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A proton-translocating H^+-ATPase is involved in C6 Glial pH regulation

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Abstract

Glial cells extrude acid equivalents to maintain pH_i. Although four mechanisms have been described so far, pH_i-control under physiological conditions is still not sufficiently explained. We therefore investigated whether a H^+-translocating ATPase is involved in glial pH_i homeostasis using an established glial cell line (C6 glioma). In the absence of bicarbonate, the inhibition of H^+-ATPases by NEM led to a pH_i decrease. The application of a more specific inhibitor (NBD-Cl) showed that the H^+-ATPase involved is of the vacuolar type. Inhibition went along with delayed cell swelling. Together with the fact that glial acidification was far more pronounced in Na^+-free media, this may serve as evidence for a secondary activation of Na^+/H^+-exchange once an activation setpoint is reached, which in turn causes secondary swelling from Na^+-uptake. Stimulation of Na^+/H^+-exchange by PMA can increase the setpoint. pH_i-recovery after an acid load was blocked by the inhibition of v-type H^+-ATPase, if pH_i did not reach 6.6 during the acid load. The inhibition of Na^+/H^+-exchange by amiloride inhibited recovery only if acidification was below the threshold. Finally, in bicarbonate-free media a v-type H^+-ATPase contributes to pH-regulation in glial cells, especially during pH-homeostasis at physiological conditions, while Na^+/H^+-exchange gains significance during severe acid loads.

Keywords: Astrocyte; C6 glioma; pH_i-regulation; Cell volume; Vacuolar type H^+-ATPase

1. Introduction

Intracellular pH (pH_i) is a parameter of vital significance for all cells, with many cellular functions being pH-dependent such as energy metabolism, enzyme activities and cell proliferation. Astroglial cells, in addition, are involved in the control of extracellular fluid pH [1], thereby possibly modulating neuronal activity [2]. It is, therefore, not surprising that efficient homeostatic mechanisms are available for glial pH maintenance: In a recent study, Shrode and Putnam [3] described four pH_i-regulating systems. According to their analysis, pH_i under physiological conditions is largely controlled by Na^+/H^+-exchange and Na^+-dependent Cl^-/HCO_3^--countertransport; the Na^+-independent Cl^-/HCO_3^--exchanger is thought to become active after intracellular alkalization, whereas Na^+/HCO_3^--cotransport plays a major role in invertebrate glial cells [4].

This concept, however, still leaves some questions open. It is unclear why the inhibition of Na^+/H^+-ex-

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change in the absence of bicarbonate ions does not regularly cause failure of pH-regulation [5,6], and why this system is considered the prime transporter of acid equivalents at physiological pH although in astrocytes it becomes fully activated only at pH_i-values far below 7.0, and, in the case of C6 glioma cells it is virtually inactive at physiological pH_i [7].

The problem might be solved by the inclusion of a fifth transport system into the framework of glial pH-control, a proton-translocating H^+-ATPase which has been recently described for glial cells [8]. H^+-ATPases so far have been studied best in epithelial cells which are capable to translocate protons by directed transport. An example is the brush border membrane of renal cells [9]. H^+-ATPases, however, are also responsible for pH-regulation of macrophages [10]. Interestingly, in these cells the H^+-ATPase is the primary pH-regulating system at physiological pH_i [11], whereas Na^+/H^+-exchange is activated only at more acidotic pH_i-levels.

This work, therefore, was designed to study the contribution of H^+-ATPases to glial pH_i regulation in more detail. An in vitro system was used, which allows the investigation of the contribution of single, defined transport mechanisms under controlled environmental conditions. As in previous studies [7,12–16], C6 glioma cells [17] were employed as model cells with many properties typical for astroglial cells in order to obtain a sufficient number of homogeneous cell cultures for statistical evaluation of the results.

There are several subtypes of H^+-ATPases (vacuolar-type H^+-ATPases, F_0F_1-ATPase, localized, e.g., in the mitochondrial membrane, and the E_1E_2-ATPases, e.g., gastric H^+/K^+-ATPase). Consequently, specific inhibitors were used in order to discriminate between these subtypes. Since the activation setpoints for various pH_i-regulating systems in glia are not known in detail, an additional attempt was made to define such thresholds for Na^+/H^+-exchange, the postulated 'physiological' pH-controlling transport system in the absence of bicarbonate. Cell volume is a sensitive parameter reflecting the net effect of ion fluxes across the cell membrane. It was also evaluated in this study (in addition to the determination of pH_i) since pH-control by Na^+/H^+-exchange differs in one essential point from the effect of H^+-ATPase: it is accompanied by changes of cell volume due to the gain of osmotically active sodium ions in exchange for protons which come from intracellular buffers and, therefore, are thought to be osmotically inactive [18].

2. Material and methods

2.1. Chemicals and solutions

Amiloride, *N*-ethylmaleimide (NEM), 7-chloro-4-nitroben-2-oxa-1,3-diazole (NBD-Cl), oligomycin, phorbol-12-myristate-13-acetate (PMA), vanadate and nigericin were purchased from Sigma (St. Louis, MO, USA). All other chemicals were obtained from Merck (Darmstadt, FRG). The HEPES-buffered experimental medium had the following composition: 130 mM NaCl, 3 mM KCl, 0.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM glucose and 40 mM HEPES. If sodium-free medium was required, NaCl was replaced by choline chloride.

The pH of the media was adjusted with HCl and NaOH or choline base in case of sodium-free medium and the osmolality was adjusted to 300 mOsm using NaCl or choline chloride.

2.2. Cell culture

C6 glioma cells were cultivated in petri dishes (Falcon Optilux 3003, Becton Dickinson, Plymouth, UK) using Dulbecco's modified Eagle's medium (DMEM; Boehringer Mannheim, FRG) with 100 IU/ml Penicillin G, 50 μg/ml Streptomycin and 10% fetal calf serum (FCS, Gibco, Paisley, UK). The cells were grown in humidified room air with 5% CO_2 at 37°C. Subcultivation was carried out daily after detachment of the cells from the petri dish using 0.05% trypsin/0.02% EDTA in phosphate-buffered saline (PBS).

For pH_i measurements, petri dishes with confluent cultures were inserted into a measurement chamber allowing for the control of temperature (37°C) and gas supply. Within this chamber, petri dishes were superfused with fresh medium using a peristaltic pump (IPS 4, Ismatec, Wertheim, FRG).

2.3. Measurement of intracellular pH

For pH_i measurement the pH-sensitive fluorescent dye 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein

(BCECF) was used. Adherent cells were incubated for 15 min with 4 μM BCECF-acetoxymethylester (BCECF-AM, Molecular Probes, Eugene, OR, USA), which diffuses into the cells and is hydrolyzed to BCECF by cytosolic esterases. The cultures were then washed and fresh medium was added.

In order to measure pH_i, cells were alternately illuminated at 442 nm and 492 nm (50 Hz) using an LTI Multiscan 2 system (AMKO, Hamburg, FRG). To prevent the dye from bleaching, the cells were illuminated only for 4 s each measurement, and kept dark otherwise. The emitted light was quantified by a photomultiplier (Leitz, Wetzlar, Germany). An A/D-board was used for on-line averaging of the signals for the two excitation wavelengths. The further evaluation of the accumulated data was performed by a PC using self-programmed BASIC software. The intracellular pH was calculated from the intensity ratio of the emitted fluorescence at two excitation wavelengths, thus correcting for inhomogeneous distribution of the dye, loss of dye, or photobleaching.

To calibrate the measurements, the nigericin method was used [19]. BCECF-loaded cells were incubated in isotonic media containing 120 mM K^+ and 7.5 μM nigericin. Calibration measurements were performed over a range of pH 6.0–8.0.

2.4. Acid loading

Acid loading was performed through the exposure of cells at 37°C to isoosmotic, HEPES-buffered media containing 25 mM NH_4Cl for 20 min followed by a rapid wash with NH_4Cl-free media. Typically, cells alkalize during NH_4Cl exposure, followed by a slow pH_i-normalization. At NH_4Cl withdrawal, the pH_i process becomes rapid (the so-called acid-load). pH_i-regulatory mechanisms were studied in the next phase of the experiment, using inhibitors of mechanisms that may be involved in pH_i-regulation. In respective experiments, NBD-Cl (100 μM) or amiloride (1 mM) were applied 10 s before and after NH_4Cl withdrawal.

2.5. Measurement of cell volume

For cell volume measurements cells were harvested, suspended in the medium, which was later used for the different experimental setups, and preincubated in a chamber placed directly under the measuring capillary of the cell analysis system (described below). This incubation chamber allows us to perform the experiments with close control over extracellular pH, temperature, O_2, CO_2 and N_2. A magnetic stirrer prevents cell sedimentation. The experiments were performed at 37° C.

Samples for cell volume measurement are directly drawn from the chamber into the 'Cell Analyser System CASY1 TTC' (Schärfe System, Reutlingen, Germany). The principle of volume measurement is based on an advanced Coulter method combined with modern signal processing procedures termed 'pulse area analysis' [20], allowing the discrimination of particle sizes with a dynamic range of 1:27.000 (smallest vs. largest particle).

2.6. Statistics

Results are expressed as mean $\pm$ standard error of the mean (SEM). The data were tested for statistical significance by the Kruskal–Wallis test for nonparametric one-way analysis of variance and multiple comparisons on ranks for unpaired samples using SigmaStat software (Jandel Scientific, Erkrath, FRG). Statistical significance was accepted at an error probability of $p < 0.05$.

3. Results

3.1. pH_i under physiological conditions

At an extracellular pH of 7.4, C6 glioma cells maintained their pH_i over 80 min at values slightly below the pH_e ($n = 5$, Fig. 1). Under control conditions pH_i in HEPES-media was 7.11 ± 0.07 ($n = 41$). Changing to Na^+-free HEPES-buffered media ($n = 7$) after a 15-min control period led to a slow, continuing decrease in pH_i from 7.06 ± 0.03 before Na^+-removal to 6.87 ± 0.04 after 60 min of Na^+-removal. From 30 min after Na^+-removal the decrease of pH_i was statistically significant as compared to control experiments without omission of Na^+. Application of 1 mM NEM, an inhibitor of H^+-ATPases, was followed by a quick decrease of pH_i ($n = 5$). After 15 min pH_i was reduced from 7.04 ± 0.01 to $6.70 \pm$

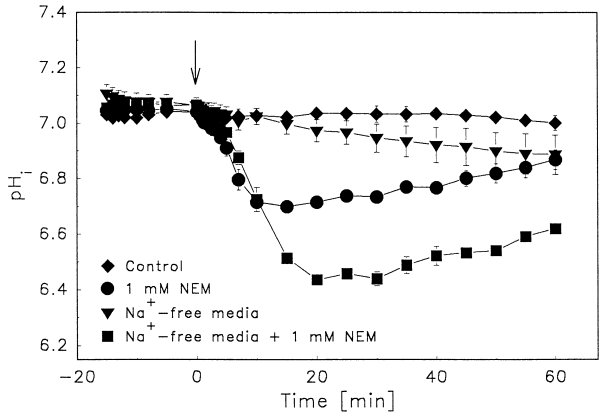

Fig. 1. The pH_i of C6 glioma cells after different treatments. In one group, the H^+-ATPase inhibitor NEM was applied, in the second, e.c. Na^+-ions were withdrawn, and in the third, both treatments were combined. Treatments were initiated at time 0 (arrow), all media were buffered by HEPES ($pH_e = 7.4$) (means $\pm$ SEM).

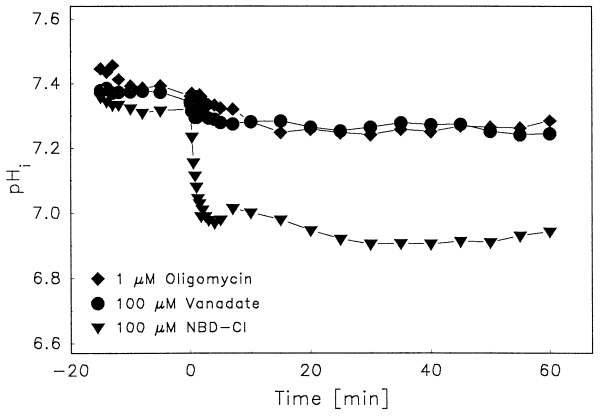

Fig. 2. The pH_i of C6 glioma cells after the application of inhibitors of different types of H^+-ATPases at time 0 (arrow) in HEPES-buffered media ($pH_e = 7.4$). A total of 100 μM NBD-Cl was used to inhibit v-type H^+-ATPase, 1 μM Oligomycin B to block F_0F_1-H^+-ATPases and 100 μM Na_3VO_3 for inhibition of E_1E_2 H^+-ATPases.

0.01. In the further course of the experiment a slight pH_i-recovery was observed, and after 60 min pH_i was 6.87 ± 0.04 (Fig. 1). Compared to the control group the change of pH_i is statistically significant between 5 and 50 min after NEM application. If NEM was applied in Na^+-free media ($n = 5$), the pH_i-decrease was even more pronounced. Within 20 min, pH_i decreased from 7.07 ± 0.02 to 6.44 ± 0.02. A slight recovery was seen during the following measurements, and after 60 min pH_i was 6.62 ± 0.02 (Fig. 1).

Since the specificity of the inhibitory action of NEM for vacuolar type H^+-ATPase remains unresolved, more specific inhibitors [21] were used in addition. Administration of NBD-Cl, an inhibitor of vacuolar (v-)type H^+-ATPases also caused a fast pH_i-decrease comparable to that observed after application of NEM, while there was no difference between 50 μM (data not shown) and 100 μM NBD-Cl (Figs. 2 and 4). In both cases pH_i was reduced after 4 min for 0.31 ± 0.06 (50 μM) or 0.35 ± 0.10 pH units (100 μM).

No effect was observed after the application of oligomycin or vanadate, which inhibited the F_0F_1-ATPase and E_1E_2-ATPases, respectively (Fig. 2).

If the pH_i decrease after the application of NEM or NBD-Cl is really caused by an inhibition of proton-translocating ATPases, then the withdrawal of ATP also should cause a pH_i decrease. Therefore, in an additional set of experiments iodoacetate (2.5 mM), an inhibitor of energy metabolism, was applied to the cells ($n = 7$). This resulted in a pH_i decrease from 7.27 ± 0.03 to 6.89 ± 0.03 within 20 min (Fig. 3). From 10 min after iodoacetate application pH_i was statistical significantly lower ($p < 0.05$) than in a control group without iodoacetate ($n = 6$).

In further experiments, it was tested whether a stimulation of Na^+/H^+-exchange would counteract a

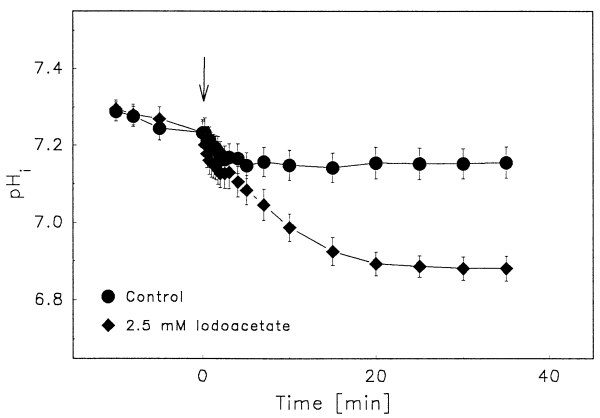

Fig. 3. The effect of inhibition of energy metabolism and, therefore, ATP-supply by 2.5 mM iodoacetate at time 0 (arrow) on pH_i of C6 glioma cells. Experiments were performed in HEPES-buffered media (means $\pm$ SEM).

NBD-Cl induced acidification. Hence, cells were preincubated with PMA (50 nM), a phorbol ester which activates Na^+/H^+-exchange via protein kinase C stimulation [22]. Incubation with PMA did not alter baseline pH_i which was 7.15 ± 0.03 ($n = 6$) and 7.16 ± 0.05 in the control group without preincubation ($n = 6$). After application of NBD-Cl, pH_i diminished in both groups (Fig. 4), but PMA pretreatment reduced the effect of NBD-Cl significantly ($p < 0.01$), with pH_i dropping to only 6.84 ± 0.06 30 min after addition of NBD-Cl as compared to 6.63 ± 0.03 in the non-pretreated group.

3.2. Cell volume

Upon suspension, the volume of C6 glioma cells decreased slightly before it stabilized 15 min after suspension in the incubation chamber. The average of three cell volume measurements taken during the final 15 min of the control period served as reference volume for each experiment. The thus determined baseline cell volume at pH 7.4 was 621 ± 5.01 fl. Incubation of the cells in DMEM for up to 150 min was accompanied by a minor volume decrease of $1.73 \pm 0.42\%/h$ ($n = 7$, Fig. 5). If 100 μM NBD-Cl was applied, cell volume began to increase with a 30-min delay, finally reaching a maximum at $137.20 \pm 0.98\%$ of control after 85 min of NBD-Cl exposure ($n = 7$, Fig. 5). Differences were statistically significant ($p < 0.05$) as compared to the baseline cell volume during the last 70 min of NBD-Cl exposure. In the absence of extracellular Na^+ cell swelling was significantly attenuated. Cell size remained at $98.64 \pm 2.16\%$ of the control volume ($n = 5$; Fig. 5).

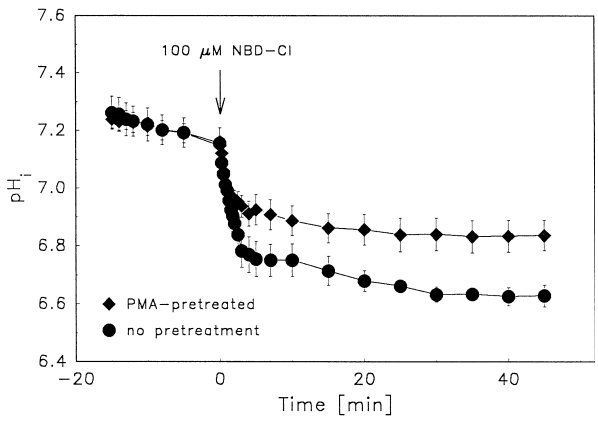

Fig. 4. The activation of Na^+/H^+-exchange by preincubation with 50 nM PMA attenuates the acidifying effect of 100 μM NBD-Cl applied at time 0. Experiments were performed with C6 glioma cells in HEPES buffered media.

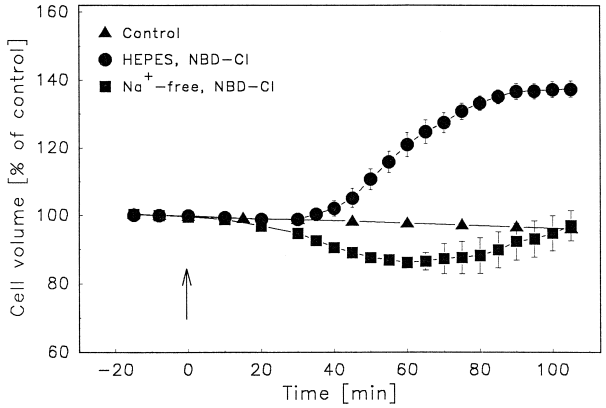

Fig. 5. Volume of C6 glioma cells after the application of 100 μM NBD-Cl and variation of the buffer conditions and e.c. Na^+-concentration at time 0 (arrow), respectively, compared to a control group without NBD-Cl (means ± SEM). Cells were suspended in an incubation chamber permitting a close control of extracellular conditions with $pH_e = 7.4$ and osmolality 300 mOsm.

3.3. pH_i recovery from an intracellular acid load

The application of 25 mM NH_4Cl goes along with a fast intracellular alkalization. pH_i then begins to decrease to reach the original level within 20 min. Removal of extracellular NH_4Cl now leads to a rapid drop in pH_i ('acid load'). pH_i thereafter normalizes as a result of pH_i-regulatory mechanisms (Fig. 6). Application of NBD-Cl inhibited this recovery completely ($n = 4$, Fig. 6). pH_i was 6.55 ± 0.04 at the end of the measurement as compared to 6.99 ± 0.09 in the untreated group ($n = 6$).

After exposure to amiloride (1 mM), which inhibits Na^+/H^+-exchange, an unexpected observation was made. In some experiments the pH_i recovery after an acid load was virtually unaffected, whereas in others almost no recovery was seen (Fig. 7). A closer look at the data revealed that in the subgroup showing a good recovery the degree of acidification was less pronounced than in the non-recovering subgroup. This can be concluded from parallel control experiments without inhibitor. In the first amiloride-treated subgroup ($n = 6$), 2 min 30 s after NH_4Cl

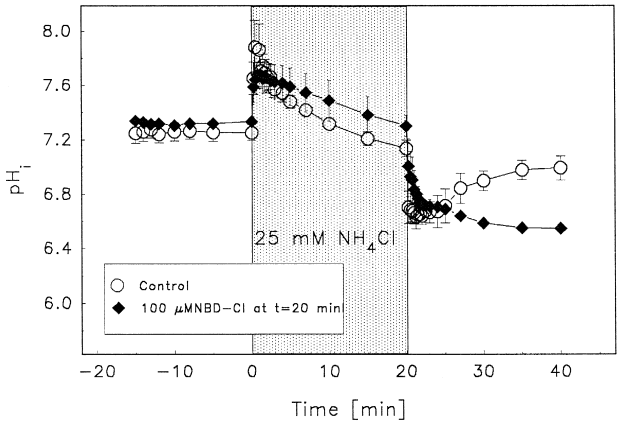

Fig. 6. The effects of 100 μM NBD-Cl on pH_i recovery after acid-loads with NH_4Cl in HEPES-buffered media (the shaded area indicates the presence of 25 mM NH_4Cl). Results are compared to matched controls without NBD-Cl. pH_i recovery after acid-loads could be prevented by NBD-Cl.

removal pH_i had reached its minimum (6.55 ± 0.06) and began to recover afterwards to finally 6.96 ± 0.15. In the second subgroup ($n = 4$) pH_i continued to decrease until a plateau was reached after 7 min. pH_i then remained stable and was 5.70 ± 0.20 at the end of the observation.

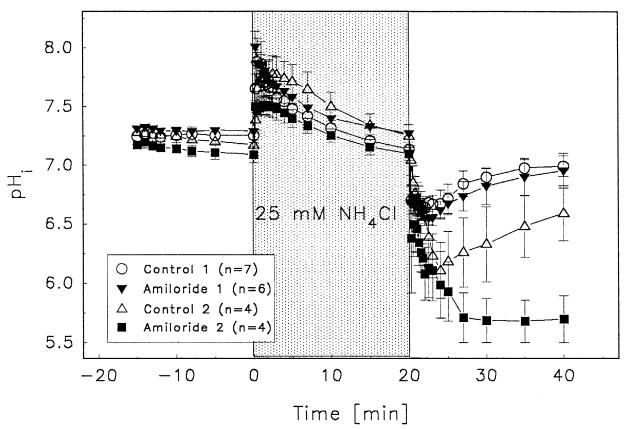

Fig. 7. The effects of 1 mM amiloride on pH_i recovery after acid-loads with NH_4Cl in HEPES-buffered media (the shaded area indicates the presence of 25 mM NH_4Cl). Results from two spontaneously formed experimental subgroups using cells grown from the same clone of C6 glioma were used for the amiloride experiment and compared to matched controls without amiloride. Amiloride could inhibit pH-recovery only if the acid-load caused pH_i to drop below 6.6.

4. Discussion

The data confirm that at a physiological pH_e protons leak into glial cells, a process which requires continuous active pH_i regulation. This is clearly evidenced by the drop of pH_i after either Na^+ withdrawal or exposure to specific inhibitors of H^+-ATPases. In the absence of active pH_i regulation the pH_i would follow a Donnan equilibrium, and should therefore be calculated according to Roos and Boron [23] as 6.59 for C6 glioma cells assuming a temperature of 37°C, a pH_e of 7.4 and a membrane potential of -50 mV [24].

Although the present results confirm that the Na^+/H^+-exchanger is involved in the regulation of pH_i in bicarbonate-free media, the contribution of the Na^+/H^+ exchanger to pH_i-control appears relatively low at physiological pH_i levels. This is suggested by the moderate decrease of pH_i after extracellular Na^+ removal, and is in good correspondence with results of Sapirstein and Benos [14], who observed only a low activity of the Na^+/H^+ exchanger under physiological conditions. The activity of H^+-ATPases in comparison is significantly higher during maintenance of pH_i at a physiological level, as expressed by the fast and pronounced pH_i decrease after application of NEM or NBD-Cl (Figs. 1 and 2). H^+-ATPases involved are likely to be of the vacuolar type, since NBD-Cl, which is commonly used as a specific inhibitor of v-type ATPases [for review, see Ref. [21]] had a similar effect as NEM, while oligomycin B, which inhibits the F_0F_1-ATPase, and vanadate, an antagonist of E1E2-ATPases, did not alter pH_i.

Following the inhibition of H^+-ATPases in HEPES buffered media pH_i decreases rapidly without, however, reaching pH_i values predicted by the Donnan equilibrium: there was even a partial pH_i recovery once a pH_i-threshold of approximately 6.7 had been reached (Figs. 1 and 2). Only in the absence of Na^+-ions, which prevented Na^+/H^+-exchange activity, glial acidification continued (Fig. 1). This indicates, that the effect of NEM is not caused by inhibition of the Na^+/H^+ exchanger. As these experiments were performed in HCO_3^--free HEPES-buffered media it can also be excluded that inhibition of HCO_3^--dependent mechanisms are responsible for the observed results. The assumption that the NEM or NBD-Cl induced acidification in fact is caused by

inhibition of H^+-ATPases is further supported by the observation, that inhibition of the ATP supply by iodoacetate also induces acidification (Fig. 3). As iodoacetate is not a selective inhibitor and has several unspecific effects, this observation is certainly no conclusive evidence. On the other hand, we have gained quite some experience with this substance and are confident that energy production is completely blocked [13]. Inhibition of glycolysis could lead to an intracellular acidification regardless of the H^+-ATPase, because elimination of energy supply also inhibits Na^+/K^+-ATPase, thus resulting in a decrease of the Na^+-gradient, which is the driving force for other pH-regulating systems (Na^+/H^+-exchange, Na^+/HCO_3^--cotransport or Na^+-dependent Cl^-/HCO_3^--exchange). But these mechanisms would not fail before the Na^+-gradient has decreased considerably. It is unlikely that the observed rapid acidification is due to a loss of the Na^+-gradient since a sufficient gradient is preserved in C6 cells for at least 30 min after inhibition of Na^+/K^+-ATPase [13]. Therefore the acidification during exposure to iodoacetate is in line with the hypothesis that in the absence of bicarbonate pH_i is controlled by a vacuolar type H^+-ATPase which can only function if sufficient ATP is available.

The partial pH_i recovery after NEM induced acidosis in the presence of Na^+ (Fig. 1) suggest that pH-regulating systems other than H^+-ATPases took over at that point. The Na^+/H^+ exchanger is an excellent candidate since in other cell types such as lymphocytes a set-point of $pH_i = 6.8$ has been postulated to activate the transporter via an internal allosteric 'modifier' site [25]. In consequence Na^+/H^+-exchange in some cell types is virtually inactive at near neutral pH [26]. This explanation is emphasized by the observation that stimulation of the Na^+/H^+-exchanger by PMA reduces NBD-Cl induced acidification (Fig. 4), suggesting that activation of protein kinase C by PMA results in an increased setpoint for Na^+/H^+-exchange, while baseline activity of the exchanger remains unchanged. Similar observations have been made in fibroblasts, where PMA application alone did not alter baseline pH_i [27].

The partial pH_i recovery after NEM application in Na^+-free media (Fig. 1) is not easily explained. In those experiments pH_i decreased by approximately 1 unit below pH_e, which may go along with membrane depolarization, and, therefore, a reduction of the calculated pH-gradient which could lead to a passive recovery on pH_i. Another explanation might be a gradual pH-normalization due to endogenous production of bicarbonate.

The postulated role of H^+-ATPases in the absence of bicarbonate ions receives further support by the peculiar kinetics of NBD-Cl induced glial swelling, which occurred after a significant delay following NBD-Cl application (Fig. 5). According to the view proposed herewith, the pH_i-decrease from H^+-ATPase inhibition during NBD-Cl exposure activates Na^+/H^+-exchange as soon as its setpoint has been reached, thus causing an increase of intracellular Na^+. The increased intracellular osmolarity is followed by an influx of water, thus causing swelling [12,15,28]. In good agreement with this explanation is the observation of reduced swelling in HCO_3^--free HEPES-buffered media which was even more reduced if no Na^+ at all was present in the media. Compared to the kinetics of acidification, the delay of the swelling may appear somewhat surprising. On the other hand, activation of the Na^+/H^+-exchanger will not take place immediately after NBD-Cl application, but only after pH_i has fallen below a critical value. Swelling is expected to occur once intracellular Na^+ is increased significantly, which likely requires some time after activation of Na^+/H^+-exchange, and may explain the delay of the swelling response. Besides, the cell volume measurements were performed using suspended cells which, in contrast to adherent cells exhibit a rounded shape with retracted processes and a decreased surface/volume ratio which might influence the kinetic properties of transport systems involved in pH-regulation.

Assuming a Na^+/H^+-exchanger that is activated at a fairly acidic pH_i also provides for a good explanation of the challenging observations made in the current study after an acid-load with NH_4Cl, where 1 mM amiloride blocked pH-recovery only in a subpopulation of cells (Fig. 7). Similar findings with amiloride have been reported by Mellergard et al. [5], who found amiloride inhibition of pH-regulation only in 5 out of 12 astrocytes tested. In the subgroup where no inhibitory effect was found, this may be due to a moderate acid-load which remained sub-threshold and, hence, did not activate Na^+/H^+

exchange. Indeed the respective control experiments of that subgroup had a less severe acid load than those from the second sub-group. The pH-recovery in the amiloride insensitive subgroup should, therefore, be managed mainly by the H^+-ATPase. In order to prove this, measurements of oxygen consumption might be helpful in future studies. In the other spontaneously formed subgroup, where pH_i had further decreased after the acid-load, Na^+/H^+ exchange became fully activated, and, therefore, should be more sensitive to inhibition by amiloride. On the other hand, this would require that H^+-ATPases are fully active at physiological pH_i with far less activity below the threshold. This is in good agreement with the effects of NBD-Cl after an acid load. In those experiments where pH_i did not fall below 6.6 during the acid-load, recovery was totally prevented by NBD-Cl (Fig. 6). In some individual experiments (data not shown) the acid-load was larger with pH_i decreasing to 6.4, a level that should activate Na^+/H^+ exchange. Only then a partial recovery was found in the presence of NBD-Cl. One might argue, that a decreasing activity of H^+-ATPases at very low pH_i values appears to be quite inappropriate for a system destined to prevent intracellular acidification. On the other hand, such low pH_i values in vivo only occur under pathological conditions such as cerebral ischemia, where ATP supply cannot be maintained and H^+-ATPases will not function anyway. The Na^+/H^+-exchanger, however, can extrude protons even under these conditions as long as a Na^+ gradient remains.

Although the current results provide answers to open questions in glial pH-control, there are reports in the literature which are in conflict with our observations. In particular the far more rapid decline of pH_i after Na^+-withdrawal sometimes observed in astrocytes [3] points to a predominantly active Na^+/H^+-exchange under physiological conditions in those cultures. Considering, however, the broad variation of glial subtypes and specialized functions attributed to them, it is not unlikely that there are also differences in acid-extrusion mechanisms. Those glial cells in particular which serve as an interface between the cerebral parenchyma and the vasculature should possess special capabilities to modulate extracellular pH in the vicinity of resistance vessels, and, therefore, are most likely to contain H^+-ATPase activity. In fact proton-translocating ATPases have been identified in rat cerebral microvessels as well as the surrounding parenchyma [29] which is known to contain an abundance of glial endfeet.

Furthermore, inconsistent use of the pathways of pH_i-regulation may result from the peculiarities of in vitro processing of cultured cells, as it is known that the culture conditions can influence the activity of pH-regulating mechanisms. Especially the Na^+/H^+-exchanger is known to be stimulated by a host of protein kinase C stimulating agents such as phorbol esters [26] but also growth factors [25,30] which are abundant in most culture media. The highly active Na^+/H^+-exchange sometimes observed under physiological conditions may hence result from such culture conditions.

Taken together, it can be hypothesized that in glial cells H^+-ATPases of the vacuolar type constitute a major pH_i regulating mechanism at physiological pH_i, a condition where ATP is available to fuel the pump, while Na^+/H^+ exchange (which is driven by the sodium gradient) gains significance once pH_i has dropped below a setpoint of approximately 6.7. On the other hand, the gradual decrease of pH_i after Na^+ removal in virtually bicarbonate-free solutions suggests a persisting low activity of Na^+/H^+ exchange at pH_i values above that setpoint. In addition, astrocytes possess three bicarbonate requiring pH-regulatory systems—Na^+-dependent Cl^-/HCO_3^--countertransport, Cl^-/HCO_3^--exchange and Na^+/HCO_3^--cotransport—[3] which were not part of this investigation. Their presence in glia, however, is an indicator for the central role of astrocytes in cerebral pH-regulation. The apparent significance of H^+-ATPases for regulation of pH_i in glia might be comparable to that in renal epithelial cells [9] or macrophages [10,11,31]. These cell types fulfill special requirements of pH control. Renal epithelia transport protons to the urine. Macrophages are confronted with large pH_i changes during the respiratory burst. Astrocytes not only regulate their internal pH, but in addition also have specialized functions for pH homeostasis of the brain extracellular fluid. Moreover, astrocytes are anatomically separating neurons and the microcirculation. Hence, they are a dividing element between two compartments, just as renal epithelial cells are in the kidney. Like them, H^+-ATPases could enable astrocytes to perform a di-

rected transport of protons, i.e., from the cerebral parenchyma to blood vessels, thus facilitating acid removal but also the communication of high neuronal activity to the microcirculation by local periarteriolar acidification.

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