Evidence that glial cells modulate extracellular pH transients induced by neuronal activity in the leech central nervous system

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1. The role of the giant neuropile glial cells in the buffering of activity-related extracellular pH changes was studied in segmental ganglia of the leech *Hirudo medicinalis* L. using pH-sensitive microelectrodes and a slow, two-electrode voltage-clamp system. Neuronal activity was induced by electrical stimulation of a ganglionic side nerve (20 Hz, 1 min).

2. In CO_2–HCO_3^--buffered saline the glial cells were depolarized by $6{\cdot}5 \pm 2{\cdot}3$ mV and alkalinized by $0{\cdot}024 \pm 0{\cdot}006$ pH units (mean ± S.D.) during the stimulation. The stimulation induced an acidification of $0{\cdot}032 \pm 0{\cdot}006$ pH units in the extracellular spaces (ECS).

3. Voltage clamping the glial cells suppressed the stimulus-induced glial depolarization and turned the intraglial alkalinization into an acidification of $0{\cdot}045 \pm 0{\cdot}021$ pH units ($n = 6$) that closely resembled the acidification observed in the presence of the anion transport blocker DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid, 0·5 mM), and in CO_2–HCO_3^--free saline.

4. Voltage clamping the glial cell resulted in the appearance of a distinct stimulus-induced extracellular *alkalinization* of $0{\cdot}024 \pm 0{\cdot}013$ pH units at the onset of the stimulation, as also observed during DIDS application and in the absence of CO_2–HCO_3^-.

5. The results suggest that glial uptake of bicarbonate is mediated by depolarization-induced activation of the electrogenic Na^+–HCO_3^- cotransport, which suppresses the profound alkalinization of the ECS during neuronal activity. This is the first *direct* evidence that glial cells actively modulate extracellular pH changes in a voltage-dependent manner.

In recent years evidence has accumulated that glial cells not only mediate extracellular potassium homeostasis (Coles, 1989), but also actively contribute to extracellular pH changes like those observed during activation of neurones (Chesler, 1990; Deitmer, 1992; Ransom, 1992). In general, these activity-induced extracellular pH transients consist of a biphasic alkaline–acid shift or of an acidification alone (Chesler, 1990). They are thought to arise from acid/base fluxes through transmitter-gated ion channels (Chesler & Kaila, 1992), and cellular acid secretion and/or interstitial CO_2 accumulation (Voipio & Kaila, 1993). The extracellular pH shifts are accompanied by an acidification of neurones (Ahmed & Connor, 1980; Rose & Deitmer, 1994), and by an intraglial alkalinization (Chesler & Kraig, 1989). Observations on the developing rat optic nerve and the rat spinal cord suggest that the glial alkalinization might acidify the extracellular spaces of the neuropile (ECS), thereby leading to a dampening of the activity-induced extracellular alkalinization (Ransom, Carlini & Connors, 1986; Sykova, Jendelova, Simonova & Chvatal, 1992). As extracellular acidifications of only 0·1 pH units have been shown to reduce neuronal excitability in the mammalian hippocampus (Balestrino & Somjen, 1988; Jarolimek, Misgeld & Lux, 1989), a glial acid secretion into the ECS during neuronal activity could dampen neuronal excitability (Deitmer, 1992; Ransom, 1992).

In order to study the mechanism of the glial cell contribution to extracellular H^+ homeostasis in the adult nervous system, we have investigated pH transients evoked by repetitive nerve stimulation (20 Hz, 1 min) in both neuropile glial cells and in the extracellular spaces of isolated segmental ganglia of the leech *Hirudo medicinalis* with double-barrelled pH-sensitive microelectrodes. We

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tried to elucidate the effect of the glial electrogenic Na^+–HCO_3^- cotransport (Deitmer & Schlue, 1989; Deitmer, 1991) on the stimulus-evoked intraglial and extracellular pH shifts by (1) voltage clamping the glial cell at its resting potential during the nerve stimulation, (2) applying the anion transport blocker DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid), and (3) comparing the pH changes in the presence and in the absence of CO_2–HCO_3^-.

METHODS

Preparation and nerve stimulation

The experiments were performed at room temperature (22–24 °C) in the anterior neuropile glial cell and in the ECS of isolated segmental ganglia of the leech *Hirudo medicinalis* L. The methods for dissection procedures and identification of the cells have been reported in detail previously (Deitmer & Schlue, 1989; Deitmer, 1991). Briefly, the isolated ganglia were pinned by their connectives to the silicone rubber base of an experimental chamber with a volume of about 0·2 ml that was grounded via an Ag–AgCl pellet. Measurements on single glial cells were performed with ganglia pinned ventral side upwards; for simultaneous measurements in both glial cells and the ECS, ganglia were positioned dorsal side upwards. To reduce the influence of contractions of smooth muscle fibres on the microelectrode recordings, the outer ganglion capsule was cut from posterior to anterior without damaging the neuronal cell bodies in the ganglion periphery.

The ganglia were superfused with leech saline at a rate of 2 ml min^{-1}. For electrical stimulation, an anterior ganglionic side nerve root was positioned in a glass suction electrode that was connected by two silver wires to an isolation unit of a stimulator (DS2, D100; Digitimer Ltd, Welwyn Garden City, UK). The stimulus parameters were adjusted to 5 V, 20 Hz and 1 min to obtain maximal pH responses.

Solutions

The CO_2–HCO_3^--buffered leech saline was continously bubbled with 5% CO_2–95% O_2 and had the following composition (mM): 63 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 24 $NaHCO_3$ and 5 Hepes. The nominally CO_2–HCO_3^--free leech saline was buffered with 20 mM Hepes and contained 72 mM NaCl to obtain iso-osmolarity. The pH of all solutions was adjusted to 7·40 with NaOH or HCl.

The stilbene DIDS (0·5 mM) was obtained from Sigma (Buchs, Switzerland) and added to the saline shortly before use.

Intra- and extracellular pH recording

Reference potential and pH were measured with neutral carrier, pH-sensitive microelectrodes, which were constructed, calibrated and tested as described before (Deitmer, 1991). The H^+ cocktail (Hydrogen Ionophore I, Cocktail A, Fluka 95291; Fluka Biochemika, Buchs, Switzerland) was covered by 0·1 M sodium citrate (pH 6·0) and the reference barrel was filled with 3 M KCl. The electrodes responded with a slope of, on average, 55 mV per pH unit and the resolution was usually around 0·3 mV, which corresponds to about 0·005 pH units. A response of up to 3 mV to CO_2–HCO_3^--buffered solutions of the same pH was accepted.

The electrometer outputs were low-pass filtered (5 Hz) and were recorded by a skript chart (Kontron 520).

Glial voltage-clamp recording

Single-barrelled microelectrodes (from 1·5 mm glass capillaries) were filled with 3 M KCl for current injection and voltage recording and connected to the headstages of an Axoclamp-2A amplifier (Axon Instruments Inc., Foster City, CA, USA). Membrane currents were recorded by the built-in current measurement circuit of the headstages. For combining the voltage clamp with the intracellular pH-sensitive microelectrode measurements, the double-barrelled pH-sensitive microelectrodes 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.

The neuropile glial cell has a cell body of 60–100 μm in diameter, from which numerous wide and fine processes extend over a neuropile hemisphere. The electrical input resistance of this glial cell is relatively low (around 500 kΩ). Therefore, the control of the membrane potential in our slow, high-gain voltage clamp is presumably incomplete with regard to the fine cellular branches (Munsch & Deitmer, 1994). However, an influence of glial voltage clamping on the stimulus-evoked pH transients in the ECS could be observed when the extracellular pH-sensitive microelectrode was positioned close to the glial cell body. In the cell body, the stimulus-evoked depolarization was suppressed to less than half a millivolt during voltage clamp.

The data are presented as means ± S.D. and were statistically analysed by a modified t test after Dixon & Massey (1969; $P < 0·05$).

RESULTS

The intracellular pH (pH_i) of leech neuropile glial cells is mainly regulated by an electrogenic Na^+–HCO_3^- cotransport, which operates at a stoichiometry of 2 HCO_3^-:1 Na^+ (Deitmer & Schlue, 1989; Deitmer, 1991). Since its reversal potential is close to the resting membrane potential (Deitmer, 1991; Munsch & Deitmer, 1994), membrane depolarizations lead to an inward transport and membrane hyperpolarizations to an outward transport of Na^+ and HCO_3^- (Deitmer & Szatkowski, 1990).

In order to study the role of glial Na^+–HCO_3^- cotransport in the regulation of *extracellular* pH, we investigated the influence of voltage clamping the neuropile glial cells near their resting potential on the stimulus-evoked intraglial and extracellular pH transients.

In saline buffered with 5% CO_2–24 mM HCO_3^- (pH 7·4), the neuropile glial cells had a resting membrane potential of −72·4 ± 6·7 mV (mean ± S.D.) and an intracellular pH (pH_i) of 7·23 ± 0·08 ($n = 14$). At the onset of electrical nerve stimulation the glial cells depolarized by 6·5 ± 2·3 mV ($n = 14$), presumably due to the transient rise in extracellular potassium (Rose & Deitmer, 1994). When the stimulation was discontinued, the glial membrane potential repolarized towards the resting membrane potential; ten out of fourteen cells transiently hyperpolarized by 1·5 ± 0·8 mV (Fig. 1*A* and *B*). The stimulation induced an intraglial alkalinization of 0·024 ± 0·006 pH units that lasted during the entire stimulation period and

then recovered within 10–60 s ($n = 14$; Fig. 1*A*). In nine out of fourteen experiments, a weak intraglial acidification of 0·017 ± 0·007 pH units was observed when the stimulation was discontinued.

When the glial cells were voltage clamped by a slow, two-electrode voltage-clamp system, the stimulus-evoked glial depolarization was suppressed to less than 0·5 mV, and an average inward current of −76·3 ± 45·4 nA was recorded. An outward current of 11·7 ± 7·4 nA, corresponding to the membrane after-hyperpolarization in unclamped cells, was elicited when the stimulation was discontinued ($n = 14$; Figs. 1*A* and *B*).

During voltage clamp, the stimulus-evoked glial alkalinization seen in the control was reversibly changed into an intracellular acidification of 0·045 ± 0·021 pH units ($n = 6$). The acidification reached its peak 30–60 s after the stimulation ended, and then slowly recovered towards the resting pH_i (Fig. 1*A*). After releasing the voltage clamp, the stimulation again induced a glial membrane depolarization and an intraglial alkalinization as before (Fig. 1*A*).

Voltage clamping the glial cells in the presence of CO_2–HCO_3^- not only influenced the intraglial stimulus-evoked alkalinization, but also significantly changed the stimulus-evoked pH changes in the ECS recorded in close vicinity to the glial cell body.

In the control, the steady-state extracellular pH (pH_e), which was 7·35 ± 0·08 pH units, decreased by 0·036 ± 0·010 pH units during the electrical stimulation. This stimulus-evoked extracellular acidification reached its peak during the stimulation (Fig. 1*B*). In one out of eight experiments a small extracellular alkalinization (< 0·01 pH units) preceded the acidification at the onset of the nerve stimulation.

In contrast, during glial voltage clamp, an extracellular alkaline shift of 0·024 ± 0·013 pH units became apparent at the onset of the nerve stimulation ($n = 8$). This alkalinization lasted about 10 s and was followed by an acidification, the amplitude of which was not significantly different from the control (Fig. 1*B*). These effects of glial voltage clamping on the stimulus-evoked extracellular pH shifts were reversible. After releasing the voltage clamp, the extracellular alkalinization disappeared and the nerve stimulation again evoked an acidification alone (Fig. 1*B*).

In experiments where the pH-sensitive microelectrodes were not positioned close to the glial cell body, but elsewhere in the ECS, no change in the extracellular stimulus-evoked pH shifts was observed during glial voltage clamp as compared to the control ($n = 10$).

We repeated the nerve stimulation before and during application of the anion transport blocker DIDS (0·5 mM), which inhibits the electrogenic Na^+–HCO_3^- cotransport in this and other preparations (Deitmer & Schlue, 1989; Newman & Astion, 1991). DIDS itself led to a glial membrane depolarization by 3–4 mV and to a steady pH_i decrease of about 0·2 pH units. The amplitude and time course of the stimulus-evoked glial depolarization was

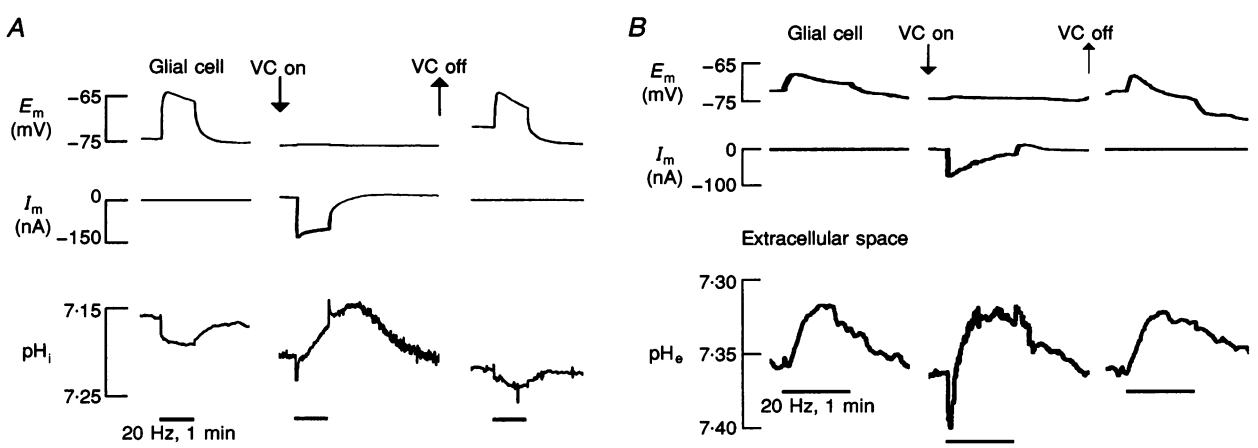

Figure 1. Effects of voltage clamping neuropile glial cells on intraglial and extracellular pH transients

A, the nerve stimulation evoked a membrane depolarization and an intracellular alkalinization of the neuropile glial cells (left). During suppression of the stimulus-evoked depolarization by voltage clamping the glial cell, an inward current was recorded, and the intraglial alkalinization was reversibly turned into an acidification (middle). In these experiments, stimulus artifacts presumably produced by intracellular coupling between the current injection electrode and the high-resistance pH electrode were observed at the onset and at the ending of the nerve stimulation. The effects of glial voltage clamping were reversible (right). *B*, voltage clamping the glial cells reversibly influenced the stimulus-evoked pH changes in the extracellular spaces: instead of an extracellular acidification alone (left and right), in addition a distinct alkalinization preceded the acidification when the stimulus-evoked glial depolarization was suppressed (middle). Bars indicate the duration of the nerve stimulation; E_m, glial membrane potential; I_m, membrane current; pH_i, intraglial pH; VC on/off, voltage clamp on/off; and pH_e, extracellular pH.

virtually unchanged by DIDS ($n = 7$; Fig. 2*A*). DIDS, however, led to similar changes in the stimulus-evoked intraglial pH shift as observed during glial voltage clamp. Instead of an alkalinization, the stimulation now elicited an intraglial acidification, which amounted to 0·024 ± 0·008 pH units and only partially recovered ($n = 7$; Fig. 2*A*).

During the DIDS application no change in the extracellular baseline pH was observed. However, a distinct extracellular alkalinization of 0·034 ± 0·012 pH units was evoked at the onset of the electrical stimulation in the presence of DIDS. This alkaline shift lasted 10–15 s and was followed by an acidification of 0·039 ± 0·039 pH units ($n = 8$; Fig. 2*B*). The amplitude of this acidification was not significantly different from that in the control; however, the recovery tended to occur more slowly.

To suppress glial Na^+–HCO_3^- cotransport directly, we also performed experiments in Hepes-buffered, nominally CO_2–HCO_3^--free saline. In this saline the glial membrane potential was −63·5 ± 5·9 mV, and the pH_i amounted to 6·93 ± 0·07 ($n = 10$). The glial hyperpolarization and alkalinization in the presence as compared with the absence of CO_2–HCO_3^- is due to the activation of glial Na^+–HCO_3^- cotransport (Deitmer & Schlue, 1989).

The glial membrane potential changes during nerve stimulation remained virtually unchanged in the absence as compared with the presence of CO_2–HCO_3^- (Fig. 2*C*); however, they were accompanied by a transient intraglial acidification. This acidification reached its peak of 0·026 ± 0·007 pH units at the end of the stimulation, and then recovered within 2–4 min (Fig. 2*C*). The acidification was preceded by a short alkalinization of 0·012 ± 0·002 pH units in five out of ten experiments.

In CO_2–HCO_3^--free saline, which was buffered with 20 mM Hepes to obtain a comparable pH_e as in the presence of CO_2–HCO_3^-, the stimulation led to a fast alkalinization of the ECS by 0·053 ± 0·034 pH units, followed by an acidification of 0·034 ± 0·018 pH units ($n = 5$) that lasted several minutes (Fig. 2*D*). The extracellular pH changes were thus similar to those recorded when the glial cell was voltage clamped or in the presence of DIDS.

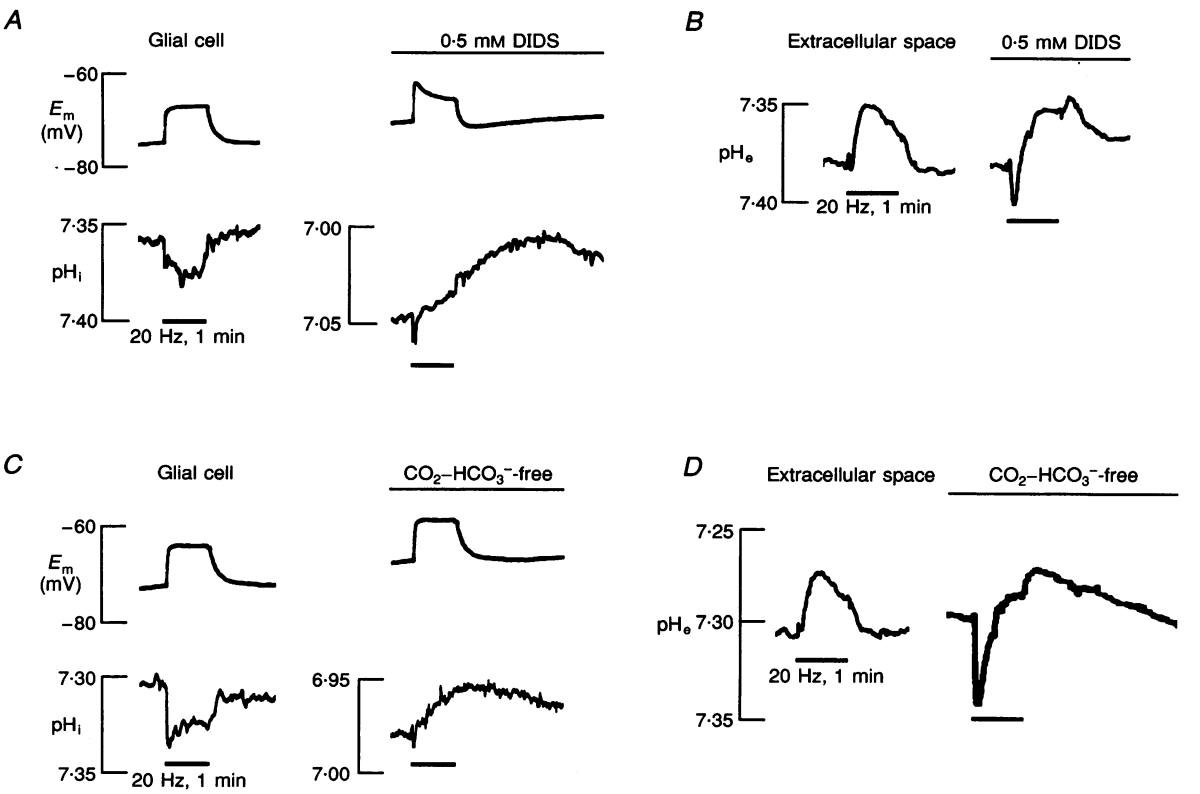

Figure 2. Effects of the stilbene DIDS and of CO_2–HCO_3^--free saline on intraglial and extracellular pH transients

A, the intraglial alkalinization during the nerve stimulation (left) was turned irreversibly into an acidification by DIDS (0·5 mM; right). *B*, DIDS led to the appearance of a distinct extracellular alkalinization at the onset of the electrical nerve stimulation (right) instead of an acidification alone (left). *C*, the stimulus-evoked intraglial alkalinization elicited in the presence of CO_2–HCO_3^- (left) was turned into an intraglial acidification in the absence of CO_2–HCO_3^- (right). *D*, nerve stimulation induced an extracellular acidification in CO_2–HCO_3^--buffered saline (left), and a biphasic extracellular alkaline–acid shift in CO_2–HCO_3^--free, Hepes-buffered saline (right). Bars indicate duration of the nerve stimulation; E_m, glial membrane potential; pH_i, intraglial pH; and pH_e, extracellular pH.

DISCUSSION

Our study provides the first direct evidence that glial cells actively regulate extracellular pH changes related to neuronal activity. The results demonstrate that the CO_2–HCO_3^--dependent, intraglial alkalinization observed during nerve stimulation is caused by depolarization-induced activation of inwardly directed, electrogenic glial Na^+–HCO_3^- cotransport. This interpretation is supported by the evidence that the stimulus-evoked intraglial alkalinization turned into an acidification (a) when the glial stimulus-induced depolarization was suppressed during glial voltage clamp, (b) during pharmacological inhibition of Na^+–HCO_3^- cotransport by the stilbene DIDS, and (c) in the nominal absence of CO_2–HCO_3^- (the substrate of the transporter). Moreover, these experimental protocols, preventing the activation of inward glial Na^+–HCO_3^- cotransport, de-masked an additional alkaline transient in the ECS at the onset of the nerve stimulation. This suggests that the depolarization-induced glial uptake of bicarbonate leads to the suppression of an extracellular alkalinization. A similar mechanism might also be present in vertebrate nervous systems, where evidence for electrogenic Na^+–HCO_3^- cotransport was recently given for several glial cell types (Newman & Astion, 1991; Brune, Fetzer, Backus & Deitmer, 1994). Indeed, stimulus-evoked alkaline shifts were also enhanced in mammalian preparations when glial depolarization was prevented in the presence of Ba^{2+} (Chesler & Kraig, 1989), or when the stilbene SITS (4-acetamido-4′-isothiocyanatostilbene-2,2′-disulphonic acid) was added to the superfusate (Sykova *et al.* 1992).

The stimulus-evoked extracellular pH changes in our study amounted to about 0·04 pH units. It appears likely, however, that the actual ion changes during neuronal activity were significantly larger, since the tips of the ion-sensitive microelectrodes (approx. 1 μm) would lead to some widening of the ECS. In the mammalian hippocampus, extracellular acidifications of only 0·1 pH units have been shown to decrease neuronal excitability, whereas alkalinizations increase the excitability (Balestrino & Somjen, 1988; Jarolimek *et al.* 1989). By reducing the alkalinization, and/or by increasing the acidification in the ECS during neuronal activity, glial uptake of bicarbonate could therefore mediate a decrease in neuronal excitability in a feedback-like manner (Ransom, 1992), and may also help to counteract neuronal hyperexcitability as observed during epileptiform discharges (Aram & Lodge, 1987).

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