant difference from control for low-pH medium 6.0.
the Bonferroni post hoc multiple-comparison test. Statistical analysis. The results are expressed as means ± SE, and comparison was made by one-way analysis of variance followed by the Bonferroni post hoc multiple-comparison test.

RESULTS

The stilbene derivative DIDS (4,4′-diisothiocyanato-2,2′-disulfonic stilbene, 100 µM) reduced the rate of ouabain-sensitive 86Rb uptake by ~70%. In the presence of 10 µM PP2, the effect of DIDS on ouabain-sensitive 86Rb uptake was abolished (Fig. 1). Added alone, PP2 did not significantly alter the rate of ouabain-sensitive 86Rb uptake.

In separate experiments, intact cells were exposed to either DIDS or SITS for 10 min; the cells were then homogenized and used for measurements of ouabain-sensitive ATP hydrolysis (Na-K-ATPase activity). The rate of ouabain-sensitive ATP hydrolysis was reduced by ~40% in samples obtained from cells that had been exposed to DIDS (Fig. 2). DIDS-induced reduction of Na-K-ATPase activity was abolished by PP2 (10 µM; Fig. 2). The magnitude of Na-K-ATPase activity inhibition observed in cells exposed to DIDS for 10 min was similar to the Na-K-ATPase activity inhibition in cells exposed to a different stilbene derivative, SITS (100 µM; Fig. 3).

The detection of reduced ouabain-sensitive ATP hydrolysis in homogenized material obtained from cells exposed to DIDS points to an intrinsic change of Na-K-ATPase activity that is maintained following homogenization, centrifugation, and freezing. Evidence of SFK activation was observed in DIDS-treated cells (Fig. 4A). Increased SFK phosphorylation at Y416, a modification that signifies SFK activation, was significant in cells that had been exposed to DIDS for 10 min. The increase in SFK phosphorylation was not observed in protein samples obtained from cells that were exposed to DIDS in the presence of 10 µM PP2 (Fig. 4B). The principal phospho-SFK band appeared at ~60 kDa, but faint phospho-SFK bands at lower molecular weights were also observed.

DIDS is a recognized inhibitor of anion transport, and in some cells, DIDS exposure has been reported to alter cytoplasmic pH. Since lowering cytoplasmic pH has been shown to cause SFK activation, we conducted studies to test whether DIDS lowers pH in BCECF-loaded cells. This was the case. DIDS (100 µM) reduced cytoplasmic pH (Fig. 5). A cytoplasmic pH reduction of comparable magnitude was observed in cells exposed to acidic extracellular medium (pH 6.5 and 6.0; Fig. 5). Interestingly, exposing intact cells to low-pH medium (pH 6.5) also caused a decrease in the rate of ouabain-sensitive 86Rb uptake (Fig. 6) and PP2 eliminated the 86Rb uptake response.

In the continued presence of PP2 (10 µM) for 40 min before being exposed to low-pH medium (pH 6.5) also caused a decrease in the rate of ouabain-sensitive 86Rb uptake (Fig. 6) and PP2 eliminated the 86Rb uptake response. Moreover, significant inhibition of ouabain-sensitive ATP hydrolysis (~50%) was observed in protein samples obtained from cells that had previously been subjected to low-pH medium for 5 min (Fig. 7). In contrast, ouabain-sensitive ATP hydrolysis was unchanged in cells that had been pretreated with PP2 (10 µM) and then exposed to low-pH medium in the presence of PP2 (Fig. 7). An increase in SFK phosphorylation at Y416 was observed in cells exposed to low-pH medium (Fig. 8A). The ability of PP2 to prevent the Na-K-ATPase activity response to low-pH medium was consistent with its ability to prevent an increase in SFK phosphorylation (Fig. 8B). In a different set of experiments, cytoplasmic pH was lowered by a different maneuver. Cells were exposed to 20 mM ammonium chloride for 5 min and then subjected to ammonium chloride withdrawal in sodium-free Krebs solution.

It has been established earlier that the inability of Na-dependent transporters to operate under sodium-free conditions prevents pH recovery from ammonium chloride exposure-induced acid load, causing sustained cytoplasmic acidification. Typical traces of pH responses in normal and in Na+-free Krebs solution are shown in Fig. 9A. An increase in SFK phosphorylation was observed in cells examined 1.0 min after ammonium chloride withdrawal and replacement with Na+-free Krebs (Fig. 9B).
DIDS caused an increase in ERK1/2 phosphorylation (Fig. 10A) that could be prevented entirely by U0126, a recognized ERK1/2 inhibitor (Fig. 10B). Importantly, DIDS-induced ERK1/2 phosphorylation also was abolished when cells were exposed to DIDS in the presence of the SFK inhibitor PP2 (Fig. 10C). This suggests that SFK phosphorylation may be a required step for ERK1/2 activation. Low-pH medium similarly induced robust ERK1/2 phosphorylation that could be prevented by PP2 (Fig. 11) and U0126 (data not shown). ERK1/2 activation has been reported to lead to reduction of Na-K-ATPase activity in other tissues (18, 39). For this reason, Na-K-ATPase activity was measured in cells that had been exposed to DIDS in the presence of UO126. Although U0126 did not prevent the Na-K-ATPase response to DIDS (Fig. 12).

**DISCUSSION**

DIDS and other disulfonic stilbenes, such as SITS, have been widely used as inhibitors of anion transporters (3, 28). They also have been recognized as inhibitors of Na-K-ATPase activity in preparations such as microsomal material obtained from turtle bladder and electric eel organ. In regard to Na-K-ATPase inhibition, DIDS and SITS were considered to be ineffective when applied to the exterior of intact cells because they do not readily penetrate the plasma membrane. This reasoning was based on the notion that their principle mechanism of action was a direct inhibitory effect caused by interaction with a Lys residue on the cytoplasmic portion of P-type ATPases (27, 38). In the present study we show evidence that DIDS is able to cause Na-K-ATPase inhibition in ocular nonpigmented ciliary epithelium (NPE) by a mechanism that is associated with SFK activation.

DIDS and SITS caused marked inhibition of ouabain-sensitive ⁸⁶Rb uptake by intact cells, and the SFK inhibitor PP2 prevented the response. Moreover, diminished Na-K-ATPase activity, along with evidence of SFK phosphorylation at Y416, was observed in homogenates obtained from intact cells that had been exposed to DIDS for 5 min or more, and PP2 prevented both responses. SFK phosphorylation at Y416 has been widely recognized to indicate SFK activation (29, 37). The findings are consistent with a response that involves activation of SFK and SFK-dependent inhibition of Na-K-ATPase.

It should be noted that the magnitude of inhibition caused by DIDS was different for ouabain-sensitive ⁸⁶Rb uptake and ouabain-sensitive ATP hydrolysis. It is difficult to make a direct comparison of the magnitude of the DIDS inhibitory effect in the two experiments. Although they both relate to the same mechanism, ⁸⁶Rb uptake and Na-K-ATPase activity measurements are different in important respects. Ouabain-sensi-
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The DIDS solution, subjected to homogenization, centrifuged, and then assayed at a later time for Na-K-ATPase activity under \( V_{\text{max}} \) conditions.

DIDS caused cytoplasmic acidification. To examine this aspect of the response, we subjected cells to cytoplasmic acidification caused by low-pH medium. This also elicited inhibition of Na-K-ATPase activity that could be prevented by the SFK inhibitor PP2. SFK phosphorylation at Y416 was evident in cells that had been subjected to cytoplasmic acidification either by exposure to low-pH medium or by ammonium chloride acid load (the addition then subsequent removal of extracellular ammonium chloride). Importantly, the SFK inhibitor PP2, which suppressed SFK phosphorylation, was found to prevent the inhibition of ouabain-sensitive \(^{86}\text{Rb}\) uptake and Na-K-ATPase activity caused by exposure to low-pH medium. Taken together, the findings are consistent with the notion that lowering cytoplasmic pH is associated with SFK activation that leads to Na-K-ATPase inhibition. Reduction of cytoplasmic pH in a proximal renal tubule cell line has previously been shown to activate Src (42). Indeed, Src activation by intracellular acidification appears to be an important mechanism in modulating a number of cellular functions. For example, Src activation triggers Pyk2, a member of the focal adhesion kinase (FAK) family of tyrosine kinases (21), has been demonstrated as a required step in the activation of the sodium/hydrogen exchanger NHE3 in kidney tubule cells when exposed to acidic medium (22).

The cytoplasmic acidification observed upon DIDS exposure likely stems from the fact that DIDS is a potent inhibitor of anion transport (3, 28). However, DIDS also has been shown to inhibit carbonic anhydrase (CA) (13), and the cytoplasmic pH fall could be the combined result of inhibition of anion transporters and CA. Inhibition of anion transporters, and membrane-bound carbonic anhydrase (CA4), will interfere with base (\( \text{HCO}_3^-/\text{CO}_2^- \)) transport across the cell membrane and thus has the potential to lower cytoplasmic pH.

Previous studies have linked inhibition of Na-K-ATPase activity to tyrosine phosphorylation. In rabbit NPE, dopamine agonists were found to cause inhibition of ouabain-sensitive \(^{86}\text{Rb}\) uptake in a response that could be abolished by genistein, a nonselective tyrosine kinase inhibitor. SFK itself is activated by tyrosine phosphorylation, at residue 416, and in the intact porcine lens, stimulation of ET receptors by endothelin-1 was reported to cause reduction of Na-K-ATPase activity by a mechanism that could be prevented by PP2, the selective inhibitor of SFK activation (24). The role of SFK activation is complex and still poorly understood because it also can trigger Na-K-ATPase stimulation. SFK activation that occurs following purinergic receptor activation in lens epithelium was linked to an increase in Na-K-ATPase activity (36). How SFK can be linked to either inhibition or activation of Na-K-ATPase, depending on the stimulus, is still under investigation and falls beyond the scope of the present study. There are nine tyrosine kinases in the SFK family and the appearance of faint phospho-SFK bands in addition to the principal ~60 kDa band in DIDS-treated cells suggests that more than one SFK might be activated. It is possible that different patterns of SFK activation could elicit different downstream responses. The key finding in this study is that DIDS-mediated Na-K-ATPase inhibition depends on SFK activation. The evidence is consistent with a mechanism that hinges on SFK activation associated with a reduction of cytoplasmic pH.
It is noteworthy that cells exposed to either DIDS or low-pH medium displayed significant ERK1/2 phosphorylation. The ability of the SFK inhibitor PP2 to prevent such ERK1/2 phosphorylation indicates that ERK1/2 activation under these conditions is SFK dependent. However, DIDS-induced ERK1/2 activation did not play an obvious role in modulating Na-K-ATPase activity since the selective ERK1/2 inhibitor U0126 prevented ERK1/2 phosphorylation but failed to prevent DIDS-induced reduction of Na-K-ATPase activity. These findings are in contrast to reports that ERK1/2 activation leads to a reduction of Na-K-ATPase activity in opossum kidney cells and lung alveolar epithelium (18, 39) and causes Ser phosphorylation of the Na-K-ATPase α1-subunit in vitro (2), and internalization of Na-K-ATPase α1-polypeptide (19). The downstream consequences of ERK1/2 activation in DIDS-treated NPE cells remain to be determined, but SFK activation is clearly an early step in this part of the DIDS response as well as the Na-K-ATPase inhibition response.

While the findings in this study point to SFK activation and ERK1/2 activation associated with a reduction of cytoplasmic pH, DIDS and low-pH medium caused sustained lowering of cytoplasmic pH but transient SFK activation and ERK1/2 activation. Transient (terminated rapidly within minutes) activation has been reported widely for SFK and ERK1/2 in a variety of cells subjected to a range of stimuli. For example, geldamycin transiently activates Src in human embryonic kidney cells as well as in several cancer cells, including T24 bladder cancer, MCF7 breast cancer, and PC-3 prostate cancer cells (20). Additionally, rapid and transient activation of ERK1/2 in response to macrophage migration inhibitory factor has been reported to be dependent on Src tyrosine kinase activation (23). However, there also are reports of sustained
SFK (17) and ERK1/2 (16) activation. The physiological significance of transient versus sustained activation of SFK and ERK1/2 signaling pathways is a topic for further study. Temporal regulation is an important feature of cellular signaling and depends on the stimulus, the specific receptors activated, as well as the presence of diverse additional modulators (1). Transient activation is a characteristic feature of other signaling pathways. For instance, calcium signaling commonly adheres to a pattern where an agonist-induced rise of cytoplasm calcium is followed quickly by a reduction in calcium concentration even though the cell remains exposed to the agonist.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

M.S. and N.A.D. conception and design of research; M.S. and G.W. performed experiments; M.S. analyzed data; M.S. interpreted results of experiments; M.S. prepared figures; M.S. drafted manuscript; M.S. and N.A.D. edited and revised manuscript; M.S. and N.A.D. approved final version of manuscript.

REFERENCES


REMARKS

Figs. 11 and 12: The influence of U0126 on Na-K-ATPase activity in cells exposed to DIDS. Na-K-ATPase activity (ouabain-sensitive ATP hydrolysis) was measured in samples obtained by homogenizing cells that had been preincubated with U0126 (10 µM) for 20 min before being exposed to DIDS (100 µM) for 10 min in the continued presence of PP2. Values are means ± SE of results from 4–6 samples, each pooled from 2 cell monolayers. ***P < 0.001, significant difference from control; ##P < 0.01, significant difference from U0126 treatment.

Fig. 11. The influence of PP2 on ERK1/2 phosphorylation in cells exposed to low-pH medium. Cells were preincubated with PP2 (10 µM) for 40 min before being exposed to low-pH medium (pH 6.5) for 5 min in the continued presence of PP2. A typical Western blot result is shown together with a bar graph of pooled band density from 3 independent experiments (means ± SE). *P < 0.05, significant difference from control.


