Sodium–bicarbonate cotransport current in identified leech glial cells

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1. The membrane current associated with the cotransport of Na^+ and HCO_3^- was investigated in neuropil glial cells in isolated ganglia of the leech *Hirudo medicinalis* L. using the two-electrode voltage-clamp technique.

2. The addition of 5 % CO_2–24 mM HCO_3^- evoked an outward current, which slowly decayed, and which was dependent upon the presence of external Na^+. Removal of CO_2–HCO_3^- elicited a transient inward current. Re-addition of Na^+ to Na^+-free saline in the presence of CO_2–HCO_3^- also produced an outward current. Under these conditions an intracellular alkalinization and a rise in intracellular [Na^+] were recorded using triple-barrelled, ion-sensitive microelectrodes. Addition or removal of HCO_3^-, in the absence of external Na^+, caused little or no change in membrane voltage, membrane current and intracellular pH, indicating that the glial membrane has a very low HCO_3^- conductance.

3. Voltage steps revealed nearly linear current–voltage relationships both in the absence and presence of CO_2–HCO_3^-, with an intersection at the assumed reversal potential of the HCO_3^--dependent current. These results suggest a cotransport stoichiometry of 2 HCO_3^- : 1 Na^+. The HCO_3^--dependent current could be inhibited by diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS).

4. Simultaneous recording of current and intracellular pH showed a correlation of the maximal acid–base flux with the transient HCO_3^--dependent current during voltage steps in the presence of CO_2–HCO_3^-. The maximum rate of acid–base flux and the HCO_3^--dependent peak current showed a similar dependence on membrane voltage. Lowering the external pH from 7·4 to 7·0 produced an inward current, which increased twofold in the presence of CO_2–HCO_3^-. This current was largely inhibited by DIDS, indicating outward-going electrogenic Na^+–HCO_3^- cotransport during external acidification.

5. When external Na^+ was replaced by Li^+, a similar outward current and intracellular alkalinization were observed in the presence of CO_2–HCO_3^-. The Li^+-induced intracellular alkalinization was not inhibited by amiloride, a blocker of $Na^+(Li^+)$–H^+ exchange, but was sensitive to DIDS. These results suggest that Li^+ could, at least partly, substitute for Na^+ at the cotransporter site.

6. Our results indicate that the Na^+–HCO_3^- cotransport produces a current across the glial cell membrane in both directions with a reversal potential near the membrane resting potential, rendering pH_i a function of the glial membrane potential.

Glial cells play an important role in maintaining ion homeostasis in nervous systems. We have recently shown that glial cells actively regulate their intracellular pH (Deitmer & Schlue, 1987, 1989) and also participate in pH regulation of extracellular spaces in the nervous tissue by uptake and secretion of bicarbonate (Deitmer, 1991). The latter may increase the effective buffering capacity in these extracellular spaces and thereby counteract extracellular acidosis or alkalosis (Deitmer, 1992*a*).

The transport mechanism responsible for the efficient shift of acid–base equivalents across the glial membrane is a Na^+–HCO_3^- cotransport, which has been identified in epithelial tissue (see Boron & Boulpaep, 1989), in glial cells of invertebrates (Deitmer & Schlue, 1987, 1989) and vertebrates (Astion & Orkand, 1988; Kettenmann & Schlue, 1988; Newman, 1991; Chow, Yen-Chow, White & Woodbury, 1991), and in cardiac tissue (Dart & Vaughan-Jones, 1992; Lagadic-Gossman, Buckler & Vaughan-Jones, 1992).

In most epithelial and glial cells, this Na^+–HCO_3^- cotransport is electrogenic, and hence sensitive to the membrane potential (Boron & Boulpaep, 1983; Jentsch, Keller, Koch & Wiederholt, 1984; La Cour, 1989; Hughes, Adorante, Miller & Lin, 1989; Deitmer & Szatkowski, 1990; Fitz, Lidofsky, Xie & Scharschmidt, 1992). In retinal Müller glial cells, the current underlying the Na^+–HCO_3^- cotransport was measured using whole-cell patch-clamp recordings (Newman & Astion, 1991; Newman, 1991). These studies indicated a stoichiometry of the cotransport of one Na^+ with three HCO_3^- ions. This agrees well with most studies on epithelial tissues but disagrees with the stoichiometry of one Na^+ to two HCO_3^- ions that we reported for leech neuropil glial cells using ion-sensitive microelectrodes to measure intracellular pH and Na^+ (Deitmer & Schlue, 1989; Deitmer, 1992*b*). In cultured oligodendrocytes (Kettenmann & Schlue, 1988), sheep cardiac Purkinje fibres (Dart & Vaughan-Jones, 1992) and guinea-pig ventricular myocytes (Lagadic-Gossmann *et al.* 1992), the Na^+–HCO_3^- cotransport appeared to be electroneutral.

The stoichiometry of the cotransport is, however, crucial for its mode and function, because it determines, together with the electrochemical gradients for Na^+ and HCO_3^-, the membrane potential sensitivity and the transport direction. With a stoichiometry of one Na^+ and two HCO_3^- ions, which we suggested from steady-state measurements of intracellular Na^+ and pH in leech glial cells, the cotransport would reverse close to the membrane resting potential. In this study we have therefore examined the current underlying the operation of the carrier in the membrane of identified leech glial cells, using a slow two-electrode voltage clamp. A preliminary report of some of the results has been communicated to the Physiological Society (Deitmer & Munsch, 1993).

METHODS

Preparation of glial cells

The experiments were performed on isolated ganglia of the leech *Hirudo medicinalis* L. Briefly, individual ganglia were pinned in a Sylgard-coated Perspex chamber in a modified Leibovitz (L-15) tissue culture medium. The ventral ganglionic capsule was removed mechanically with fine forceps. The ganglia were then incubated for 1 h in 2 mg ml^{-1} collagenase–dispase containing modified L-15 medium at room temperature (20–25 °C). After thoroughly washing the ganglia with enzyme-free medium, neurones overlying the two neuropil glial cells of each segmental ganglion were removed by suction into a glass micropipette, thereby exposing the glial cells (Munsch & Deitmer, 1992). For experimentation ganglia were then transferred into leech saline.

Solutions

Modified L-15 medium was prepared by dilution (1:3) of original L-15 medium (Gibco, Eggenstein, Germany) with a salt solution of the following composition (in mmol l^{-1}): $CaCl_2$, 6·87; $MgCl_2$, 2·51; KCl, 3·32; sodium malate, 20·1; sodium pyruvate, 12·5; Hepes, 15; glucose, 15; gentamycin (10 mg ml^{-1}), 0·3 %; adjusted to pH 7·4 with NaOH.

In experiments in which intracellular pH and Na^+ were measured with ion-sensitive microelectrodes, a standard physiological leech saline was used, which contained (in mmol l^{-1}): NaCl, 85; KCl, 4; $CaCl_2$, 2; $MgCl_2$, 1; Hepes, 10; pH adjusted to 7·4 with 3–5 mM NaOH. In CO_2–HCO_3^--buffered saline 24 mM NaCl was replaced by equimolar amounts of $NaHCO_3$ and bubbled with 5 % CO_2–95 % O_2. When the pH of salines was adjusted to 7·0, Hepes was replaced by 3-(*N*-morpholino) ethanesulphonic acid (Mops; 10 mM) and 10 mM NaCl was replaced by 10 mM $NaHCO_3$. In Na^+-free salines, Na^+ was replaced by *N*-methyl-D-glucamine (NMDG) or Li^+. In Na^+-free CO_2–HCO_3^--buffered leech saline, 4 mM KCl was additionally replaced by 4 mM $KHCO_3$. The pH was first adjusted to 7·0 and then the saline was bubbled for several hours (> 3 h) with 5 % CO_2–95 % O_2 to generate NMDG-HCO_3. Finally the pH was adjusted to 7·4 with HCl. Amiloride (2 mM) and DIDS (diisothiocyanatostilbene-2,2′-disulphonic acid, 0·3–0·5 mM) were added to solutions shortly before use.

For voltage-clamp measurements, the standard physiological leech saline was modified to reduce the large background conductance of the glial membrane. This modified saline contained (in mmol l^{-1}): sodium gluconate, 75; potassium gluconate, 4; calcium gluconate, 15 (Ca^{2+} elevated to compensate for its binding with gluconate; Vaughan-Jones, 1979); magnesium gluconate, 5; $BaCl_2$, 3; Hepes, 10; pH adjusted to 7·4 with NaOH (4–5 mM). As in the salines used for ion-sensitive microelectrode measurements, Hepes was replaced by Mops when the pH was changed to 7·0. In CO_2–HCO_3^--buffered saline 24 mM sodium gluconate was replaced by $NaHCO_3$ and the solution was bubbled with 5 % CO_2–95 % O_2. In a CO_2–HCO_3^--buffered saline with pH 7·0, 10 mM sodium gluconate was replaced by 10 mM $NaHCO_3$. Sodium-free, Hepes-buffered salines were prepared by replacing sodium gluconate by equivalent amounts of NMDG (75 mM) or Li_2SO_4 (37·5 mM). The pH of Na^+-free salines was adjusted to 7·4 with gluconic acid. In CO_2–HCO_3^--buffered Na^+-free salines potassium gluconate was additionally replaced by $KHCO_3$ (4 mM) and bubbled with 5 % CO_2–95 % O_2 for several hours.

Voltage clamp

Microelectrodes for voltage clamp (made from single-barrelled 1·5 mm glass capillaries, Clark Electromedical GC 150F-15) were filled with 4 M potassium acetate for voltage recording and 2 M potassium citrate for injecting current. Current electrodes were additionally dry-bevelled before filling (Kaila & Voipio, 1985) and shielded with silver paint which was connected to ground. For voltage clamping, both microelectrodes were connected to the headstages of an Axoclamp-2A (Axon Instruments, Foster City, CA, USA). Membrane currents were recorded by the built-in current measurement circuit of the headstages. The experimental bath was grounded via a Ag–AgCl pellet.

For combining the voltage clamp with ion-sensitive microelectrode measurements, single-barrelled microelectrodes filled with 2 M potassium citrate were used as current electrodes and double-barrelled pH-sensitive microelectrodes (Deitmer, 1991) were used for voltage recording and pH measurement. The reference barrel was connected to one headstage of an Axoclamp-2A amplifier and the ion-sensitive barrel to an electrometer input (npi, Tamm, Germany). The voltage signal of the reference barrel was used for voltage clamping and subtracted from the signal of the pH-sensitive barrel to obtain direct readings of pH.

Ion-sensitive microelectrodes

Triple-barrelled pH- and Na^+-sensitive microelectrodes were prepared as described previously (Deitmer, Schneider & Munsch, 1991; Deitmer, 1992*b*). Briefly, θ-type capillaries were fused with

a single glass capillary, and the two barrels of the θ capillary were silanized using a drop of 5% tri-*N*-butylchlorosilane (Fluka) mixed in 99·9% pure carbon tetrachloride which was backfilled into the tips. The pipette was then baked on a hot plate at 470 °C for 4–5 min. For the pH-sensitive barrel, H^+ cocktail (Fluka 95291) was backfilled into the tip of one silanized barrel and then filled up with 0·1 M sodium citrate, pH 6·0. The Na^+-sensitive barrel was backfilled with a Na^+ cocktail based on the Na^+ ionophore VI (Fluka 71739) and filled up with 0·1 M NaCl + 10 mM Mops, pH 7·0. The reference barrel was filled with 3 M KCl. The electrodes were calibrated in leech salines with different pH and Na^+ concentrations. The pH-sensitive barrel responded on average with 52 mV for a unit change in pH. Electrodes were accepted when they responded with at least 47 mV for one pH unit and with less than 3 mV to 5% CO_2. The Na^+-sensitive barrel responded on average with 53 mV between 90 and 9 mM Na^+ concentration. Measurements of Na^+ were presented as Na activity (a^i_{Na}, using an activity coefficient of 0·75 for Na^+.

Each channel of the triple-barrelled microelectrodes was connected via chlorided silver wires to a high-impedance input of a four-channel electrometer amplifier (electronics workshop of Kaiserslautern University). The voltage of the reference barrel was subtracted from voltages of the pH- and the Na^+-sensitive barrels to obtain direct readings of pH and Na^+ alone. The electrometer outputs were recorded on chart paper for display.

Statistics

All averages were quoted as means ± standard deviation (S.D.). Significance was tested using a modified *t* test after Dixon & Massey (1969).

RESULTS

HCO_3^- and Na^+ dependence

Introduction of CO_2–HCO_3^- to the saline results in a hyperpolarization of the glial cell membrane (Deitmer & Schlue, 1987), due to an inwardly directed, electrogenic Na^+–HCO_3^- cotransport across the membrane (Fig. 1, and Deitmer & Schlue, 1989). When the glial membrane potential is clamped near its resting level (−65 to −75 mV), an outward current is recorded upon addition of CO_2–HCO_3^- (Fig. 1*A*). This current quickly rises to a peak amplitude of 20–30 nA (24·8 ± 2·1 nA, $n = 5$), before it decays with a maximum rate of 10–20 nA min^{-1}. An apparent steady-state outward current is usually obtained, measuring up to + 20 nA (17·6 ± 4·5 nA, $n = 5$) with respect to the holding current in the absence of CO_2–HCO_3^-. After the removal of CO_2–HCO_3^- the current reversed and became transiently inward, before it returned to its initial level (Fig. 1*A*).

When external Na^+ was removed, the large membrane outward currents were absent, and only a small transient depolarization and a small transient inward current remained upon addition of CO_2–HCO_3^- (Fig. 1*B*). When external Na^+ was re-added in the continuous presence of CO_2–HCO_3^-, again a large outward current was elicited with an amplitude of up to 50 nA (43·6 ± 6·9 nA, $n = 3$) (Fig. 1*C*). The current subsided with a similar rate to before during the CO_2–HCO_3^- exposure to a level of up to + 30 nA (30·3 ± 8·3 nA, $n = 3$). When external Na^+ was again removed, the current reversed transiently to an inward current, before returning to its initial level.

When changing to current-clamp conditions, the membrane hyperpolarized from − 86 to − 110 mV during the short exposure to Na^+-containing saline (Fig. 1*C*). This is considerably more negative than the K^+ equilibrium potential, which is around − 80 mV (Walz & Schlue, 1982), confirming that the large negativity is produced by an electrogenic process rather than an ion conductance change of the membrane.

Under very similar conditions, we have also simultaneously measured the intracellular pH, pH_i, and the intracellular Na^+ activity, a^i_{Na} (Fig. 2). The membrane still hyperpolarized and a^i_{Na} decreased due to removal of external Na^+. Addition of 5% CO_2–24 mM HCO_3^- did not markedly alter the course of the membrane potential and a^i_{Na}, and decreased pH_i by about 0·04 pH units. This indicates that external Na^+ is needed to produce the potential and ion changes described previously upon addition of CO_2–HCO_3^- (see also Deitmer & Schlue, 1989; Deitmer, 1992*b*).

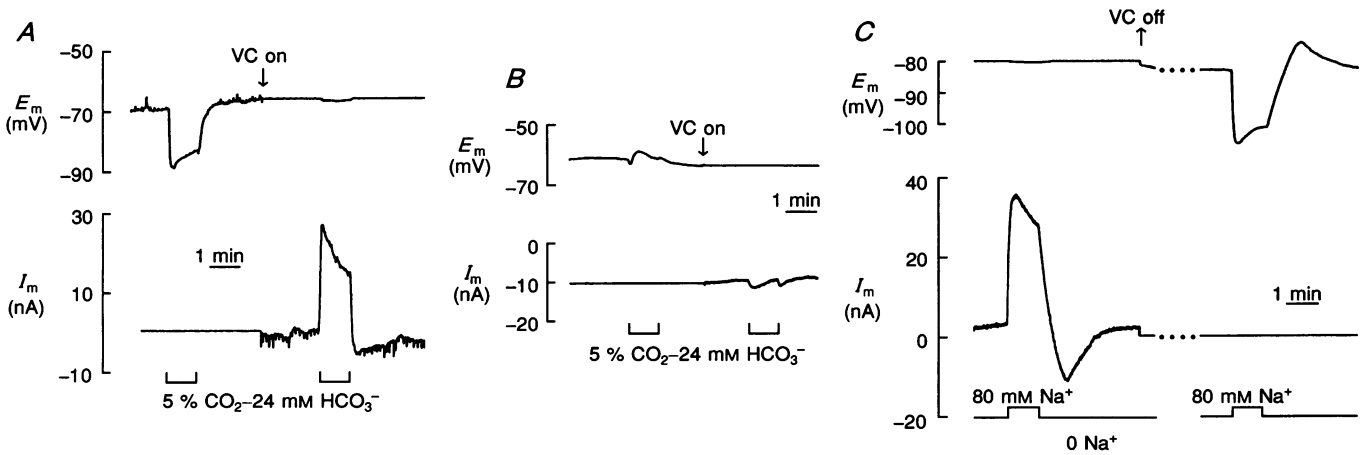

Figure 1.
Membrane potential (V_m) changes (upper traces) and membrane currents (I_m, lower traces) during membrane voltage clamp (VC) evoked by CO_2–HCO_3^- in the presence of external Na^+ (*A*), in Na^+-free saline (*B*) and after addition of external Na^+ in CO_2–HCO_3^--buffered saline (*C*).

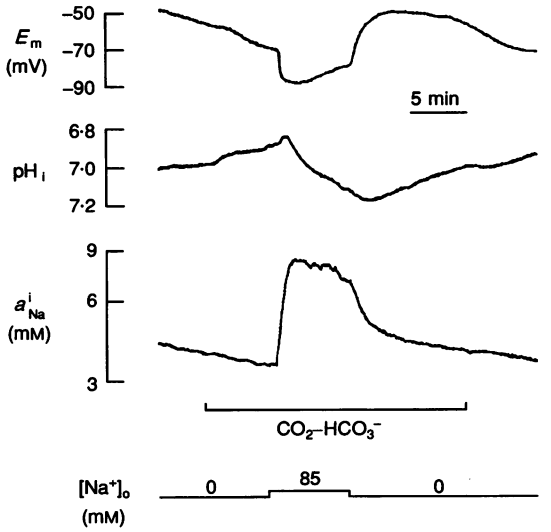

Figure 2.
Changes of intracellular Na^+ (a^i_{Na}, lower trace), intracellular pH (pH_i, middle trace) and membrane potential (E_m, upper trace) during brief re-addition of Na^+ to a Na^+-free saline; Na^+ was replaced by *N*-methyl-D-glucamine and records were made simultaneously with a triple-barrelled ion-sensitive microelectrode.

This experiment also suggests that the glial membrane has no significant HCO_3^- conductance, because this would tend to hyperpolarize (transiently) and alkalinize the cell due to the large inward gradient of HCO_3^- at the time of CO_2–HCO_3^- addition. In the voltage-clamp recordings (Fig. 1*B*), the addition of CO_2–HCO_3^- also provided no evidence for a HCO_3^- conductance, which should show up as a transient *outward* current due to HCO_3^- influx. However, a small inward current was recorded under these conditions.

A short exposure to Na^+-containing saline in the presence of CO_2–HCO_3^- produced a membrane hyperpolarization, an intracellular alkalinization and a substantial rise in a^i_{Na} (Fig. 2). Removal of CO_2–HCO_3^- in the absence of external Na^+ again produced only very small or no changes in membrane potential, pH_i and a^i_{Na}, indicating that external Na^+ is also necessary for the reversal of the membrane potential and ion changes (see also Deitmer, 1992*b*). Addition of CO_2–HCO_3^- in the presence of external Na^+ produced an average intracellular alkalinization from 6·96 to 7·25 and a rise of a^i_{Na} from 5·2 to 7·4 (Deitmer, 1992*b*).

Both types of experiments confirm that the electrogenic Na^+–HCO_3^- cotransport is stimulated under these conditions.

Accordingly, the Na^+- and HCO_3^--dependent currents measured are believed to be due to the stimulation of this electrogenic Na^+–HCO_3^- cotransport. Addition of CO_2–HCO_3^- stimulates inward flow of more HCO_3^- than Na^+, which is indicated by the rapid outward current. The partial recovery of this outward current presumably reflects the

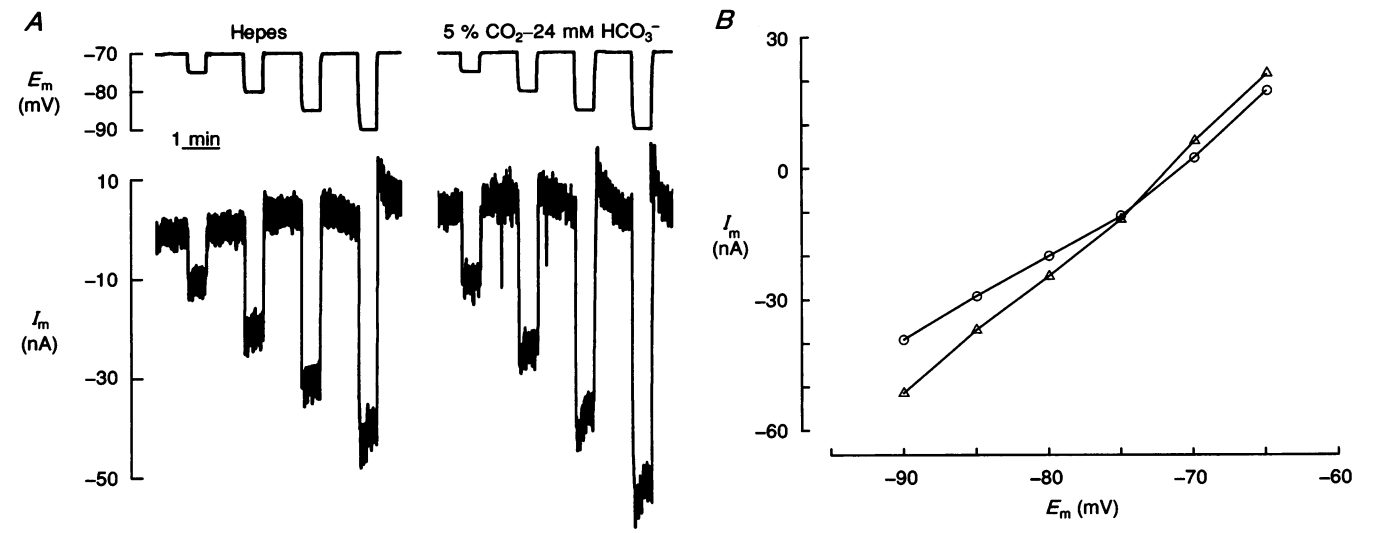

Figure 3.
A, membrane currents (I_m, lower traces) during slow, 30 s voltage steps (upper traces) between −70 and −90 mV in CO_2–HCO_3^--free (left) and CO_2–HCO_3^--buffered saline. Note the increase in the total current in the presence of CO_2–HCO_3^-. *B*, voltage relationship of the total current at the end of the 30 s voltage pulses from the experiment shown in *A* in the absence (○) and presence (△) of CO_2–HCO_3^-. The current–voltage curves intersect at the reversal of the CO_2–HCO_3^--dependent current component at −74 mV.

decay of the Na^+ and HCO_3^- influx. Similarly, upon addition of Na^+ to a Na^+-free saline, this outward current is linked to the activation of the inward-going Na^+–HCO_3^- cotransport. Upon removal of CO_2–HCO_3^- or Na^+ from the saline, the cotransporter is transiently *reversed* (Deitmer, 1991) and carries more HCO_3^- than Na^+ out of the cell. This is confirmed by the rapid fall of a^i_{Na} and pH_i.

Voltage dependence of the current

We stepped from a holding potential of between −60 to 80 mV for 30 s to different potentials and recorded the equivalent membrane currents in the absence and in the presence of CO_2–HCO_3^-. Figure 3*A* shows an experiment, where the cell membrane was held at −70 mV and changed in 5–20 mV steps to more negative potentials. The total current measured was larger in saline containing CO_2–HCO_3^-. The current–voltage relationships in the two different salines are plotted in Fig. 3*B*. The two curves display almost linear current–voltage relationships. They intercept at the reversal of the HCO_3^--dependent current, which was −74 mV in this experiment. On average, a reversal potential (E_{rev}) of −74·2 ± 10·9 mV was measured in fourteen cells. This corresponds well with the mean equilibrium potential of the Na^+–HCO_3^- cotransporter as determined by the steady-state ion activities of Na^+ and HCO_3^- ($E_{NaHCO_3} = -74$ mV, for a stoichiometry of 2 HCO_3^-: 1 Na^+; Deitmer, 1992*b*), whereas the measured E_{rev} is clearly different from the theoretical value of −41 mV for a stoichiometry of 3 HCO_3^- : 1 Na^+ (Deitmer, 1992*b*).

Since we had shown that the Na^+–HCO_3^- cotransport is sensitive to the stilbene DIDS (Deitmer & Schlue, 1989; Deitmer, 1991), we applied 0·5 mM DIDS during the voltage-clamp experiments after introducing CO_2–HCO_3^- (Fig. 4*A*). While in the presence of CO_2–HCO_3^-, the currents increased during the voltage steps; DIDS reversed this increase, indicating inhibition of HCO_3^--dependent current. In Fig. 4*B* the HCO_3^--dependent currents of two different experiments were plotted against the membrane voltage before and after application of DIDS. It shows that up to 90 % of the HCO_3^--dependent current is blocked by DIDS, and that the DIDS-resistant current was voltage insensitive over the range tested. Similar results were obtained in a total of three similar experiments, with holding potentials of either −60 or −70 mV.

Using DIDS as a tool for dissecting a Na^+–HCO_3^--cotransporter current could also be a method of determining E_{rev}, because DIDS would block all carrier current, even the residual current in HCO_3^--free solutions. We preferred the method of determing E_{rev} by using the presence/nominal absence of HCO_3^- since DIDS also blocks different anion exchangers and chloride channels in glial membranes (Gray & Richtie, 1986). Hence DIDS is not a very specific tool for dissecting a Na^+–HCO_3^- cotransporter current.

HCO_3^--dependent current and pH_i changes

In a series of experiments we have measured changes in pH_i of glial cells voltage clamped to different membrane potentials (Fig. 5). In the experiment shown, the glial

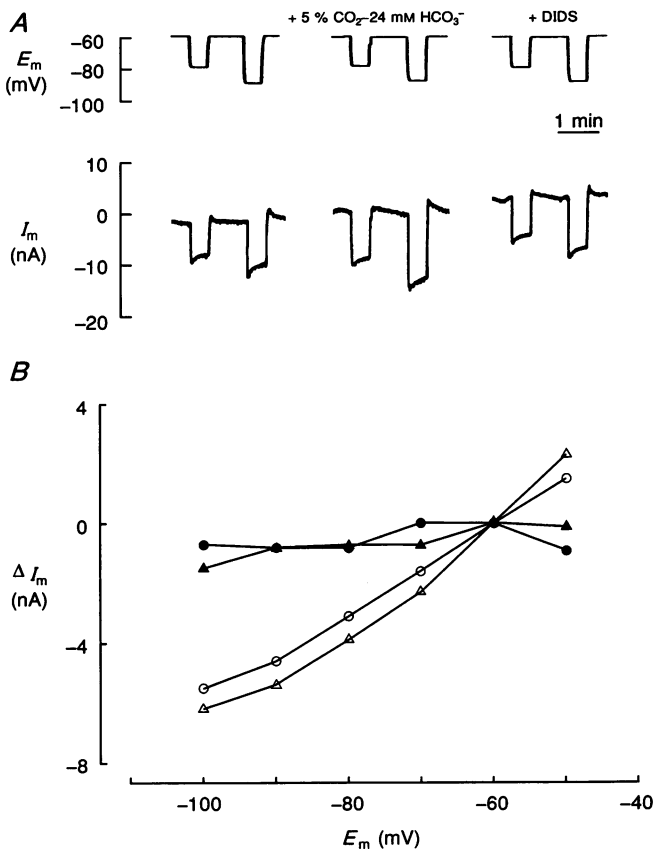

Figure 4.
The effect of the anion carrier blocker DIDS (diisothiocyanatostilbene-2,2′-disulphonic acid, 0·5 mM) on membrane currents evoked by slow, 30 s voltage pulses in the presence of CO_2–HCO_3^-. *A*, currents (lower traces) during two voltage steps from −60 to −80 and −90 mV in CO_2–HCO_3^--free saline (left) and in CO_2–HCO_3^--buffered saline in the absence (middle) and in the presence (right) of DIDS. Note that the CO_2–HCO_3^--induced increase in the total membrane current is reversed by DIDS. *B*, voltage relationships of the CO_2–HCO_3^--dependent current (I_m) obtained by subtracting the membrane current in CO_2–HCO_3^--free saline from that in CO_2–HCO_3^--buffered saline, from two experiments indicated by the different symbols (circles, triangles) in the absence of DIDS (○,△) and after addition of 0·5 mM DIDS (●,▲).

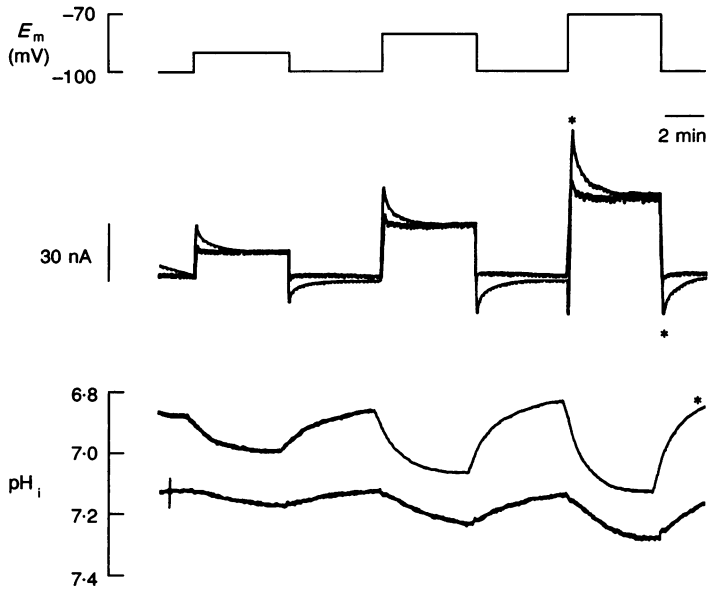

Figure 5.
Recording of intracellular pH (lower traces) and membrane current (middle traces, superimposed) during slow, 5 min voltage steps between −70 and −100 mV (upper trace) in the absence and in the presence (*) of CO_2–HCO_3^-. Note the additional, largely transient, extra currents and the increased pH_i changes in the saline containing 5 % CO_2–24 mM HCO_3^-.

membrane potential was held at −100 mV. The steady-state pH_i was lower in the presence of CO_2–HCO_3^- presumably due to the negative holding potential of −100 mV, but pH_i changed much faster and to a larger extent when the holding potential was changed than in nominally CO_2–HCO_3^--free saline. Upon a 20 mV change from −100 to −80 mV, pH_i changed by 0·23 pH units with a maximum rate of 0·13 pH units min^{-1} in the presence and by 0·1 pH units with a maximum rate of 0·03 pH units min^{-1} in the absence of CO_2–HCO_3^-. The mean steady-state pH_i values were 7·11 ± 0·03 ($n = 3$) for a holding potential of −100 mV and 7·26 ± 0·11 ($n = 4$) for −80 mV in the absence of CO_2–HCO_3^-. After addition of CO_2–HCO_3^- the respective values were 6·89 ± 0·01 ($n = 3$) for −100 mV and 7·14 ± 0·14 ($n = 4$) for −80 mV. The difference in acid–base flux is even larger when the increased intracellular buffer capacity in CO_2–HCO_3^--buffered saline is taken into account (see Deitmer, 1991). The rate of acid–base flux ($J_{a/b}$) is given by

$$J_{a/b} = \Delta pH_i\ min^{-1} \times \beta_t, \quad (1)$$

where β_t is the total buffering power, which is the sum of the intrinsic buffering power (β_i) and the CO_2–HCO_3^--dependent buffering power (β_{CO_2}). With the values for β_t from Deitmer (1991) the maximum rate of acid–base flux in the presence of CO_2–HCO_3^- increased in an approximately linear manner with the size of the voltage step and amounted to about 2·7 mM min^{-1} per 10 mV change in membrane potential between −100 and −70 mV (Fig. 6). In contrast, in the nominal absence of CO_2–HCO_3^-, this maximum rate of acid–base flux was only 0·4 mM min^{-1} per 10 mV. Thus, the acid–base flux in a nominally HCO_3^--free solution could amount to about 15 % of the value for CO_2–HCO_3^--buffered solutions. The difference of 2·3 mM min^{-1} is thus due to the presence of HCO_3^-, indicating that the acid–base flux was carried by HCO_3^-. During the depolarizing steps from −100 mV this would mean HCO_3^- influx, resulting in the intracellular alkalinization.

An additional, largely transient current was associated with the pH_i changes in the presence of CO_2–HCO_3^-. This peak current also increased linearly with larger changes of the membrane potential and displayed a voltage relationship very similar to that of the maximum rate of acid–base flux

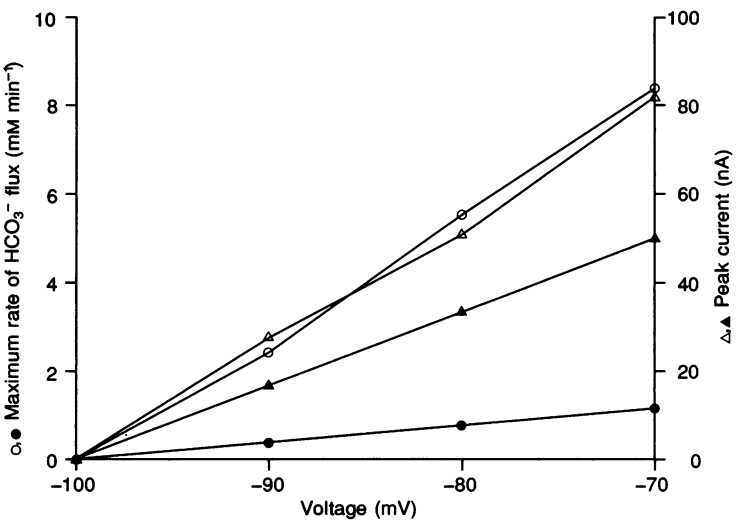

Figure 6.
Maximum rate of acid–base flux (left) and peak current (right) plotted *versus* the membrane voltage during 5 min voltage-clamp steps (for the experiment shown in Fig. 5) in the presence (○,△) and absence (●,▲) of 5 % CO_2–24 mM HCO_3^-.

(Fig. 6). If the peak current in the absence of CO_2–HCO_3^- was subtracted, a value of about 12 nA per 10 mV membrane potential change was obtained. This value is equivalent to the HCO_3^--dependent component of the total current.

The decay of the transient HCO_3^--dependent current was mono-exponential with a time constant of 34 ± 5·3 s (± S.D., $n = 9$). The rates of decay were similar for all depolarizing voltage steps between −100 and −50 mV.

In summary, the correlation between HCO_3^--dependent peak current and the maximum rate of acid–base flux, which is largely a HCO_3^- influx during depolarizing voltage steps, demonstrates the presence of an electrogenic Na^+–HCO_3^- symport in the glial cell membrane.

Reversal of the Na^+–HCO_3^- cotransport by lowering pH_o

We have previously shown that lowering the pH of the saline reverses the Na^+–HCO_3^- cotransport, so that net transport is outward (Deitmer, 1991). This produces secretion of HCO_3^-, which would help to counteract an acidosis in the extracellular spaces (Deitmer, 1992*a*). We have now recorded the current initiated by reducing the external pH from 7·4 to 7·0 (Fig. 7). An inward current was evoked by lowering pH_o both in the absence and in the presence of CO_2–HCO_3^- (Fig. 7*A*). In CO_2–HCO_3^--free saline the inward current rose slowly and amounted to 7·6 nA (mean = 15·4 ± 7·7 nA, $n = 11$). In the presence of CO_2–HCO_3^-, the inward current increased more quickly and had an amplitude of 19 nA (mean = 29·2 ± 10·0 nA, $n = 11$). The mean values for pH_o-activated currents in the nominal absence (15·4 ± 7·7 nA) and in the presence of CO_2–HCO_3^- (29·2 ± 10 nA) were significantly different ($P < 0·01$, modified t test after Dixon & Massey, 1969). Additionally, this current showed a voltage dependence upon changing the holding potential from −65 to −80 mV, as was expected for the Na^+–HCO_3^--cotransporter current (Fig. 7*B*). Upon returning to normal pH_o (7·4), the current slowly decayed to the initial level in the absence of CO_2–HCO_3^-, whereas it reversed transiently to an outward current in the presence of CO_2–HCO_3^- (Fig. 7*A*, *B* and *C*). These currents during the exposure to pH 7·0 were largely inhibited in the presence of 0·5 mM DIDS (Fig. 7*C*), leaving an inward current of 2·5 ± 1·7 nA (± S.D., $n = 4$). This residual current showed *no* voltage dependence upon changing the holding potential from −65 to −80 mV (Fig. 7*D*), which indicates that DIDS also blocked some of the inward current at pH_o 7·0 flowing in the nominal *absence* of CO_2–HCO_3^-. We do not know the origin of this inward current, but it may be partly due to remnants of outward Na^+–HCO_3^- cotransport, as intracellular HCO_3^- can amount to a few hundred micromolar originating from atmospheric CO_2 dissolving in the saline, or due to inhibition of a voltage-dependent Cl^- current by DIDS.

The effect of substituting Li^+ for Na^+

In these experiments we substituted equivalent amounts of Li_2SO_4 for sodium gluconate to test whether Li^+ could replace Na^+ at the Na^+–HCO_3^- cotransporter site. In a CO_2–HCO_3^--buffered solution, removal of external Na^+ caused the membrane to depolarize (Figs 8*A*, 1*C* and 2). Brief re-addition of external Na^+ or Li^+ produced a large membrane hyperpolarization, indicative of activation of an inwardly directed electrogenic $Na^+(Li^+)$–HCO_3^- cotransporter (Fig. 8*A*). Under voltage clamp, addition of Li^+ or Na^+ to a Na^+-free solution resulted in similar outward currents of 25 nA amplitude (Fig. 8*B*). In five experiments the outward current amplitude in Li^+ was 119·8 ± 19·5 % (± S.D.) of that recorded in Na^+, but was not *significantly* different from the Na^+-induced current.

We have also measured pH_i and a^i_{Na} in the absence of external Na^+, which was replaced either by NMDG or by Li^+ (Fig. 9). The saline contained 2 mM amiloride to inhibit Na^+–H^+ exchange. Addition of Li^+ resulted in a rapid

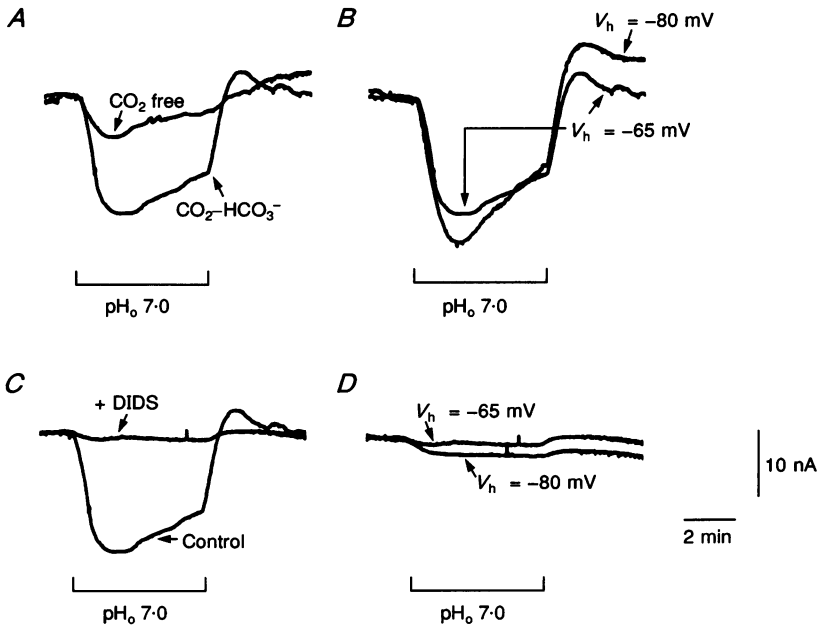

Figure 7.
Membrane inward currents evoked by the reduction of the external pH from 7·4 to 7·0, in CO_2–HCO_3^--free saline (*A*), and in CO_2–HCO_3^--buffered saline corresponding to a reduction of the external HCO_3^- concentration from 24 to 10 mM (at a constant CO_2 level of 5 %), at different holding potentials of −65 and −80 mV (*B* and *D*) and in the presence of 0·5 mM DIDS (*C* and *D*). Note that the large inward current and the transient, overshooting outward current following the return to pH 7·4 (and 24 mM HCO_3^-) are inhibited by DIDS (*C*).

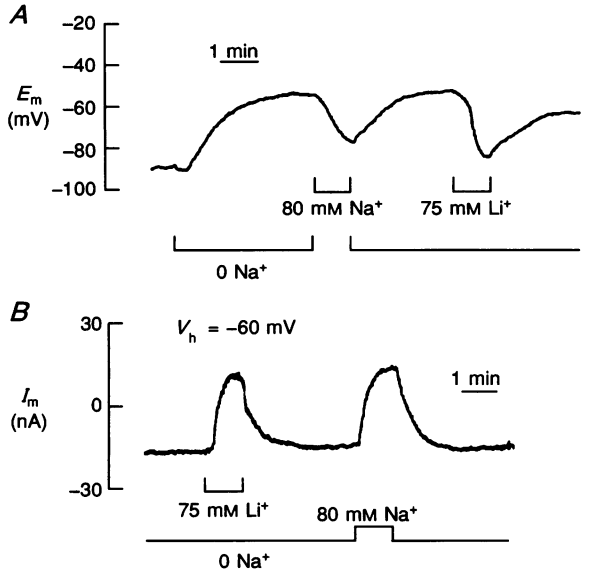

Figure 8.
Changes in membrane potential (*A*) and in membrane current (*B*) during 1 min exposures to Li^+ or Na^+, added to a Na^+-free saline (Na^+ replaced by *N*-methyl-D-glucamine).

intracellular alkalinization of about 0·35 pH units within 8 min. This response is similar to that observed when Na^+ was re-added (see Fig. 2, and Deitmer & Szatkowski, 1990), corroborating the conclusion that Li^+ can indeed substitute for Na^+ at the cotransporter site. The stilbene DIDS, which inhibits the Na^+–HCO_3^- cotransport, substantially reduced the pH_i change upon the addition of Li^+ (Fig. 9, $n = 3$).

Due to the absence of external Na^+, a^i_{Na} was very low. The electrode output corresponded to an a^i_{Na} of 1 mM, which may be partly due to some interference from other cations in the cell, in particular K^+. During the exposure to the Li^+-containing saline, a^i_{Na} apparently fell to even lower levels. However, this may indicate a reduction of the interference from other ions rather than reduction of a^i_{Na}.

It is readily apparent that the membrane potential changed only little during the exposure to Li^+. This is in contrast to the experiment shown in Fig. 8*A*, but, as previously stated, the Cl^- and K^+ conductances of the cell membrane were greatly reduced in those experiments. It appears that Li^+ may modify some of these ion conductances and thereby counteract the hyperpolarizing effect of the inwardly directed $Na^+(Li^+)$–HCO_3^- cotransport. This is supported by the fact that in the presence of DIDS a small depolarizing action of Li^+ is revealed, while in the absence of DIDS the membrane first hyperpolarized by 2–3 mV before it then slowly depolarized (Fig. 9).

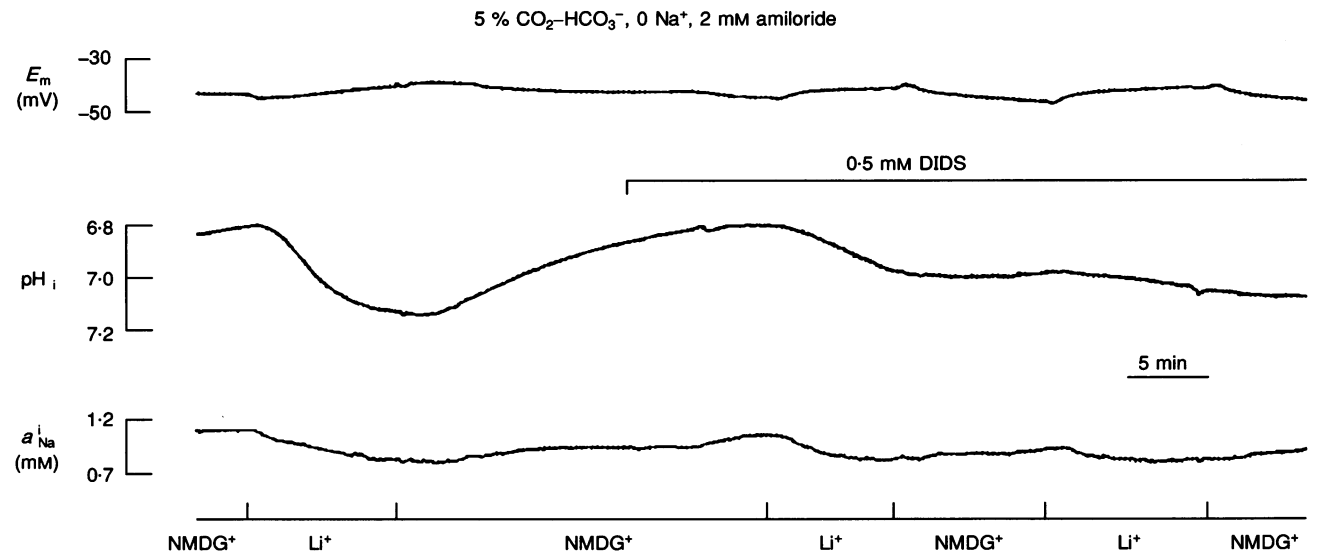

Figure 9.
Intracellular pH (pH_i, middle trace) recorded together with intracellular Na^+ (a^i_{Na}, lower trace) and membrane potential (E_m, upper trace) in Na^+-free, amiloride-containing saline, alternating between $NMDG^+$ and Li^+ as Na^+ substitutes before and after the addition of DIDS. Note the marked intracellular alkalinization in the presence of Li^+, which is inhibited by DIDS.

DISCUSSION

The present study has identified a Na^+- and HCO_3^--dependent current, which is sensitive to the stilbene DIDS, and which is accompanied by changes in intracellular pH and Na^+. The current is outwardly directed upon addition of CO_2–HCO_3^- and Na^+ (Li^+), and inwardly directed upon reducing the external pH (HCO_3^-) or Na^+. The current displayed a reversal potential near −74 mV, flowing outward at more positive potentials and inward at more negative potentials. The maximum rate of the HCO_3^- flux and the peak current showed a similar dependence on the membrane potential. In the absence of external Na^+, HCO_3^- had no effect on membrane potential, membrane current or pH_i, indicating that a HCO_3^- conductance, if present, is rather small and not detectable in our experiments. These results suggest that the current is due to the electrogenic Na^+–HCO_3^- cotransport previously described in these cells (Deitmer & Schlue, 1987, 1989; Deitmer & Szatkowski, 1990; Deitmer, 1991).

Voltage clamp of the glial cell

The neuropil glial cell has a cell body of 60–100 μm in diameter (79 ± 15 μm, $n = 37$; Pfeiffer, 1992), from which numerous wide and fine processes extend over a neuropil hemisphere. Hence, the cell membrane surface is very large. The electrical input resistance of this glial cell is relatively low (200–500 kΩ), due to large resting conductances to K^+ and Cl^- (Walz & Schlue, 1982; Ballanyi & Schlue, 1990; Wuttke, 1990). In order to improve the space clamp, we reduced the K^+ and Cl^- conductances, achieving a 2- to 3-fold increase in resting input resistance. Nevertheless, the control of the membrane potential in our slow, high-gain voltage clamp may be incomplete with regard to the fine cellular branches. It is therefore problematic to impose fast voltage changes, and to quantify early kinetics and absolute amplitudes of the current recorded. However, over the small voltage range measured, usually between −50 and −100 mV, the currents that were dependent on Na^+ and HCO_3^- and sensitive to DIDS showed a large reproducibility in amplitude and kinetics and had nearly linear voltage relationships (Figs 3 and 4).

Stoichiometry of the cotransport

The reversal potential of −74 mV determined by the voltage-clamp pulses in this study corresponds well to the equilibrium potential (E_{NaHCO_3}) calculated from the steady-state concentrations of extracellular and intracellular H^+ (HCO_3^-) and Na^+ (Deitmer, 1991, 1992*b*) according to the equation:

$$E_{NaHCO_3} = \frac{RT}{(n-1)F} \ln \frac{[Na^+]_o [HCO_3^-]_o^n}{[Na^+]_i [HCO_3^-]_i^n}, \quad (2)$$

where R, T and F have their usual thermodynamic meanings, and n is the HCO_3^- : Na^+ stoichiometry.

The value of −74 mV indicates that the reversal potential is very close to the resting membrane potential measured in these cells in intact ganglia (−73 mV; Deitmer 1992*b*). This implies that the cotransporter operates near its equilibrium at normal resting potential. Hence, our voltage-clamp experiments confirm the 2:1 stoichiometry, indicating that depolarization of the glial membrane from a resting value near −73 mV stimulates inward flux of Na^+ and HCO_3^-, while hyperpolarization drives Na^+ and HCO_3^- efflux. The linear I–V relationship is in accordance with a constant stoichiometry over this potential range.

The error in determining E_{rev}, as estimated from the difference of the voltage-clamp experiments in CO_2–HCO_3^--buffered saline and the experiments in nominally CO_2–HCO_3^--free saline could be as large as 10 mV, assuming a residual HCO_3^- flux in nominally CO_2–HCO_3^--free saline of 15 % of that in CO_2–HCO_3^--buffered saline. This would still favour a stoichiometry of 2 HCO_3^- : 1 Na^+, because the E_{rev} would still be more negative than −60 mV, i.e. about 20 mV more negative than −41 mV, which is the calculated reversal potential for a 3:1 stoichiometry.

Previous measurement of pH_i and a^i_{Na} were obtained in intact ganglia and in solutions containing normal leech saline (Deitmer, 1991, 1992*b*). In the present experiments, the ganglion was opened (see Methods) and the glial cell exposed *in situ*. This clearly shows that the mode of operation of the cotransporter is virtually unchanged when the neuropil and their two glial cells were exposed.

The reduction of the Cl^- concentration in the saline to 6 mM, to reduce the Cl^- conductance of the glial membrane, appeared to have no effect on the reversal potential of the cotransporter. This confirms that Cl^- is not involved in the cotransporter (Deitmer & Schlue, 1989). Similarly, the reduction of the K^+ conductance by Ba^{2+} had no impact upon reversal potential and stoichiometry of the Na^+–HCO_3^- cotransporter (see also Deitmer & Schlue, 1989). Both manipulations, however, lead to an increase in the membrane potential changes produced by activation of the electrogenic Na^+–HCO_3^- cotransporter, as for example during application of CO_2–HCO_3^- or Na^+ (Li^+), due to the increased membrane input resistance.

In an electroneutral cotransport, Na^+ and HCO_3^- would be carried into the cell due to the large inwardly directed Na^+ gradient, which is about twice that of the HCO_3^- outward gradient. Only under extreme conditions, i.e. in high intracellular Na^+ and/or an increased HCO_3^- gradient (i.e. high intracellular pH, low extracellular pH) could the cotransport with a stoichiometry of 1:1 eventually carry Na^+ and HCO_3^- *out* of the cell. This would lower intracellular pH and increase the extracellular HCO_3^- and buffer capacity. An electroneutral Na^+–HCO_3^- cotransport was reported for cultured oligodendrocytes (Kettenmann & Schlue, 1988) and recently in mammalian cardiac muscle fibres (Dart & Vaughan-Jones, 1992; Lagadic-Gossmann *et al.* 1992).

With a stoichiometry of 1 Na^+:3 HCO_3^-, the outwardly directed HCO_3^- gradient and the negative membrane potential would overcome the large inwardly directed Na^+ gradient and extrude Na^+ and HCO_3^-. Hence, activation of the cotransport would acidify the cytoplasm and depolarize the cell membrane. At the basolateral membrane of the proximal kidney tubule, this mode of cotransport returns

base equivalents into the blood (Boron & Boulpaep, 1983). Only large depolarizations of the cell membrane well beyond −40 mV would reverse the cotransport to an inwardly directed mode.

In frog retinal epithelium, the stoichiometry was determined to be 1 Na^+ : 2 HCO_3^-, as in leech glial cells, but with a more negative reversal of near −114 mV (Hughes *et al.* 1989). Here, the driving force for fluid absorption between the photoreceptors and the choroidal blood supply is delivered by the HCO_3^- transport.

The apparent difference in stoichiometry of Na^+–HCO_3^- cotransporters reported for different tissues and species points to the existence of different numbers of anion binding sites and/or transport modes, such as for the monovalent anion HCO_3^- or the ion pair $NaCO_3^-$. The selectivity of the anion binding site appears to be much higher for HCO_3^- (or CO_3^{2-}) than for Cl^- in all epithelial and glial cells studied so far, independent of the stoichiometry found.

HCO_3^--dependent current and flux

The simultaneous recording of the pH_i changes and the peak currents in the presence of CO_2–HCO_3^- showed that both the HCO_3^--dependent maximum rate of acid–base flux and the HCO_3^--dependent peak current are linearly dependent upon the membrane potential. There was a good correlation between the voltage dependence of flux rate and the peak current, corroborating the conclusion that they are both produced by the same process.

The HCO_3^- current is not likely to be due to a HCO_3^- conductance, since in Na^+-free saline HCO_3^- had no effect on membrane potential and current, and also did not alkalinize the glial cell. Unless there is a Na^+-dependent HCO_3^- conductance, it must be concluded that the major portion, if not all, of the HCO_3^--dependent current is carried by the electrogenic Na^+–HCO_3^- cotransporter.

It appears unvalid to quantitatively compare the values of acid–base flux and current calculated in terms of charge movement with a given stoichiometry of the underlying process, since volume and membrane surface of the giant glial cell cannot be estimated with reasonable accuracy due to the numerous cell processes, membrane infoldings, etc. Since the changes in pH_i (and hence $[HCO_3^-]_i$) greatly depend upon the membrane surface-to-cell-volume ratio, it is likely that the impact of the cotransporter is much larger in the glial cell processes with a larger surface-to-volume ratio. This, in turn, makes some assumptions about the homogeneity of the cotransporter in the glial cell membrane, as was shown in retinal Müller glial cells (Newman, 1991). With our recording site of the pH-sensitive microelectrode somewhere in the cell body, and the imperfect voltage clamp along all the cell processes, no clues can be obtained to the density and distribution of the electrogenic cotransporter in this glial cell. Application of the faster fluorescence ratio technique to meaure pH_i in smaller and more definite volumes, such as, for example, in the cable-like processes extending from the cell body, may therefore be necessary in combination with a fast and tight voltage clamp.

Li^+ substitution for Na^+ at the cotransporter

Our experiments have shown that Li^+ can substitute for Na^+ to activate the cotransporter. When Li^+ was added to a Na^+-free saline in the presence of CO_2–HCO_3^-, an outward current was recorded, and the intracellular pH increased, indicative of accumulation of intracellular HCO_3^-.

In kidney epithelial cells, Li^+ was also found to maintain the cation–base cotransporter, suggesting that Li^+ can, at least partly, substitute for Na^+ at the cotransporter site (Soleimani, Lesoine, Bergman & Aronson, 1991). In sheep heart Purkinje fibres, however, where an electroneutral Na^+–HCO_3^- cotransport was recently described (Dart & Vaughan-Jones, 1992), Li^+ did not replace Na^+. This may reflect differences in the selectivity of the cation binding site of the carrier protein. It is also possible that the efficacy of Li^+ in substituting for Na^+ may be due to a $NaCO_3^-$ ion pair moving across the glial membrane. Li^+ is known to form $LiCO_3^-$ ions (Garrells, Thompson & Siever, 1961; Becker & Duhm, 1978), which may readily substitute for $NaCO_3^-$ at the anion binding site of the transporter. The ion pair hypothesis does not rule out the 1:2 stoichiometry of the transporter, because in this case the $NaCO_3^-$ ($LiCO_3^-$) ion pair would be the only substrate. Only a kinetic analysis of the $NaCO_3^-$ ion pair dependence of initial HCO_3^- fluxes at different extracellular pH and HCO_3^- concentrations would enable us to distinguish between Na^+–HCO_3^- cotransport or $NaCO_3^-$ ion pair transport as shown for frog retinal pigment epithelium, where evidence against an ion pair was found (La Cour, 1991).

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