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Characterization of Cl^-/HCO_3^- exchange in A10 vascular smooth muscle cells using $^{36}Cl^-$

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Recently we have reported evidence for Cl^-/HCO_3^- exchange in A10 vascular smooth muscle cells (ATCC CRL 1476) by means of continuous intracellular pH measurements (Korbmacher, C., Helbig, H., Stahl, F. and Wiederholt, M. (1988) Pflügers Arch. 412, 29–36). In the present study we used $^{36}Cl^-$ to further characterize Cl^-/HCO_3^- exchange in confluent monolayers of A10 cells. Under the chosen experimental conditions, $^{36}Cl^-$ uptake was inhibited by more than 90% in the presence of the stilbene derivatives SITS (1 mM) and DIDS (1 mM), known inhibitors of Cl^-/HCO_3^- exchange. The IC_{50} for DIDS was about 5 μM. Ethacrynic acid was also an effective inhibitor of $^{36}Cl^-$ uptake with an IC_{50} of about 20 μM. 'Loop diuretics' (furosemide, bumetanide) and 'Cl^--channel blockers' (DPC, A9C, NPPB) had only small effects on $^{36}Cl^-$ uptake in concentrations in which they are thought to act specifically on $Na^+/K^+/2Cl^-$ cotransport or on chloride channels, respectively. DIDS-sensitive $^{36}Cl^-$ uptake was a saturable function of the extracellular chloride concentration with an apparent K_m for extracellular chloride of about 45 mM. $^{36}Cl^-$ uptake was inhibited by the presence of extracellular HCO_3^-, Br^-, I^-, acetate$^-$ and NO_3^-. $^{36}Cl^-$ uptake could be stimulated by bicarbonate-preloading of the cells. Intracellular alkalinization during the uptake period (induced by application of NH_4^+) also stimulated $^{36}Cl^-$ uptake. $^{36}Cl^-$ efflux was greater in the presence than in the absence of extracellular chloride or bicarbonate. $^{36}Cl^-$ efflux was diminished in the presence of 1 mM DIDS. In conclusion our data are compatible with a DIDS-sensitive Cl^-/HCO_3^- exchange which is stimulated by intracellular alkalinization.

Introduction

The transport of chloride across cell membranes plays an important role in a number of physiological regulatory mechanisms. Studies of ion transport across vascular smooth muscle plasma membranes have mainly been concerned with the movement of cations such as Na^+, K^+ and Ca^{2+}. Less attention has been paid to anion transport. However, transmembrane movements of anions may have an important impact on vascular smooth muscle tone by influencing a variety of cellular phenomena such as intracellular ion concentrations, membrane voltage and intracellular pH (for a review, see Ref. 1). We have recently reported evidence for a DIDS-sensitive Cl^-/HCO_3^- exchange in A10 vascular smooth muscle cells [2] by means of continuous intracellular pH measurements [3]. In the present study we have used $^{36}Cl^-$ to further characterize Cl^-/HCO_3^- exchange in confluent monolayers of A10 cells. Parts of this work have previously been published in abstract form [4].

Methods

Cell culture. The A10 cells [2] were obtained at passage 16 from American Type Culture Collection (ATCC No.: CRL 1476; lot No. 5590), Rockville, MD, U.S.A. They were maintained and subcultured as described previously [3]. Cells were seeded in 25 cm^2 tissue culture flasks and were used after having formed a confluent monolayer usually 7–10 days after seeding.

Abbreviations: DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid; DPC, diphenylamine-2-carboxylate; A9C, anthracene-9-carboxylate; NPPB, nitro-2-(3-phenylpropylamino)benzoate.

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Cells used in this study were from passages 17–45. We have previously demonstrated by means of electrophysiological studies that A10 cells maintain vascular smooth muscle membrane properties under longterm cell culture conditions [3,5].

$^{36}Cl^-$ experiments. $^{36}Cl^-$-uptake studies were performed essentially as described previously [6–8]. In short, confluent monolayers of A10 cells were preincubated at 37°C in solutions containing different ionic concentrations as indicated in the figure legends. The uptake-solution contained 5–10 kBq/ml $^{36}Cl^-$. The ionic composition of the uptake solution and the inhibitor concentrations are given in the figure legends. In the case of bicarbonate-containing solutions flasks were gassed at each solution change with an appropriate CO_2/air mixture to maintain the pH near 7.4. Experiments were performed at 37°C. Uptake was terminated by aspirating the labelled solution and three rapid washes with ice-cold isotonic $MgCl_2$ solution (buffered with Hepes-Tris to pH 7.4). Cells were dissolved in 0.1% sodium dodecyl sulfate (SDS) solution. The radioactivity of the lysate was subsequently measured using Pico fluor 40* (Packard Instruments, IL) scintillation gel and a β counter. Uptake was referred to the surface area and expressed as $^{36}Cl^-$ uptake per cm^2 of a surface covered by a confluent monolayer. Cell density was about $(0.5–1) \cdot 10^5$ cells/cm^2.

$^{36}Cl^-$-efflux experiments were performed as previously described [7,8]. Cells were loaded with $^{36}Cl^-$ by 90 min preincubation in nominally bicarbonate free saline containing 5–10 kBq $^{36}Cl^-$ per ml, 10 mM Cl^- and 10^{-4} M ouabain. Ouabain was included in the preincubation media since a previous study had shown that ouabain increases steady-state accumulation of $^{36}Cl^-$ in other cultured cells [7]. Prior to the efflux period, the radioactive solution was aspirated and cells were rinsed with a solution of identical composition to the efflux solution but containing no isotope. Subsequently they were incubated for the indicated period of time in efflux saline the composition of which is indicated in the figure legend. Efflux was stopped by rinsing with ice-cold stop solution as described above. Radioactivity remaining in the cells was determined and expressed as the percentage of initial radioactivity accumulated in the cells after $^{36}Cl^-$ preincubation prior to the efflux period.

Values are given as arithmetic means ± S.E. After testing for equality of variances using the *F*-test, significances were evaluated by the appropriate version of the unpaired *t*-test.

Solutions. The standard chloride and nominally bicarbonate free saline, from which the other solutions were derived, contained 10 mM Hepes (adjusted to pH 7.4) 151 mM Na^+, 5 mM K^+, 1.7 mM Ca^{2+}, 1 mM Mg^{2+}, 158.4 mM cyclamate (as a replacement for Cl^-), 1 mM SO_4^{2-}, 1 mM $H_2PO_4^-$, and 5 mM glucose. All nominally bicarbonate free solutions were equilibrated with room air *. When other anions were present (such as Cl^-, HCO_3^-, Br^-, I^-, NO_3^- or acetate$^-$) an equivalent amount of cyclamate was replaced by the respective anion. Solutions containing 10 mM, 28 mM, 46 mM or 75 mM bicarbonate were gassed with 2%, 5%, 10% and 15% CO_2/air mixture, respectively, to maintain the pH near 7.4. Sodium free solutions were obtained by replacing sodium either by *N*-methyl-D-glucamine (NMDG) or by potassium. Potassium free solutions were obtained by replacing potassium by sodium. In solutions designed to change pH_i, an equivalent amount of NaCl was replaced by 20 mM NH_4Cl.

Materials. Cell culture media and supplements were purchased from Biochrom KG, Berlin, F.R.G., and tissue culture plasticware was from Nunc A/S, Roskilde, Denmark. 4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS), probenecid, ethacrynic acid and furosemide were purchased from Sigma, Munich, F.R.G., Bumetanide was kindly provided by Dr. Karl Thomae GmbH, Biberach, F.R.G. Diphenyl-2-carboxylate (DPC) was obtained from EGA-Chemie, Steinheim/Albuch, F.R.G., and anthracene-9-carboxylate (A9C) was from Aldrich-Chemie, Steinheim, F.R.G. Nitro-2-(3-phenylpropylamino)benzoate (NPPB) was a gift from Dr. Greger [9]. DIDS and SITS are readily water soluble. The other inhibitory compounds were dissolved in dimethylsulfoxide. The final dimethylsulfoxide concentration did not exceed 0.3%. In control experiments it was verified that dimethylsulfoxide (up to 0.3%) had no detectable effect on $^{36}Cl^-$ uptake under our experimental conditions. $^{36}Cl^-$ was obtained from New England Nuclear (Boston, MA) as HCl.

Results

Stimulation of DIDS-sensitive $^{36}Cl^-$ uptake by bicarbonate preincubation

Preincubation in a chloride-free solution containing a high concentration of bicarbonate was chosen to stimulate $^{36}Cl^-$ uptake via DIDS-sensitive Cl^-/HCO_3^- exchange. Fig. 1 shows the time course of $^{36}Cl^-$ uptake in confluent monolayers of A10 cells. The cells were preincubated for 30 min at 37°C in chloride-free saline solution either in the nominal absence of bicarbonate or in the presence of 75 mM HCO_3^- (gassed with 15%

* Although these solutions were nominally bicarbonate free, the actual $[HCO_3^-]$ is expected to be about 0.15 mM when solutions with pH 7.4 are equilibrated with room air. This can be calculated from the Henderson-Hasselbalch equation assuming that air contains 0.033% CO_2 by volume, the solubility of CO_2 is 0.03 mM/Torr, and the p*K* of $H_2CO_3 \rightleftharpoons HCO_3^- + H^+$ is 6.1. However, the actual $[HCO_3^-]$ in the vicinity of the cells may be even higher due to cellular generation of CO_2.

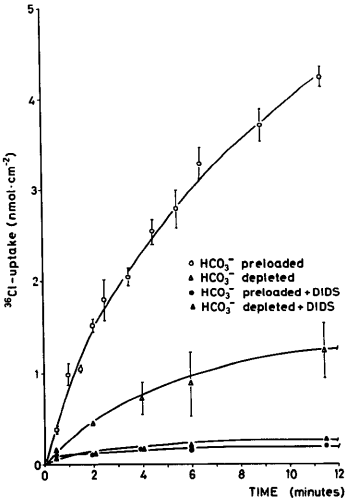

Fig. 1. Time-course of $^{36}Cl^-$ uptake in confluent monolayers of A10 vascular smooth muscle cells. Cells were preincubated for 30 min in chloride-free saline (pH 7.4), which was either nominally bicarbonate-free (▲, △) or contained 75 mM bicarbonate (gassed with 15% CO_2/air to pH 7.4) (●, ○). Uptake solution was nominally bicarbonate free (pH 7.4) and contained 10 mM Cl^- and 5–10 kBq $^{36}Cl^-$ per ml. Experiments were performed either in the absence (○, △) or presence of 1 mM DIDS (●, ▲) during the uptake period. Bars indicate S.E. values; measurements were performed in quadruplicate.

CO_2). The uptake saline was nominally bicarbonate-free and contained 10 mM chloride. The uptake was performed either in the presence or in the absence of 1 mM DIDS. $^{36}Cl^-$ uptake was several fold higher in bicarbonate-preloaded cells (preincubation in the presence of 75 mM HCO_3^-/15% CO_2) as compared to $^{36}Cl^-$ uptake in bicarbonate-depleted cells (preincubation in the nominal absence of bicarbonate). In both instances $^{36}Cl^-$ uptake was almost completely inhibited by 1 mM DIDS, indicating that a DIDS-sensitive process is mainly responsible for $^{36}Cl^-$ uptake under these experimental conditions. The findings are compatible with the presence of a DIDS-sensitive Cl^-/HCO_3^- exchange in A10 vascular smooth muscle cells [3], which can be stimulated by an outwardly directed bicarbonate gradient.

Chloride dependence of DIDS-sensitive chloride uptake

In experiments such as those shown in Fig. 2A we investigated the chloride dependence of the DIDS-sensitive (1 mM DIDS) chloride uptake in bicarbonate-preloaded A10 cells. The time of uptake in these experiments was 2 min. DIDS-sensitive chloride uptake is a saturable function of the extracellular chloride concentration. The linear transformation of the data shown in Fig. 2A according to Lineweaver-Burk, revealed an apparent K_m of 34.7 mM (Fig. 2B). The mean K_m value derived from a set of three experiments like that shown in Fig. 2A averaged 44.8 ± 11.3 mM (mean ± S.E., $n = 3$). For these calculations we assumed a linear $^{36}Cl^-$ uptake over the two minutes of the uptake period. This assumption seems to be reasonable according to the time course of $^{36}Cl^-$ uptake shown in Fig. 1. However, it should be noted that any uncertainty on this point affects the accuracy with which the kinetic parameters are estimated.

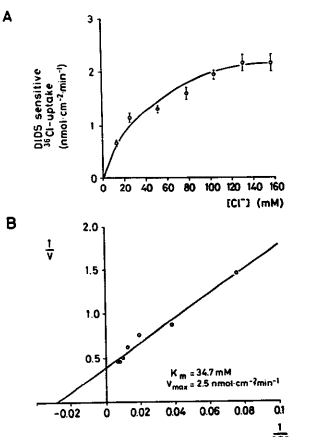

Fig. 2. Cl^- dependence of DIDS-sensitive Cl^- uptake. Cells were preincubated for 30 min in chloride-free saline containing 75 mM HCO_3^- (15% CO_2/air mixture). Uptake was measured in the presence and absence of 1 mM DIDS in nominally bicarbonate free saline containing the chloride concentration as indicated. Uptake time was 2 min. Bars indicate S.E. values ($n = 6$). (A) DIDS-sensitive Cl^- uptake is given as a function of the extracellular chloride concentration. (B) Lineweaver-Burk plot of the data from (A).

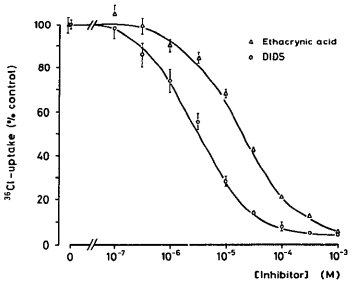

Fig. 3. Dose-response curves for DIDS (○) and ethacrynic acid (△) on $^{36}Cl^-$ uptake in bicarbonate-preincubated A10 cells. Uptake solution was nominally bicarbonate-free and contained 5–10 kBq $^{36}Cl^-$ per ml. Uptake time was 2 min, bars indicate S.E. values ($n = 3$–8).

Effect of inhibitors

Fig. 3 shows that DIDS inhibition of $^{36}Cl^-$ uptake is dose-dependent. The dose-response curve reveals an IC_{50} for DIDS of about 5 μM. Fig. 3 also shows the dose-dependent inhibitory action of ethacrynic acid on $^{36}Cl^-$ uptake in bicarbonate-preloaded A10 cells. The IC_{50} value for ethacrynic acid is about 20 μM.

Table I summarizes the effects of various inhibitors on $^{36}Cl^-$ uptake in bicarbonate-preloaded A10 cells. 1 mM SITS, another stilbene derivative, reduced $^{36}Cl^-$ uptake by more than 90%. Probenecid (10^{-4} M) inhibited $^{36}Cl^-$ uptake by about 30%.

Bumetanide (10^{-5} M) a known inhibitor of $Na^+/K^+/2Cl^-$ cotransport did not significantly affect

TABLE I

Effect of various inhibitors on $^{36}Cl^-$ uptake in HCO_3^--preloaded A10 cells

Probabilities (P) refer to control versus experimental values.

Inhibitor	Concn. (M)	$^{36}Cl^-$ uptake ($\bar{x} \pm$S.E. in % control)	n	P
SITS	10^{-3}	7.0±0.4	7	< 0.001
Probenecid	10^{-4}	71.6±1.0	4	< 0.01
Bumetanide	10^{-5}	100.3±6.6	4	n.s.
	10^{-4}	85.2±2.2	25	< 0.001
Furosemide	10^{-4}	72.0±3.0	4	< 0.001
	10^{-3}	36.3±1.7	16	< 0.001
DPC	10^{-4}	80.4±4.5	12	< 0.01
A9C	10^{-4}	89.7±3.0	13	< 0.05
NPPB	10^{-7}	101.5±6.0	5	n.s.
	10^{-6}	95.9±3.3	5	n.s.
	10^{-5}	83.8±1.6	5	< 0.01
	10^{-4}	34.1±1.5	12	< 0.001

^{36}Cl-uptake in bicarbonate-preloaded A10 cells (Table I). Bumetanide (10^{-4} M) inhibited $^{36}Cl^-$ uptake in bicarbonate-preloaded A10 cells by about 15%. Furosemide, another loop diuretic, reduced ^{36}Cl-uptake by more than 25% and more than 60% at concentrations of 10^{-4} M and 10^{-3} M, respectively (Table I).

Three 'Cl^--channel blockers' were tested on $^{36}Cl^-$ uptake in bicarbonate-preloaded A10 cells. Anthracene-9-carboxylate (A9C) 10^{-4} M and diphenyl-2-carboxylate (DPC) 10^{-4} M inhibited $^{36}Cl^-$ uptake by some 10–20% (Table I). Nitro-2-(3-phenylpropylamino)benzoate (NPPB) did not significantly affect $^{36}Cl^-$ uptake in concentrations of 10^{-7} and 10^{-6} M. High concentrations of NPPB (10^{-5} M and 10^{-4} M) inhibited $^{36}Cl^-$ uptake by some 15% and 60%, respectively (Table I). However, Cl^- channels in the thick ascending limb of the loop of Henle [9] and in human colon carcinoma cells [10] have been reported to be completely blocked by NPPB in concentrations as low as 10^{-7} M. Thus, $^{36}Cl^-$ uptake in bicarbonate-preloaded A10 cells is not highly sensitive to these substances which are known to block Cl^- channels in other tissues.

Removal of monovalent cations

$^{36}Cl^-$ uptake in bicarbonate-preincubated A10 cells was not significantly altered in the absence of extracellular potassium ($n = 14$) or in the absence of extracellular sodium. Extracellular sodium was replaced either by NMDG ($n = 22$) or by potassium ($n = 22$). Sodium replacement or high extracellular potassium concentrations have been shown to depolarize membrane voltage in A10 vascular smooth muscle cells [5]. Thus, DIDS-sensitive $^{36}Cl^-$ uptake in bicarbonate-preloaded A10 cells does not seem to be dependent on changes in membrane voltage.

Effect of extracellular anions

Increasing the extracellular bicarbonate concentration to 10, 28, 46 and 75 mM reduced the $^{36}Cl^-$ uptake

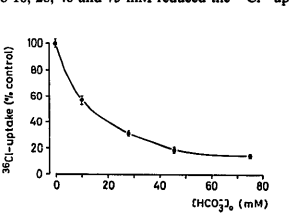

Fig. 4. Inhibition of $^{36}Cl^-$ uptake in bicarbonate-preincubated A10 cells by increasing extracellular bicarbonate concentration during the uptake period. Uptake solutions were gassed with an appropriate CO_2 concentration (see Materials and Methods). Uptake time was 2 min. Data are summarized from two separate experiments, bars indicate S.E. values (each point represents 16 individual measurements).

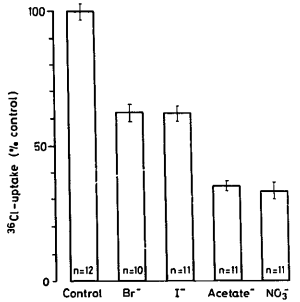

Fig. 5. Inhibition of $^{36}Cl^-$ uptake in bicarbonate-preincubated A10 cells by various extracellular anions. Uptake solution was nominally bicarbonate free and contained 50 mM of the indicated anion and 5–10 kBq $^{36}Cl^-$ per ml. Uptake time was 2 min, bars indicate S.E. values.

as compared to control conditions in nominally bicarbonate free uptake saline. Fig. 4 reveals an apparent IC_{50} for extracellular bicarbonate of about 12 mM. Increasing the HCO_3^- concentration during the uptake period not only reduces the transmembrane gradient for bicarbonate (hence the driving force for $^{36}Cl^-$ uptake) but also the CO_2 gradient. Therefore, CO_2 efflux and the resulting intracellular alkalinization (see below) will be reduced at higher extracellular bicarbonate concentrations. The two effects may both lead to a reduction of $^{36}Cl^-$ uptake. Furthermore, extracellular bicarbonate is presumably competing with $^{36}Cl^-$ at a common extracellular transport site.

In the experiment shown in Fig. 5 we investigated the anion specificity of the $^{36}Cl^-$-uptake mechanism in bicarbonate preloaded A10 cells. Different anions were added together with $^{36}Cl^-$ to the uptake saline. External Br^-, I^-, acetate$^-$ and NO_3^- markedly reduced $^{36}Cl^-$ uptake. This indicates that these anions may also compete with $^{36}Cl^-$ for a common transport site.

Effect of pH

Recently it has been reported that Cl^-/HCO_3^- exchange can be stimulated by intracellular alkalinization in a variety of cells [11,12]. Changing from a preincubation solution containing 75 mM HCO_3^- (gassed with 15% CO_2) to a nominally bicarbonate free uptake saline (equilibrated with room air) causes intracellular alkalinization due to rapid efflux of the membrane permeable CO_2 [13]. Thus, it is conceivable that not only the outwardly directed bicarbonate gradient but also the intracellular alkalinization may play a role in the observed stimulation of $^{36}Cl^-$ uptake in bicarbonate-preloaded cells.

This assumption is supported by the experiment shown in Fig. 6 in which the effect of intracellular alkalinization of $^{36}Cl^-$ uptake was tested. During the uptake period intracellular pH was raised (at constant extracellular pH) by exposing the cells to an uptake saline containing 10 mM $(NH_4)_2SO_4$. Addition of the weak acid NH_4^+ is known to cause a rapid rise in intracellular pH due to influx of membrane permeable NH_3 and its intracellular combination with H^+ [13]. This phenomenon has also been demonstrated in A10 cells. Application of a solution containing 20 mM NH_4^+ led to a rapid intracellular alkalinization, which persisted for several minutes in the continuous presence of NH_4^+ [3]. From these previous findings we can assume that under the chosen experimental conditions the intracellular pH was maintained well above 7.8 during the $^{36}Cl^-$-uptake period of two minutes. This intracellular alkalinization stimulated $^{36}Cl^-$ uptake in bicarbonate-depleted cells to about the same extent as bicarbonate-preloading of the cells.

Thus, intracellular alkalinization is clearly an important factor in the stimulation of $^{36}Cl^-$ uptake in A10 cells. On the other hand application of $(NH_4)_2SO_4$ had no additional stimulatory effect on $^{36}Cl^-$ uptake in bicarbonate-preloaded cells. This is not surprising since, as discussed above, bicarbonate-preloaded cells are already alkalinized during the uptake period due to the

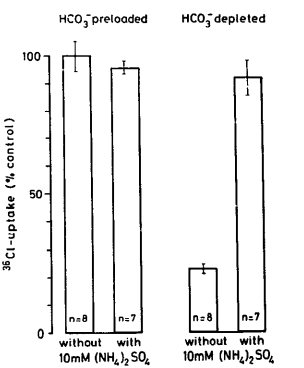

Fig. 6. Effect of intracellular alkalinization induced by addition of $(NH_4)_2SO_4$ on $^{36}Cl^-$ uptake in A10 cells, which were either preincubated in the presence of 75 mM HCO_3^- (preloaded) or in the nominal absence of HCO_3^- (depleted). Uptake time was 2 min, bars indicate S.E. values.

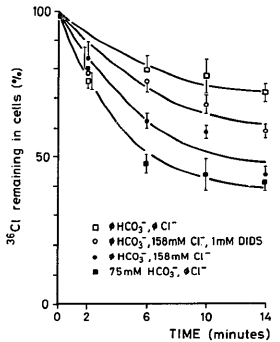

Fig. 7. $^{36}Cl^-$ efflux after preloading the cells with $^{36}Cl^-$ as described in Materials and Methods. Efflux saline was nominally bicarbonate-free and chloride-free (□), or was nominally bicarbonate-free and containing 158 mM Cl^- either with (○) or without 1 mM DIDS (●), or was chloride-free and containing 5 mM HCO_3^- (gassed with 15% CO_2/air) (■). Measurements were performed in quadruplicate; bars indicate S.E. values.

simultaneous removal of bicarbonate and CO_2. According to the law of mass action intracellular pH and bicarbonate concentration are closely linked. Raising intracellular pH also raises intracellular bicarbonate. Therefore, these experiments cannot differentiate between the stimulatory effect of intracellular alkalinization on $^{36}Cl^-$ uptake and/or the stimulatory effect of the outwardly directed bicarbonate gradient.

$^{36}Cl^-$ efflux

If a considerable portion of the chloride fluxes across the cell membrane is mediated by a DIDS sensitive Cl^-/HCO_3^- exchange, one would expect an inhibition of $^{36}Cl^-$ efflux in the absence of extracellular chloride and bicarbonate and also an inhibition in the presence of DIDS. This hypothesis was tested in $^{36}Cl^-$ efflux experiments shown in Fig. 7. In the absence of extracellular chloride and bicarbonate $^{36}Cl^-$ efflux was comparatively slow while a high extracellular bicarbonate concentration as well as a high extracellular chloride concentration enhanced $^{36}Cl^-$ efflux. This indicates that the Cl^-/HCO_3^- exchanger may also function as a Cl^-/Cl^- self-exchanger. DIDS (1 mM) diminished $^{36}Cl^-$ efflux (Fig. 7).

Discussion

Three mechanisms mediating chloride transport across the vascular smooth muscle plasma membrane have so far been proposed: (1) a conductive pathway for chloride [1,14], (2) a $Na^+/K^+/2Cl^-$ symport sensitive to loop diuretics [15,16] and (3) a DIDS-sensitive Cl^-/HCO_3^- exchange mechanism [17,3].

Evidence for the presence of a Cl^-/HCO_3^- exchange in vascular smooth muscle cells has only recently been reported from $^{36}Cl^-$ efflux studies in strips of rabbit aorta [17] and from intracellular pH measurements in A10 vascular smooth muscle cells [3]. In the present investigation we have demonstrated that DIDS-sensitive $^{36}Cl^-$ uptake in A10 cells was stimulated by an outwardly directed bicarbonate gradient and/or intracellular alkalinization. These findings are consistent with anion exchange described previously in other preparations. The exchange mode of Cl^-/HCO_3^- exchange is believed to be largely dependent on chloride and/or bicarbonate concentrations on either side of the cell membrane and it is assumed that it may also function as Cl^-/Cl^- or HCO_3^-/HCO_3^- self-exchange mechanism (for review, see Ref. 18). Furthermore, it has been demonstrated that in a number of cells Cl^-/HCO_3^- exchange is largely stimulated by intracellular alkalinization [11,12].

Under the chosen experimental conditions $^{36}Cl^-$ uptake in bicarbonate-preloaded A10 cells was inhibited by more than 90% in the presence of the stilbene derivatives SITS (1 mM) and DIDS (1 mM). DIDS is known as a potent inhibitor of the Cl^-/HCO_3^- exchange mechanisms [19,20] and other bicarbonate transporting systems such as the electrogenic Na^+/HCO_3^--symport [6]. However, DIDS and SITS have also been reported to be effective blockers of Cl^- conductances in, e.g., the electroplax organ of *Torpedo california* [21] and in chick intestinal cells [22]. Therefore, it cannot be assumed that the presence of a DIDS-sensitive Cl^--transport process is unequivocal evidence for a Cl^-/HCO_3^--exchange mechanism.

A DIDS-sensitive Cl^- conductance must be considered. However, $^{36}Cl^-$ uptake mediated by a chloride channel should be affected by changes in membrane voltage. This did not seem to be the case in bicarbonate-preloaded A10 cells. We have shown that Cl^- uptake was not affected in the presence of a high extracellular potassium concentration (sodium being completely replaced by potassium), a condition which should depolarize membrane voltage. Furthermore, that $^{36}Cl^-$ efflux is stimulated by extracellular chloride or bicarbonate is an argument in favor of Cl^-/HCO_3^- exchange rather than for a Cl^- conductance.

In A10 cells the IC_{50} value for DIDS was about 5 μM, which is similar to values reported for Cl^-/HCO_3^- exchange mechanisms in other tissues [23,8]. In contrast to this, DIDS- or SITS-sensitive Cl^--conductance pathways are thought to require rather high stilbene concentrations (100–500 μM) to achieve inhibition of conductance [22]. Furthermore, in human fibroblasts which

like vascular smooth muscle cells have a chloride conductive pathway, an anion exchange mechanism and a $Na^+/K^+/2Cl^-$ cotransport, chloride conductance has been shown to be insensitive to DIDS [24].

Another effective inhibitor of $^{36}Cl^-$ uptake in bicarbonate-preloaded A10 cells was ethacrynic acid (IC_{50} of 20 μM). Ethacrynic acid has been shown to inhibit $^{36}Cl^-/Cl^-$ self-exchange in rabbit aorta [25] and recently has been reported to selectively inhibit the Na^+-independent Cl^-/HCO_3^- exchange in Vero cells, while not affecting Na^+-dependent Cl^-/HCO_3^- exchange [26]. Similarly, probenecid has been shown to inhibit Na^+-independent Cl^-/HCO_3^- exchange [26]. In A10 cells 10^{-4} M probenecid inhibited $^{36}Cl^-$ uptake by about 30%. These findings indicate that the Cl^-/HCO_3^- exchange mechanism in A10 cells is probably Na^+-independent.

DIDS-sensitive chloride uptake was a saturable function of the extracellular chloride concentration. Saturability is a characteristic of carrier-mediated transport processes and the apparent K_m value for extracellular chloride of about 45 mM is of the same order of magnitude as K_m values previously reported for Cl^-/HCO_3^- exchange in sheep cardiac Purkinje fibre (33 mM) [27] and in red blood cells (about 20–60 mM) [19]. Extracellular anions inhibited $^{36}Cl^-$ uptake in the following order: HCO_3^-, Br^-, I^-, acetate$^-$ and NO_3^-. A similar order of inhibiting anions has previously been described in a variety of tissues including smooth muscle of the guinea-pig vas deferens [28] and is probably due to a competition of these anions with chloride at a common extracellular transport site.

DIDS-insensitive transport mechanisms accounted for less than 10% of the $^{36}Cl^-$ uptake in bicarbonate-preloaded A10 cells. This enabled us to establish an inhibitor profile for Cl^-/HCO_3^- exchange for various inhibitors known to affect chloride transport mechanisms other than Cl^-/HCO_3^- exchange. The loop diuretics bumetanide and furosemide had no or only small effects on bicarbonate-stimulated chloride uptake in concentrations in which they are known to be largely effective as inhibitors of $Na^+/K^+/2Cl^-$ transport [29,30,16]. This and the independence of $^{36}Cl^-$ uptake on extracellular sodium and potassium preclude a major role of $Na^+/K^+/2Cl^-$ transport in A10 cells under our experimental conditions. However, high concentrations of loop diuretics markedly reduced $^{36}Cl^-$ uptake, indicating that in high concentrations loop diuretics are no longer specific inhibitors of $Na^+/K^+/2Cl^-$ transport but may also affect Cl^-/HCO_3^- exchange. Similar findings, demonstrating that high concentrations of loop diuretics inhibited Cl^-/HCO_3^- exchange, have previously been reported in other tissues [19,23,8]. On the other hand the stilbene derivatives DIDS and SITS have been found to not inhibit the $Na^+/K^+/2Cl^-$-cotransport system [30].

The 'chloride-channel blockers', A9C, DPC and NPPB, did not appreciably affect ^{36}Cl-uptake at concentrations known to effectively block chloride channels in other tissues [31,1,9]. However, as in the case of the loop diuretics, high concentrations of NPPB caused a large reduction in $^{36}Cl^-$ uptake. This is probably due to a non-specific effect on Cl^-/HCO_3^- exchange, when NPPB is applied in high concentrations [9]. The inhibitory effect (about 20%) observed with 10^{-4} M DPC may also be explained by an inhibition of Cl^-/HCO_3^- exchange as has recently been proposed for *Necturus* gallbladder epithelium [32].

Thus, the specificity of the various inhibitors for only one Cl^--transport system seem to be largely dose-dependent (compare Refs. 18 and 9). Applied in sufficiently high concentrations they all may affect Cl^-/HCO_3^- exchange. Therefore these findings may be of importance in designing further studies on chloride-transport mechanisms.

Little is known as to the physiological importance of Cl^-/HCO_3^- exchange in the vascular smooth muscle cell. The importance of Cl^-/HCO_3^- exchange for the regulation of intracellular pH has been pointed out previously [27,33,34,3] and intracellular pH is believed to play an important role in the regulation of vascular tone [35]. Moreover Cl^-/HCO_3^- exchange has been shown to be involved in the maintenance of intracellular chloride activity [36,27], which has been found to be well above the electrochemical equilibrium value in smooth muscle cells [37]. Changes in intracellular chloride activity may affect membrane voltage and hence contractility.

Furthermore, in guinea-pig ileal smooth muscle, a chloride-dependent calcium pool has been proposed, which is believed to supply Ca^{2+} primarily for phasic contractions [38]. In guinea-pig myocardium SITS and DIDS have been reported to reduce contractile force in a time and concentration-dependent manner [39].

Cl^-/HCO_3^- exchange has been shown to be involved in volume regulation in other tissues [30]. In epithelial tissues, Cl^-/HCO_3^- exchange has been shown to be responsible for transepithelial transport of chloride, and also for transepithelial transport of sodium via coupling to a Na^+/H^+ exchange mechanism [23,40]. The presence of Na^+/H^+ exchange has also been demonstrated in A10 vascular smooth muscle cells [3]. Whether or not in vascular smooth muscle cells a coupled action of Na^+/H^+ exchange and Cl^-/HCO_3^- exchange plays a role not only in the regulation of intracellular pH but also in the regulation of intracellular sodium and/or chloride activity remains to be determined.

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