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Inhibition of the Na/Bicarbonate Cotransporter NBCe1-A by diBAC Oxonol Dyes Relative to Niflumic Acid and a Stilbene

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Abstract Na/HCO_3 cotransporters (NBCs) are important regulators of intracellular pH (pH_i) in a variety of organ systems where acid-base status is critical for tissue function. To characterize the pharmacology of NBCs in more detail, we used the two-electrode voltage-clamp technique to examine the effect of previously identified inhibitors of anion exchanger 1 (AE1) on the activity of rat NBCe1-A expressed in *Xenopus laevis* oocytes. NBC-expressing oocytes voltage-clamped at –60 m*V* and exposed to a 5% CO_2/33 mM HCO_3^- solution displayed NBC-mediated outward currents that were inhibited by either niflumic acid or one of the two bis-oxonol dyes diBA(3)C4 and diBA(5)C4. NBCe1-A was less sensitive to niflumic acid (apparent K_i of 100 μM) than 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS, apparent K_i of 36 μM) but more sensitive to the diBAC dyes (apparent K_i of ~10 μM). Based on current-voltage relationships, the diBAC dyes inhibited HCO_3^--induced NBCe1-mediated inward currents more so than outward currents. NBCe1 sensitivity to the dyes was (1) lower in the presence of 40 μM DIDS, (2) unaffected by changes in external HCO_3^- concentration and (3) only modestly higher at an external Na^+ concentration of 5, but not 15 or 33, mM. Therefore, the diBAC dyes compete with DIDS but not appreciably with Na^+ or HCO_3^- for binding. The mechanism of diBAC inhibition of NBCe1 appears similar to that previously reported for AE1.

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Introduction

Na/HCO_3 cotransporters (NBCs) are plasma-membrane proteins that play an essential role in transporting Na^+ and HCO_3^-/CO_3^{2-} and regulating intracellular pH (pH_i) of many tissues. pH_i regulation is particularly important because many cellular processes are sensitive to changes in pH (Roos & Boron, 1981). Our understanding of the function and localization of NBCs has advanced following the cloning of an NBC in 1996 (Romero et al., 1997). Since then, the molecular identities of electrogenic NBCs (e.g., NBCe1 and NBCe2), electoneutral NBCs (e.g., NBCn1) and Na-coupled anion exchangers (e.g., NDAE and NDCBE) have been elucidated (*see review by* Romero, Fulton & Boron, 2004). These Na-coupled HCO_3^- transporters in conjunction with anion exchangers (AEs) define a superfamily of bicarbonate transporters (BTs), which are predicted to have similar membrane topologies.

The biophysical properties and pharmacological profiles of many BTs have yet to be elucidated. Although all cloned NBCs and AEs are inhibited by stilbene derivatives, little is known about more discriminating inhibitors of HCO_3^- transporters. Transporter-specific inhibitors would be useful in cellular studies designed to characterize the function and regulation of one HCO_3^- transporter type among a heterogeneous population. In addition, such inhibitors would be useful in identifying and characterizing specific regions of a transporter protein involved in substrate binding, ion translocation or regulation. NBC-specific inhibitors might have some therapeutic value. Indeed, an antibody reported to be

effective at inhibiting human heart NBCe1 protects both systolic and diastolic functions of the rat heart during reperfusion (Khandoudi et al., 2001).

There are several classes of BT inhibitors (Cabantchik & Greger, 1992). The best-known inhibitors of Na-independent and -dependent Cl-HCO_3 exchangers, as well as electrogenic and electroneutral Na/HCO_3 cotransporters, are sulfonates such as 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), 4-acetamide-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS) and 4,4′-dinitrostilbene-2,2′ disulfonate (DNDS). Cloned NBCe1-A expressed in *Xenopus* oocytes is also sensitive to benzamil and the nonsteroidal anti-inflammatory drug tenidap (Ducoudret et al., 2001; Lu & Boron, 2007). Additional inhibitors of Na-independent Cl-HCO_3 exchangers include the noncompetitive inhibitor niflumic acid (Cousin & Motais, 1979; Knauf & Mann, 1984) and fluorescent oxonol dyes such as 3-methyl-1-*p*-sulfophenyl-5-phrazolone-(4)-(1,3 dibutylbarbituric acid)pentamethine oxonol (WW-781) (Raha, Spinelli & Knauf, 1993; Knauf, Raha & Spinelli, 2000), bis(1,3-dibutylbarbituric acid)trimethine oxonol (diBA[3]C4) (Alper et al., 1998; Knauf, Law & Hahn, 1995) and bis(1,3-dibutylbarbituric acid)pentamethine oxonol (diBA[5]C4) (Alper et al., 1998; Knauf et al., 1995). Presently, oxonal dyes are the most potent inhibitors of AEs. Furthermore, AE isoforms exhibit different sensitivities to these dyes. For example, diBA(5)C4 is a more potent inhibitor of AE1 than AE2 (Alper et al., 1998). The sensitivity of Na-coupled bicarbonate transporters to oxonols is thus far unknown.

In the present study, we determined that NBCe1-A expressed in *Xenopus* oocytes is inhibited by niflumic acid and two oxonol dyes, diBA(5)C4 and diBA(3)C4. NBCe1-A is less sensitive to niflumic acid (apparent K_i of 100 μM) than DIDS (apparent K_i of 36 μM) but more sensitive to diBA(3)C4 and diBA(5)C4 (apparent K_i of ~10 μM). NBCe1 sensitivity to oxonol dyes is lower in the presence of 40 μM DIDS, an observation consistent with overlap between the binding sites for oxonol dyes and DIDS. In contrast, NBC sensitivity to diBA(3)C4 is unaffected by changes in external HCO_3^- and increases only modestly when external Na^+ is decreased to 5, but not 15 or 33, mM. Therefore, the binding sites for oxonol dyes and the transporter substrates appear to be independent.

Methods

Solutions

ND96 (pH 7.5) contained (in mM) 96 NaCl, 2 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) and 2.5 NaOH. In the 5% CO_2/33 mM HCO_3^- solution (pH 7.5), 33 mM NaCl was replaced with 33 mM $NaHCO_3$, and the solution was equilibrated with 5% CO_2/95% O_2. Similar replacement strategies were used to generate pH-7.5 solutions containing 0.5% CO_2/3.3 mM HCO_3^-, 1.5% CO_2/10 mM HCO_3^- and 10% CO_2/66 mM HCO_3^-. Solutions containing only 5, 15 or 33 mM Na^+ were generated by replacing the removed Na^+ with an equimolar amount of *N*-methyl-D-glucammonium ($NMDG^+$).

Stock solutions of niflumic acid, diBA(3)C4 and diBA(5)C4 were prepared in dimethyl sulfoxide (DMSO). For some experiments, a stock solution of diBA(5)C4 was prepared in methanol. DIDS was powdered directly into solutions, except for the Dixon-plot experiments in which a 40-mM stock solution was made with DMSO. diBAC dyes were obtained from Molecular Probes (Invitrogen, Carlsbad, CA). Niflumic acid, 1-octanol and all other chemicals were obtained from Sigma (St. Louis, MO).

Harvesting and Injecting Oocytes

Healthy stage V/VI oocytes were harvested from female *Xenopus laevis* frogs as previously described (McAlear et al., 2006) and then incubated at 18°C in sterile ND96 (*see below*) supplemented with 10 mM Na/pyruvate and 10 mg • ml^{-1} gentamycin (Mediatech, Herndon, VA). Oocytes were injected with ~48 nl of either RNAse-free distilled water (negative control) or cRNA encoding rat kidney NBCe1-A using a Nanoject II microinjector (Drummond Scientific, Broomall, PA). Injected oocytes were incubated at 18°C in the aforementioned supplemented ND96 solution, and experiments were performed 2–7 days after injection. For some experiments, NBCe1-A contained a hemagglutinin epitope (which does not interfere with transporter activity) at residue 746 in the extracellular loop between transmembrane domains 5 and 6 (McAlear et al., 2006).

Voltage-Clamp Experiments

Generating and purifying cRNA

Rat NBCe1-A subcloned in the oocyte expression vector pTLNII (Lorenz, Pusch & Jentsch, 1996; Romero et al., 1998) was linearized with *Mlu*I and then transcribed using an SP6 transcription kit (Ambion, Austin, TX). cRNA was purified with the RNeasy® kit (Qiagen, Santa Clarita, CA) and stored at –80°C.

Using the two-electrode voltage-clamp technique

NBC-mediated currents were recording using the two-electrode voltage-clamp technique as previously described (McAlear et al., 2006). Briefly, microelectrodes were pulled from borosilicate glass capillaries using a micropipette puller from either Sutter (P-97; Novato, CA) or Na-

rishige (PC-10; Tokyo, Japan). The electrodes were filled with saturated KCl and attached to two channels of an OC-725C voltage-clamp apparatus (Warner Instruments, New Haven, CT). Microelectrode resistances were typically 0.8-3.0 MΩ for the voltage electrode and 0.2–1.0 MΩ for the current-passing electrode. Current signals acquired with pClamp 8.2 ClampEx software (Axon Instruments, Molecular Devices, San Jose, CA) were digitized with a 1322A interface (Axon Instruments, Molecular Devices) and analyzed with pClamp 8.2 ClampFit software (Axon Instruments, Molecular Devices).

During experiments, oocytes were placed in a flow-through chamber (~0.5 ml) and solution changes were made with a custom-designed solution-delivery system (McAlear et al., 2006). The solution flow rate was typically 4-5 ml • min^{-1}.

Statistics

Data are reported as mean ± standard error of the mean. Levels of significance were assessed using paired or unpaired Student's *t*-tests, and $p < 0.05$ was considered significant. For each experiment, % NBC inhibition was calculated using the equation $100 \times [1-(I_{NBC}^{inh}/I_{NBC})]$, where I_{NBC}^{inh} and I_{NBC} are the HCO_3^--induced currents in the presence and absence, respectively, of an inhibitor. An I_{NBC}^{inh} of 0 would be equivalent to 100% NBC inhibition. For the Dixon-style plots (*see below*), straight lines were fit (Microsoft Excel 2002®, Redmond, WA) to the inverse of normalized NBC currents ($[\text{Norm } I_{NBC}]^{-1}$) *vs.* oxonol concentration. Currents were normalized to the NBC-mediated outward current elicited by a 33-mM HCO_3^- solution at the beginning of each experiment in the absence of inhibitors. Fits to dose-dependence data were generated using a hyperbolic function (Michaelis-Menten enzyme kinetics model) available in Origin 7.5 software (OriginLab, Northampton, MA). Hyperbolic functions as well as associated apparent K_i values and maximum inhibitions were compared using GraphPad Prism (v4.03; San Diego, CA) software.

Results

Dose-Dependent Inhibition of NBCe1-A by DIDS and Niflumic Acid

Dose-dependent inhibition by DIDS

Rat NBCe1-A expressed in *Xenopus* oocytes has previously been shown to be inhibited by 200 μM DIDS (Sciortino & Romero, 1999; McAlear et al., 2006), and such inhibition is independent of external Na^+ concentration (up to 120 mM) and membrane potential (Sciortino & Romero, 1999). To determine the dose dependence of inhibition, we used the two-electrode voltage-clamp technique to examine the effect of different DIDS concentrations on NBC-mediated outward currents elicited by exposing oocytes to a 33-mM HCO_3^- solution. A representative experiment from an NBCe1-expressing oocyte is shown in Figure 1a. At the onset of the experiment (prior to point *a* in Fig. 1a), the oocyte was bathed in ND96 (pH 7.5) and voltage-clamped at –60 m*V*. Exposing the cell to a solution containing 5% CO_2/33 mM HCO_3^- (pH 7.5) elicited an outward current of ~1,400 nA (*ab*) due to NBC-mediated transport of net negative charge into the cell (McAlear et al., 2006). The transporter current reversed when the oocyte was returned to ND96 (point *c*). Applying

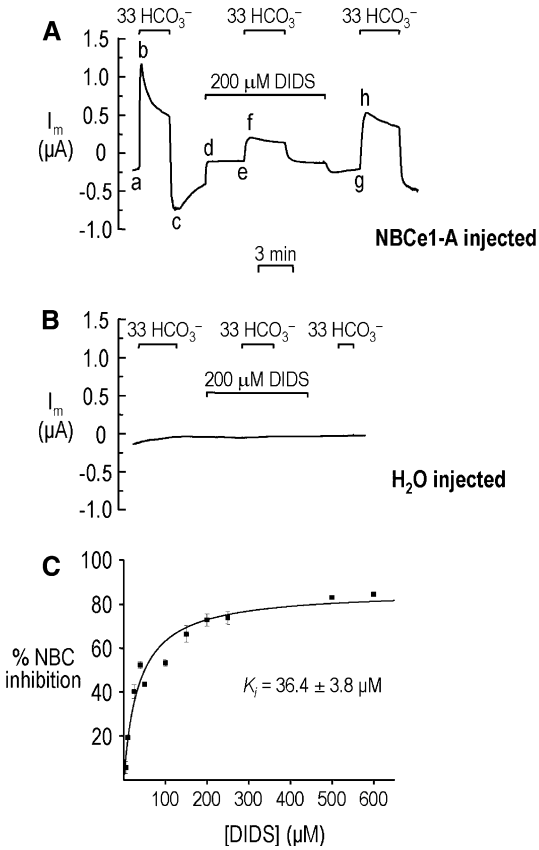

Fig. 1 Inhibition of rat NBCe1-A by DIDS. (**a**) NBCe1-A-injected oocyte voltage-clamped at –60 m*V*. Exposing the oocyte to a 5% CO_2/33 mM HCO_3^- solution elicited an outward current that was ~75% smaller in the presence of 200 μM DIDS (*ef*) than in the absence of the inhibitor (*ab*). The HCO_3^--induced outward current was partially restored (*gh*) after removing DIDS. (**b**) H_2O-injected oocyte voltage-clamped at –60 m*V*. Exposing the oocyte to the 33-mM HCO_3^- solution in the presence or absence of DIDS did not affect the current. (**c**) Dose dependence of NBC inhibition by DIDS. For each experiment similar to that shown in **a**, the HCO_3^--induced current inhibited by DIDS was plotted as a percent of the current obtained in the absence of the inhibitor. $n = 3$–6 for each symbol, and error bars are absent where smaller than the symbol size

200 μM DIDS caused a small outward current (point *d*), possibly due to inhibition of either an anionic (e.g., Cl^-) current or residual outwardly directed NBCe1 activity following HCO_3^- exposure. In the continued presence of DIDS, the HCO_3^- solution elicited a ~325-nA outward current (*ef*) that was ~75% smaller than the current observed in the absence of DIDS (*ab*). The HCO_3^--induced outward current was partially restored after removing the DIDS (*gh*). The 33-mM HCO_3^- solution in the presence or absence of DIDS did not elicit any appreciable current in an H_2O-injected oocyte (Fig. 1b).

Experiments similar to that shown in Figure 1a were performed at different DIDS concentrations. For each experiment, the outward current elicited by HCO_3^- in the presence of DIDS was compared to the paired HCO_3^--induced current at the beginning of the experiment. Oocytes were exposed to DIDS for approximately the same length of time in each experiment. The plot of percent NBC inhibition *vs.* DIDS concentration shown in Figure 1c is hyperbolic, with an apparent K_i of 36.4 ± 3.8 μM and a maximal inhibition of 86 ± 2% (n = 42 total experiments) under our experimental conditions (e.g., $[Na^+]$ = 98.5 mM and V_m = –60 m*V*). This apparent K_i is similar to the apparent K_i of ~40 μM recently reported for reversible DIDS inhibition of human NBCe1-A at 0 m*V* (Lu & Boron, 2007).

Dose-dependent inhibition by niflumic acid

Experiments similar to those described above for DIDS were performed with niflumic acid. As shown in Figure 2a, the HCO_3^- -induced current was ~75% smaller in the presence of 750 μM niflumic acid (*ef*) compared to the current in the absence of the inhibitor (*ab*). Applying niflumic acid in the nominal absence of CO_2/HCO_3^- caused a transient outward current (point *d*) similar to that seen with DIDS and likely due to the same mechanism. Note that a higher concentration of niflumic acid (750 μM) was required to inhibit NBC to the same extent (~75%) as 200 μM DIDS (Fig. 1). As shown above for DIDS, the effect of niflumic acid was partially reversible (*gh*). The HCO_3^- solution in the presence or absence of niflumic acid failed to elicit any appreciable current in an H_2O-injected oocyte (*data not shown*). The niflumic acid vehicle DMSO also had no effect on the HCO_3^--induced outward current (*data not shown*).

From experiments similar to that shown in Figure 2a, a hyperbolic dose dependence of niflumic acid inhibition was obtained (Fig. 2b) with an apparent K_i of 100 ± 21 μM and a maximal inhibition of 88 ± 5% (n = 16 total experiments) under our experimental conditions. Therefore, niflumic acid is a less potent inhibitor of NBCe1-A than DIDS.

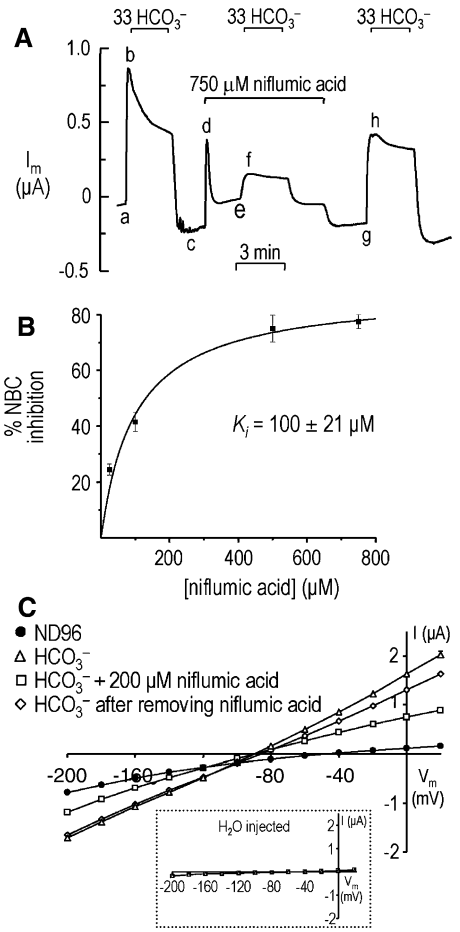

Fig. 2 Inhibition of rat NBCe1-A by niflumic acid. (**a**) NBCe1-A-injected oocyte voltage-clamped at –60 m*V*. Exposing the oocyte to the 33-mM HCO_3^- solution elicited an outward current that was ~75% smaller in the presence of 750 μM niflumic acid (*ef*) than in the absence of the inhibitor (*ab*). The HCO_3^--induced outward current was partially restored (*gh*) after removing the niflumic acid. (**b**) Dose dependence of NBC inhibition by niflumic acid. Data were obtained from experiments similar to that shown in **a**. n = 3–6 for each symbol. (**c**) Effect of niflumic acid on the current-voltage (*I-V*) relationship of rat NBCe1-A. Mean *I-V* plots were obtained from NBCe1-injected oocytes sequentially exposed to ND96 (*closed circles*), a 5% CO_2/33 mM HCO_3^- solution for 5 min (*open triangles*), HCO_3^- solution containing 200 μM niflumic acid (*open squares*) and then HCO_3^- solution again without the inhibitor (*open diamonds*). n = 5 for each data point and error bars smaller than symbols are not shown. *(Inset) I-V* plots were obtained from a single H_2O-injected oocyte exposed to the aforementioned solutions. Similar results were obtained from a second H_2O-injected oocyte

Inhibition by niflumic acid at different membrane voltages

To study the effect of niflumic acid on NBCe1-A at different membrane potentials, we obtained current-voltage relationships from NBCe1-expressing oocytes first exposed to ND96, then after 5 min in 5% CO_2/33 mM HCO_3^- (to allow for intracellular equilibration of CO_2) and finally after 2 min in CO_2/HCO_3^- solution containing 200 μM ni-

flumic acid. As shown in Figure 2c, currents from an NBCe1-injected oocyte were larger in the presence of 5% CO_2/33 mM HCO_3^- (triangles) than in ND96 (circles) due to NBCe1 activity (Sciortino & Romero, 1999; McAlear et al., 2006). The HCO_3^--induced currents at all potentials were markedly reduced in the presence of niflumic acid (squares). For example, niflumic acid reduced both the inward and outward HCO_3^--stimulated currents at $V_m \leq -160$ m*V* and at $V_m \geq -40$ m*V* by 50–66%. These currents were largely restored by removing the niflumic acid (diamonds). Neither HCO_3^--induced currents nor niflumic acid-sensitive currents were observed in an H_2O-injected control oocyte (inset). *I-V* plots looked similar for the control oocyte exposed to ND96 and CO_2/HCO_3^- ± niflumic acid. In summary, niflumic acid inhibits $HCOHCO_3^-$-induced NBCe1 activity to a similar degree at all membrane potentials.

Inhibition of NBCe1-A by diBAC dyes

Dose-dependent inhibition by diBA(3)C4 and diBA(5)C4

As described in the Introduction, both diBA(3)C4 and diBA(5)C4 are potent inhibitors of AE1. We therefore examined the effect of these two diBAC dyes on NBCe1 activity using the inhibitor assay described above for Figures 1 and 2. As shown in Figure 3a, 40 μM diBA(3)C4 inhibited ~75% of the NBC-mediated outward current elicited by the 33-mM HCO_3^- solution (compare segments *ab* and *de*). The diBA(3)C4-elicited outward current in the nominal absence of CO_2/HCO_3^- is similar to that seen with DIDS and likely due to the same mechanism. In contrast to the inhibition by DIDS and niflumic acid described above, diBAC inhibition was poorly reversible (*fg*) at the holding potential of –60 m*V*. Irreversible diBAC inhibition of NBCe1 is probably due to a low off-rate constant as reported for AE1 (Knauf et al., 1995). Interestingly, there was little inhibition of the HCO_3^--induced NBCe1 current if cells were first transiently exposed to diBAC in the nominally HCO_3^--free ND96 solution (*data not shown*). Irreversible diBAC inhibition of NBCe1 may therefore require HCO_3^- binding and/or activation of the transporter.

Because diBAC dyes are hydrophobic, we tested the possibility that diBAC-induced inhibition of NBCe1-A activity reflected a change in membrane fluidity. In experiments similar to that shown in Figure 3, we examined the effect of the hydrophobic *n*-alkanol 1-octanol at 80 μM (twice the highest concentration of diBAC used) on the HCO_3^--induced NBCe1 current. In six paired experiments, the mean HCO_3^--induced outward current was similar before (714 ± 21 nA) and during (749 ± 18 nA) exposure to 80 μM octanol (*data not shown*). Therefore, diBAC inhibition of NBCe1 is unlikely to be the result of a nonspecific effect on membrane fluidity.

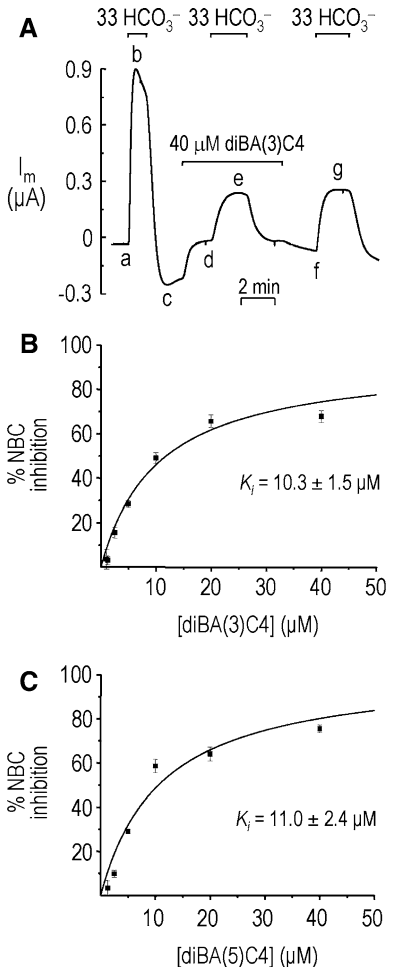

Fig. 3 Inhibition of rat NBCe1-A by diBA(3)C4 and diBA(5)C4. (**a**) NBCe1-A-injected oocyte voltage-clamped at –60 m*V*. Exposing the oocyte to the 33-mM HCO_3^- solution elicited an outward current that was ~75% smaller in the presence of 40 μM diBA(3)C4 (*de*) than in the absence of the inhibitor (*ab*). The HCO_3^--induced NBC current remained ~75% smaller (*fg*) after removing the diBAC dye. (**b**) Dose dependence of NBC inhibition by diBA(3)C4. Data were obtained from 66 experiments similar to that shown in **a.** n = 3–15 for each symbol. (**c**) Dose dependence of NBC inhibition by diBA(5)C4 from 35 experiments similar to that shown in **a.** n = 3–8 for each symbol and error bars smaller than symbols are not shown

From experiments similar to that shown in Figure 3a, a hyperbolic dose dependence of inhibition was obtained (Fig. 3b) with an apparent K_i of 10.3 ± 1.5 μM and maximal inhibition of 93 ± 6% (n = 66 total experiments) under our experimental conditions. Similar results were obtained with diBA(5)C4, and the summary data are shown in Figure 3c. For diBA(5)C4, the apparent K_i is 11.0 ± 2.4 μM and maximal inhibition is 102 ± 10% (n = 35 total experiments). In summary, both diBA(3)C4 and diBA(5)C4 are more potent inhibitors of NBCe1-A than DIDS or niflumic acid.

Inhibition by diBAC dyes at different membrane voltages

We performed experiments similar to those described above for niflumic acid in Figure 2c. As shown in Figure 4a, currents from an NBCe1-injected oocyte were larger in the presence of 5% CO_2/33 mM HCO_3^- (triangles) than in ND96 (circles). In the presence of 20 μM diBA(3)C4, (3–4 min) the HCO_3^--induced currents at all potentials were markedly reduced (squares), particularly at very negative voltages. For instance, diBA(3)C4 eliminated the HCO_3^--induced inward currents, as evident by the superimposed *I-V* plots for ND96 and diBAC + HCO_3^- at $V_m \leq -100$ m*V*. At each $V_m \leq -100$ m*V*, the mean inward currents for oocytes in the presence of HCO_3^- and diBA(3)C4 were either no different ($p \geq 0.09$) or slightly less ($p \leq 0.01$) than the mean currents for oocytes in ND96. In contrast, diBA(3)C4 inhibited only 50–70% of the outward currents stimulated by HCO_3^- at $V_m \geq -80$ m*V*.

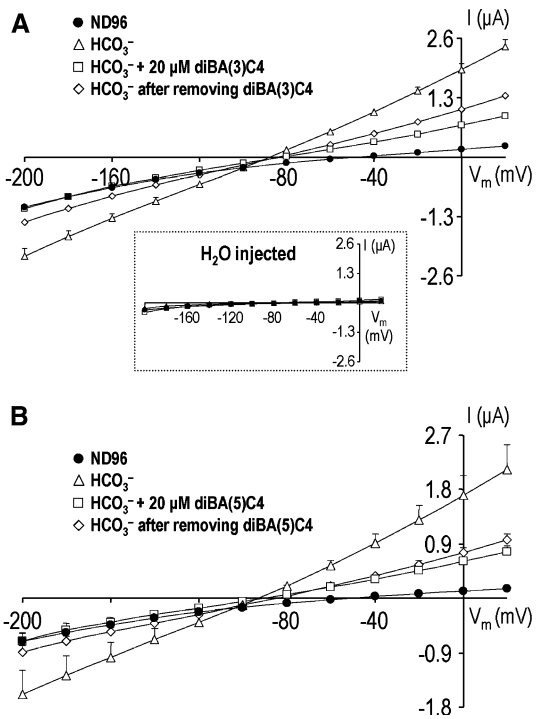

Fig. 4 Effect of diBAC dyes on the current-voltage (*I-V*) relationship of rat NBCe1-A. (**a**) diBA(3)C4. Mean *I-V* plots were obtained from NBCe1-injected oocytes sequentially exposed to ND96 (*closed circles*), a 5% CO_2/33-mM HCO_3^- solution for 5 min (*open triangles*), HCO_3^- solution containing 20 μM diBA(3)C4 (*open squares*) and then HCO_3^- solution again without the inhibitor (*open diamonds*). $n = 4$ for each data point and error bars smaller than symbols are not shown. (*Inset*) *I-V* plots were obtained from a single H_2O-injected oocyte exposed to the aforementioned solutions. Similar results were obtained from a second H_2O-injected oocyte. (**b**) diBA(5)C4. *I-V* plots were obtained from NBCe1-injected oocytes subjected to the same experimental protocol as described in **a.** $n = 3$ for each data point and error bars smaller than symbols are not shown

diBA(3)C4 is therefore a better inhibitor of HCO_3^--induced NBCe1-mediated transport out of *vs.* into cells. The NBC currents were only partially restored 3–4 min after removing the diBAC (diamonds), data consistent with some irreversible binding of the inhibitor. Neither HCO_3^--induced currents nor diBAC-sensitive currents were observed in an H_2O-injected control oocyte (inset). *I-V* plots looked similar for the control oocyte exposed to ND96 and CO_2/ HCO_3^- ± diBA(3)C4.

Nearly identical results were obtained with diBA(5)C4, and the *I-V* plots are shown in Figure 4b. diBA(5)C4 eliminated the HCO_3^- -induced inward currents at $V_m \leq -100$ m*V* and inhibited only 50-69% of the outward currents stimulated by HCO_3^- at $V_m \geq -80$ m*V*. In summary, both diBAC dyes inhibit HCO_3^--induced NBCe1 activity at all membrane but potentials, but more so at potentials favoring NBC-mediated transport out of cells.

Mechanism of Inhibition by diBAC Dyes

According to previous studies, diBA(5)C4 competes with DNDS binding, but not Cl^- binding, to AE1 in red blood cells (Knauf et al., 1995). Using similar approaches, we assessed whether the diBAC binding site of NBCe1 overlaps with the binding site for DIDS, Na^+ or HCO_3^-.

Inhibition by diBAC in the presence of DIDS

Two-electrode voltage-clamp experiments were performed on oocytes expressing NBCe1-A. Using the assay described in Figure 3a, we obtained 33 mM HCO_3^--induced NBC currents in the absence of diBAC dyes or concentrations ranging from 1.25–20 μM, and in either the presence or absence of 40 μM DIDS (the approximate apparent K_i). NBC currents in the presence of one or both inhibitors were normalized (Norm. I_{NBC}) to the NBC-mediated outward current elicited by a 33-mM HCO_3^- solution at the beginning of each experiment in the absence of inhibitors. The inverse of Norm. I_{NBC} values was computed, and mean values *vs.* [diBAC] are displayed in the Dixon-style plots shown in Figure 5. For both diBA(3)C4 (Fig. 5a) and diBA(5)C4 (Fig. 5b), the data in the presence and absence of DIDS were best fit with parallel lines.[1]. This parallel relationship is indicative of DIDS and diBAC being mutually exclusive inhibitors (Knauf et al., 1995; Fröhlich & Gunn, 1987). The most likely explanation for the mutual exclusion is overlap of the inhibitors' binding sites on

[1] In the diBA(5)C4 study, two of the six (Norm $I_{NBC})^{-1}$ values obtained at 20 μM diBAC + 40 μM DIDS were not included in our analysis because they appeared to be outliers. These two values (8.63 and 9.91) had a mean (Norm $I_{NBC})^{-1}$ of 9.27, which was 2.3-fold larger than the mean value of 3.96 obtained from the other four experiments.

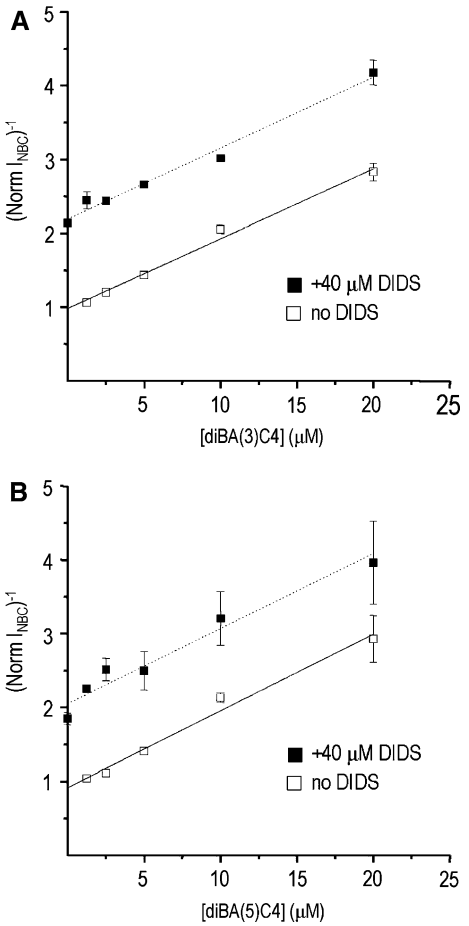

Fig. 5 Dixon-style plots of NBC inhibition by diBAC dyes. (**a**) diBA(3)C4. Experiments were performed either in the presence of 40 μM DIDS (*closed squares*) or the absence of the inhibitor (*open squares*). Linear fits to the two data sets of (Norm. $I_{NBC})^{-1}$ *vs.* diBA(3)C4 had nearly identical slopes of 0.094 μM^{-1} (without DIDS, *solid line*) and 0.096 μM^{-1} (with DIDS, *dotted line*). $n \geq 3$ for each data point and error bars smaller than symbols are not shown. (**b**) diBA(5)C4. Similar experiments were performed as described in **a.** Linear fits to the two data sets of (Norm. $I_{NBC})^{-1}$ *vs.* diBA(5)C4 had nearly identical slopes of 0.104 μM^{-1} (without DIDS, *solid line*) and 0.102 μM^{-1} (with DIDS, *dotted line*). $n \geq 4$ for each data point and error bars smaller than symbols are not shown

NBCe1, as described for DNDS and diBA(5)C4 binding to AE1 (Knauf et al., 1995).

As reviewed by Cabantchik & Greger (1992), the irreversible covalent inhibition of anion transporters by DIDS and other stilbene derivatives with similar isothiocyanate moieties is preceded by a reversible ionic inhibition. We hypothesized that DIDS inhibition during our relatively short incubations in the aforementioned experiments is primarily reversible. Indeed, in a separate study on five NBCe1-injected oocytes subjected to the same experimental protocol as shown in Figure 3a, ~5-min exposure to 40 μM DIDS inhibited the mean HCO_3^--induced outward current by 52.3 ± 1.5%, as expected. Upon removing the DIDS and repeating the HCO_3^--pulse protocol in the same experiments, the inhibition was reversed 72.6 ± 4.1%.

Inhibition by diBA(3)C4 at different substrate concentrations

If diBAC and HCO_3^- share at least part of the same binding site, then diBAC's apparent K_i should change in the same direction as the [HCO_3^-] due to competition between the two molecules. We therefore repeated the Figure 3 hyperbolic dose dependence of diBA(3)C4 inhibition at external HCO_3^- concentrations of 66, 10 and 3.3 mM.[2]

The solutions were equilibrated with appropriate CO_2/O_2 mixtures to maintain pH_o = 7.5. Compared to the apparent K_i of 10.3 μM with 5% CO_2/33 mM HCO_3^-, similar apparent K_i values were obtained with 66 mM HCO_3^-/10% CO_2 solutions (8.0 ± 2.7 μM, n = 17 total experiments), 10 mM HCO_3^-/1.5% CO_2 solutions (7.9 ± 1.2 μM, n = 16 total experiments) and even 3.3 mM HCO_3^-/0.5% CO_2 solutions (10.3 ± 1.5 μM, n = 26 total experiments). As shown in Figure 6a, the dose dependence of inhibition with 3.3 mM HCO_3^- (squares) was similar to that reported with 33 mM HCO_3^- (dotted fit replotted from Fig. 3b). The data are consistent with no overlap of the diBAC and HCO_3^- binding sites.

In a similar way, if diBAC and Na^+ share at least part of the same binding site, then diBAC's apparent K_i should change in the same direction as the Na^+ concentration. We therefore examined the dose dependence of diBA(3)C4 inhibition at external Na^+ concentrations of 33, 15 and 5 mM[2] using 5% CO_2/33 mM HCO_3^- solutions. Compared to the apparent K_i of 10.3 μM with full 98.5 mM Na^+, apparent K_i values were somewhat higher ($p \leq 0.04$) with 33 mM Na^+ solutions (24.4 ± 12.4 μM, n = 10 total experiments) and 15 mM Na^+ solutions (24.2 ± 9.0 μM, n = 32 total experiments). However, as shown in Figure 6b, at the very low external Na^+ concentration of 5 mM (squares), the apparent K_i (6.0 ± 0.8 μM, n = 23 total experiments) was ~40% lower ($p = 0.01$) than that obtained in full 98.5 mM Na^+ (dotted fit replotted from Fig. 3b). Thus, diBA(3)C4's apparent K_i increases when external Na^+ is lowered to concentrations near NBCe1-A's apparent K_M of 20-30 mM (Romero & Sciortino, 1999; McAlear et al., 2006) but modestly decreases when external Na^+ is reduced further to the very low concentration of 5 mM. The data are consistent with minimal overlap of the diBAC and Na^+ binding sites.

[2] Approximately ~7 mM HCO_3^- and 20-30 mM Na^+ are the apparent K_M values of NBCe1-A expressed in oocytes (Sciortino & Romero, 1999; Grichtchenko, Romero & Boron, 2000; McAlear et al., 2006).

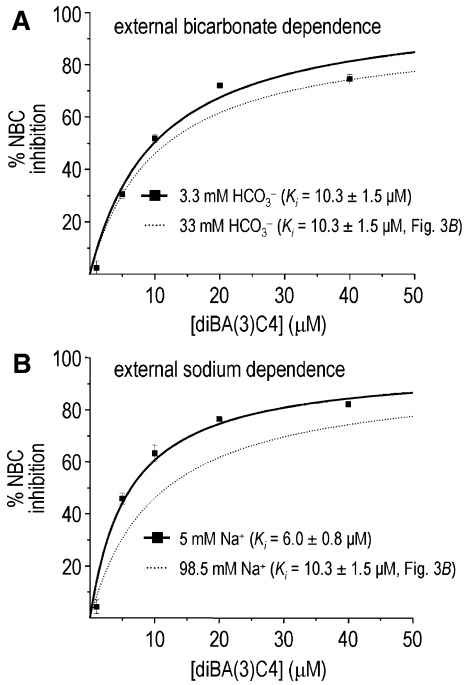

Fig. 6 Dose dependence of NBC inhibition by diBA(3)C4 at different external substrate concentrations. (**a**) External bicarbonate dependence. Dose dependence of NBC inhibition was determined from experiments similar to those reported for Figure 3, except at an external HCO_3^- concentration of 3.3 mM (*closed squares* and *solid thick trace*). For comparison, the fit to the 33-mM HCO_3^- data shown in Figure 3b is replotted (*thin dotted trace*). Maximum inhibition is 102 ± 6% for the 3.3-mM HCO_3^- data. $n \geq 3$ for each data point and error bars smaller than symbols are not shown. (**b**) Effect of Na^+. Dose dependence of NBC inhibition was determined at an external Na^+ concentration of 5 mM (*closed squares* and *solid thick trace*). For comparison, the fit to the 98.5-mM Na^+ data shown in Figure 3b is replotted (*thin dotted trace*). Maximum inhibition is 97 ± 4% for the 5-mM Na^+ data. $n \geq 4$ for each data point and error bars smaller than symbols are not shown

Discussion

Relative Potencies of DIDS, Niflumic Acid and diBAC Dyes

We used the two-electrode voltage-clamp technique to characterize the dose dependence of inhibition by DIDS, niflumic acid and two diBAC dyes of rat NBCe1-A-expressed *Xenopus* oocytes. The relative potency of NBCe1-A inhibition is niflumic acid (apparent K_i = 100 μM) < DIDS (apparent K_i = 36 μM) < diBA(3)C4 ≅ diBA(5)C4 (apparent $K_i \cong 10$ μM). Thus, the two diBAC dyes are ~4-fold more potent than DIDS and ~10-fold more potent than niflumic acid at inhibiting NBCe1-A.

One consistent observation with all three inhibitors was the inability of high concentrations to inhibit NBCe1 activity completely. For example, ~24% NBCe1 activity remained with 500 and 750 μM niflumic acid, ~16% activity with 500 and 600 μM DIDS and 25-32% activity with 40 μM diBAC. In principle, we could have examined higher concentrations of niflumic acid and DIDS, although the experimental use of such high concentrations is somewhat impractical and increases the likelihood of nonspecific effects. Because the diBAC dyes are quite hydrophobic and poorly water soluble, we did not examine concentrations higher than 40 μM. Interestingly, at high diBA(3)C4 concentrations (particularly 20 and 40 μM), we sometimes observed dye particles in our 5% CO_2/95% O_2-bubbled solutions, especially over time and with vigorous bubbling. As a result, precipitation of diBA(3)C4 may have led to an underestimation of maximum NBCe1 inhibition. Reduced dye solubility may be caused by either O_2-induced oxidation of the dye or a CO_2-induced pH decrease in microscopic pockets of solution (Zhao, Zhou & Boron, 2003). Such particles in solution were not observed with diBA(5)C4, even at the high concentrations examined.

Niflumic Acid Inhibition of NBCe1-A

Niflumic acid is a reversible, noncompetitive inhibitor of AE1 in erythrocytes, and niflumic acid and the stilbene SITS are mutually exclusive inhibitors (Cousin & Motais, 1979). Niflumic acid also inhibits NBCe1-A expressed in oocytes and to a similar extent at all membrane potentials from –200 to +20 m*V*. However, it is a relatively poor inhibitor (apparent K_i = 100 μM) compared to DIDS (apparent K_i = 36 μM) and the diBAC dyes (apparent $K_i \cong$ 10 μM). Nevertheless, NBCe1-A is pharmacologically similar to AE1 in being sensitive to niflumic acid. To our knowledge, the effect of niflumic acid on endogenous NBC activity has not been examined. However, 1 mM triflocin (4[3-trifluoromethylanilino-]nicotinic acid), an isomer of niflumic acid, has been shown to inhibit the electrogenic Na/HCO_3 cotransporter in *Necturus* proximal convoluted tubule cells (Belachgar et al., 1994). In oocytes expressing the C variant of NBCe1 (Bevensee et al., 2000), 1 mM triflocin also inhibits the NBC-mediated pH_i recovery when the cells are exposed to CO_2/ HCO_3^- (Williams, Planelles & Bevensee, *unpublished observations*). The effect of triflocin on AE activity has not been explored, and it remains to be determined if either niflumic acid or its derivatives can also inhibit other cation-dependent members of the BT superfamily, including the electroneutral NBCs and the Na-driven anion exchangers.

diBAC Inhibition of NBCe1-A Compared to AE1

Similar to that reported for AE1 in red blood cells (Knauf et al., 1995), both diBA(3)C4 and diBA(5)C4 are potent inhibitors of NBCe1-A expressed in oocytes. diBA(5)C4 also inhibits AE1 heterologously expressed in oocytes, al-

though at a somewhat lower potency than seen in red blood cells (Alper et al., 1998). Therefore, it is quite possible that the potency of diBAC inhibition of NBCe1 in native cells is greater than that observed in our oocyte expression system. It is also important to note that the two diBAC dyes are quite hydrophobic and therefore probably accumulate in the plasma membrane. Bis-barbituric acid oxonols such as diBA(3)C4 and diBA(5)C4 are slow-response membrane potential probes that exhibit an increase in fluorescence with depolarization by binding to either membranes or intracellular proteins after crossing membranes (Haugland, 2005). Therefore, in our experiments, diBAC concentrations may be somewhat higher in the oocyte plasma membrane than in the bath solution. As reported for AE1 (Knauf et al., 1995), diBAC inhibition of NBCe1 complicates the use of these dyes as potentiometric probes.

In addition to identifying diBAC dyes as inhibitors of NBCe1, we compared the mechanism of NBCe1 inhibition to that previously reported for AE1 (Knauf et al., 1995). We found the following two key similarities. First, based on the Dixon-style plots shown in Figure 5, both diBAC and stilbenes share at least part of the same external binding site. Although the apparent competition between DIDS and diBAC dyes for binding is consistent with an externally accessible diBAC binding site, it is possible that this site becomes occluded with irreversible inhibition. Another possible interpretation is that binding of one inhibitor alters the transporter's conformation and reduces the binding of the other inhibitor (Knauf et al., 1995). The second similarity between the AE1 and NBCe1 studies is that diBAC does not compete with HCO_3^- for binding.

Regarding Na^+, lowering external Na^+ from 98.5 mM to either 33 or 15 mM causes an increase in diBA(3)C4's apparent K_i, whereas lowering external Na^+ further to 5 mM causes a decrease in the apparent K_i. The explanation for this biphasic effect is not clear. However, the increase in K_i is similar to the IC_{50} increase for diBAC inhibition of AE1 with a decrease in Cl^- concentration, a finding consistent with reduced diBAC affinity in the absence of bound substrate (Knauf et al., 1995). While the decrease in diBA(3)C4's apparent K_i at low external Na^+ may be due to competition between diBAC and Na^+ for a single binding site, it was curious that the effect was only observed at a Na^+ concentration well below 20–30 mM—NBCe1-A's apparent K_M for external Na^+ (Sciortino & Romero, 1999; McAlear et al., 2006). An alternative explanation is that a marked reduction in Na^+ binding with low external Na^+ leads to an NBC conformation with increased diBAC affinity. Overall, there appears to be minimal overlap of the diBAC and Na^+ binding sites. It is important to note that our diBAC-substrate competition studies were performed only with diBA(3)C4. However, we expect that similar results would be obtained with diBA(5)C4 because both diBAC dyes have nearly identical apparent K_i values and voltage dependencies of NBC inhibition, and both compete with DIDS for binding.

Because NBCe1-A is electrogenic, we also assessed diBAC inhibition at different membrane voltages. Although diBAC inhibited transporter activity at all potentials from -200 to +20 m*V*, HCO_3^- -induced NBC-mediated inward currents (Na^+ and HCO_3^- transport out of cells) at negative potentials were more inhibited than the outward currents (Na^+ and HCO_3^- transport into cells) at more positive potentials. diBAC binding, presumably to an external site, has a greater inhibitory effect on transport out of cells *vs.* into cells. For NBCe1, our voltage dependence of diBAC inhibition is opposite to that recently reported for DIDS, which exhibits less reversible inhibition at more negative potentials (Lu & Boron, 2007). Three possible explanations for the voltage dependence of this DIDS inhibition proposed by the authors include a voltage-dependent conformational change of NBCe1, a Boltzmann effect whereby the concentration of anionic DIDS in the vicinity of binding is less at more negative potentials and displacement of bound DIDS due to Na^+/HCO_3^- cotransport out of the cells at more negative potentials. Compared to the DIDS data, the opposite voltage dependence of NBCe1 inhibition by the anionic diBAC dyes is somewhat surprising, particularly for any reversible inhibition. One potential explanation is based on outward- *vs.* inward-facing binding sites in an alternating access model of transport, such as the ping-pong mechanism described for AE1 (*see* Knauf et al., 2002) and the rocker-switch mechanism described for the lactose permease (LacY) cotransporter of *Eschericha coli* (Kaback et al., 2007; *see* DeFelice, 2004). The initial outward-facing binding site may become inward-facing after diBAC binding. Consequently, one might expect the voltage dependence of diBAC inhibition to be reversed (i.e., greater at more negative potentials), particularly if diBAC displacement is voltage-dependent.

In summary, the oxonol dyes diBA(3)C4 and diBA(5)C4 are potent inhibitors of NBCe1-A expressed in oocytes, and the mechanism of inhibition is similar to that reported for AE1. Future studies are required to examine the inhibitory effect of diBAC dyes on other members of the BT superfamily. It is possible that the aforementioned diBAC dyes and related compounds have different effects on various BTs. For example, diBA(5)C4 inhibits AE2 several hundred times less effectively than AE1 expressed in oocytes (Alper et al., 1998). Oxonol dyes and their analogs may therefore be quite useful in selectively inhibiting BTs in a heterologous transporter population. Drawbacks in using diBAC compounds as NBC inhibitors include their poor water solubility relative to other inhibitors and their tendency as dyes to stain solution-delivery lines. Nevertheless, diBAC dyes may be useful molecules for developing more discriminating inhibitors of different BTs (Alper et al., 1998).

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