Neuroscience Letters, 107 (1989) 167–172
Elsevier Scientific Publishers Ireland Ltd.

NSL 06526

Na^+/H^+ exchange in glial cells of *Necturus* optic nerve

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(Received 30 June 1989; Accepted 16 August 1989)

Key words: Neuroglia; pH regulation; Antiport; Sodium proton exchange; Brain homeostasis; pH electrode

Single and double-barreled pH-sensitive electrodes were used to study intracellular pH (pH_i) regulation in glial cells of *Necturus* optic nerve in the nominal absence of HCO_3^-/CO_2. After the cells were acidified by the addition and withdrawal of NH_4^+, the pH_i recovered toward the original steady-state pH_i. The recovery from acidification was Na^+-dependent and inhibited by 1 mM amiloride. These results suggest the existence in intact vertebrate glial cells of a Na^+/H^+ exchanger which functions in acid extrusion.

In many cells acid extrusion is mediated by a membrane process which is electroneutral, Na^+-dependent and sensitive to amiloride. These properties are indicative of a Na^+/H^+ exchanger (for reviews see refs. 2, 10). The exchanger has been described in invertebrate neurons [18] and glial cells [8], vertebrate neurons [6], and a variety of mammalian glial cells in tissue culture [9, 11, 15]. The present report describes experiments in which we looked for Na^+/H^+ exchange in glial cells of the intact optic nerve of *Necturus*. The exchanger is of interest because of its participation in intracellular pH regulation as well as its possible role in extracellular pH regulation and volume control [7, 16, 17].

The optic nerve of the mudpuppy, *Necturus maculosus*, about 0.1 mm in diameter and consisting solely of large astrocytes surrounding small unmyelinated axons, was dissected as previously described [12] and mounted in a 0.2 ml perspex chamber at room temperature (20–23°C) in flowing Ringer's solution of the following composition in mM: NaCl 110, KCl 3, $CaCl_2$ 2, HEPES 5 titrated with NaOH to pH 7.4 or 7.5. To eliminate the contribution of the HCO_3^--dependent mechanisms of pH_i regulation, all solutions were nominally free of HCO_3^-/CO_2 (i.e. in equilibrium with

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room air). For NH_4^+-containing solutions NH_4Cl replaced NaCl. Na^+-free solutions were made by replacing Na^+ with *N*-methyl-D-glucamine. Double-barreled pH-sensitive electrodes were prepared using a modification of the method of Muñoz and colleagues [13]. The tip of the sensor barrel was filled with the pH cocktail of Ammann et al. [1] (FLUKA 82500 or 95297). The sensor barrel was then backfilled with a solution containing in mM: KH_2PO_4 40, NaOH 23, NaCl 15, pH 7.0. The reference barrel was filled with 3 M KCl. Each barrel of the double-barreled electrode was connected via a Ag/AgCl wire to the input of a high-impedance amplifier (Burr Brown OPA 128 LM) which was kindly configured by Dr. J.A. Coles. The bath was grounded with a Ag/AgCl wire connected through a 3 M KCl agar bridge (0.25 mm diameter). The output of the reference channel, and the voltage difference between the reference barrel and the pH-sensitive barrel were recorded on a chart recorder (Kipp and Zonen BD41) and a digital tape recorder (Unitrade, Philadelphia). In some experiments single-barreled pH-sensitive electrodes were used to measure the sum of the membrane potential and the logarithm of H^+ activity (see below).

Stable impalements with double-barreled electrodes were difficult to obtain. For an impalement to be considered valid, pH_i and the membrane potential had to be stable for 5 min and the membrane potential had to be more negative than -70 mV. At a bath pH of 7.5, the average initial pH_i was 7.32 (S.D. 0.03, $n=6$). There was a tendency for the pH_i to slowly drift in the alkaline direction during long impalements with double-barreled electrodes. For example, the record in Fig. 1A was obtained 90 min after the cell was initially impaled. The pH_i upon impalement was 7.34. During the 90 min leading up to the record the pH_i drifted to 7.7.

To learn about pH_i regulation in the absence of HCO_3^-/CO_2, we studied how the cell recovered from acidification [18]. As shown in Fig. 1, intracellular acidification is readily produced by the addition and withdrawal of NH_4^+ [5, 14]. Addition of NH_4^+ causes a rapid increase in pH_i which slows and eventually reverses. The initial alkalinization is due to the rapid influx of NH_3 and the slowing and reversal is the result of NH_4^+ entry down its electrochemical gradient. Withdrawal of NH_4^+ leads to an acidification beyond the original baseline pH_i because the cell's membrane potential provides a larger driving force for NH_4^+ entry than NH_4^+ exit. The excess intracellular NH_4^+ acidifies the cell by dissociating into NH_3 and H^+. Following acidification, pH_i recovers towards the control level. Fig. 1A shows that the pH_i recovery is reversibly blocked by the removal of Na^+. Removal of Na^+ gave similar results in 2 other nerves.

Fig. 1B illustrates the effect of adding amiloride during the recovery from acidification. The addition of amiloride further acidified the cell and the subsequent recovery was slowed. Removal of amiloride led to a pH_i recovery rate that was faster than the rate observed before amiloride. In addition, amiloride removal resulted in a steady-state pH_i that was alkaline to the steady-state pH_i measured at the beginning of the record.

It was difficult to impale *Necturus* glial cells with double-barreled pH-sensitive electrodes; we found it easier to obtain stable impalements with single-barreled electrodes. The single-barreled pH-sensitive electrode records a signal equal to $V_m + s$ log

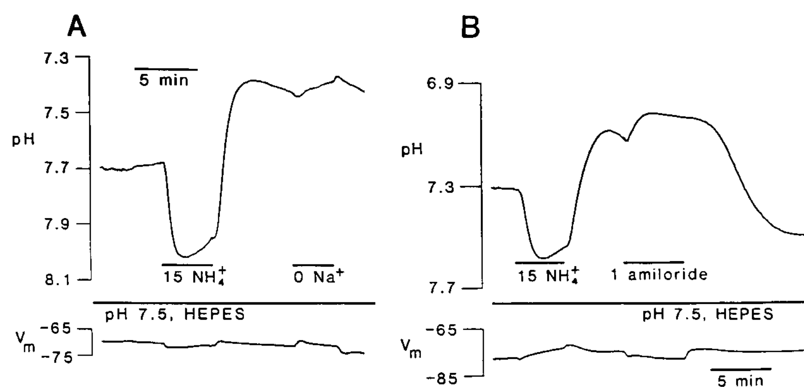

Fig. 1. A: sodium removal reversibly blocks recovery from intracellular acidication. The figure shows the pH_i (upper record) and glial membrane potential (lower record) as recorded simultaneously with a double-barreled pH-sensitive electrode. The durations of exposure to NH_4^+ (15 mM) or '0 Na^+' are indicated by the bars. The baseline solution was a HEPES-buffered Ringer's solution, pH 7.5 and nominally HCO_3^--free. Withdrawal of the NH_4^+ led to an acidification which started to recover toward the original baseline. Removal of Na^+ (replaced by *N*-methyl-D-glucamine) reversibly blocked the recovery of pH_i. The trace ends immediately before the electrode came out of the cell. B: amiloride blocks the recovery from intracellular acidification. Recordings as in A, but from a different nerve. The durations of exposure to NH_4^+ (15 mM) or amiloride (1 mM) are indicated by the bars. During the recovery from the NH_4^+ withdrawal-induced acidification, amiloride was added, leading to a further acidification and a slowing of the pH_i recovery rate.

($[H^+]_i/[H^+]_o$) where s is the response of the pH-sensitive electrode to a 1 unit change in pH (normally 58 mV at room temperature). Thus, a 0.1 pH unit change will produce a voltage change of 5.8 mV in the absence of a change in membrane potential. In 9 nerves, the average potential recorded with pH-sensitive electrodes was -82 mV (S.D. = 7). In the same series of experiments, the membrane potential recorded with 3 M KCl electrodes, which were made from the same electrode glass and with the same geometry as the pH-sensitive electrodes, was -82 mV (S.D. = 2, $n = 5$).

Fig. 2 illustrates records obtained with these two types of electrodes. The top trace was recorded with a single-barreled pH-sensitive electrode during an impalement with lasted two hours. The lower trace is a voltage recording obtained with a 3 M KCl electrode from a different nerve using the same experimental protocol. Amiloride had very little effect on membrane potential ($n = 3$). Thus, the amiloride-induced potential changes observed in the upper record can be interpreted as changes in pH_i. The addition of amiloride acidified the cell, and following amiloride withdrawal the cell became alkaline. In 3 experiments with single-barreled pH-sensitive electrodes, the potential changes relative to the pre-amiloride baseline were $+5$ mV (S.D. = 1) during exposure to amiloride, and -7.2 mV (S.D. = 2.6) in the 10–15 min after amiloride was washed out. Fig. 2 also shows that when amiloride was added as NH_4^+ was withdrawn, the resultant acidification was greater, and the pH_i recovery rate was slower than those observed in the post-amiloride control. In a total of 4 experiments, 3 with single-barreled electrodes and 1 with a double-barreled electrode (Fig. 1B), the initial pH_i recovery rate in the presence of 1 mM amiloride was an average of

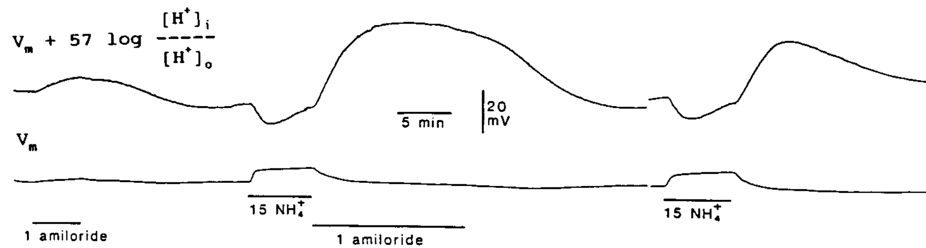

Fig. 2. Effect of amiloride on baseline pH_i and the recovery from acidification. The upper record was obtained from a single-barreled pH-sensitive electrode and the lower record was obtained from a conventional 3 M KCl electrode. The records are from different nerves, but the experimental protocol was identical. Each record is from a single impalement and the breaks in the records are 15 min. Solution changes from the HEPES-buffered Ringer's solution (pH 7.4, nominally HCO_3^- free) are indicated by the bars. The addition of 1 mM amiloride produced an intracellular acidification and has almost no effect on membrane potential. The addition of 1 mM amiloride upon withdrawal of NH_4^+ led to a larger acidification and slower pH_i recovery than seen in the control obtained after amiloride was washed out. Initial $V_m + 57 \log [H^+]_i/[H^+]_o = -77$ mV; initial $V_m = -84$ mV.

17% (S.D. = 21%) of the rate in the absence of an amiloride treatment. Furthermore, as in Fig. 1B, withdrawal of amiloride during block of the recovery from acidification led to a faster pH_i recovery rate than that observed in the absence of an exposure to amiloride. In a total of 4 experiments, 3 with single-barreled electrodes and 1 with a double-barreled electrode (Fig. 1B), the rate of pH_i recovery immediately after amiloride withdrawal was an average of 4.2 times greater (S.D. = 2.4) than the control recovery rate.

An explanation of the effects of amiloride must account for the following observations: (1) addition of amiloride during recovery from acidification decreases the pH_i recovery rate (Figs. 1B and 2); (2) addition of amiloride induces an acidification from the steady state pH_i measured in nominally HCO_3^--free solutions (Fig. 2); (3) withdrawal of the amiloride block of pH_i recovery, increases the pH_i recovery rate to above the rate observed in non-treated controls (Figs. 1B and 2); and (4) withdrawal of amiloride leads to an alkalinization of the steady-state pH_i (Figs. 1B and 2).

The first two observations are probably due to the inhibition of the Na^+/H^+ exchanger. Hypothetically, the effects of amiloride withdrawal, observations 3 and 4, could be due to either a decrease in acid loading or an increase in acid-extrusion. A decrease in acid loading could result from a decrease in the rate of an acid-generating reaction. An increase in acid extrusion could be due to activation of acid efflux mechanisms like the Na^+/H^+ exchanger. Amiloride withdrawal effects similar to the ones we observed have been described in red blood cells of *Amphiuma* [16, 17]. In these cells, Na^+ uptake in response to osmotically induced shrinkage is increased by withdrawal of an amiloride treatment.

The mechanism of operation of the Na^+/H^+ exchanger has been studied in detail in a number of cells (for review see ref. 2). The exchanger uses the inwardly directed concentration gradient for Na^+ to drive the efflux of H^+. Thermodynamically, the exchanger is at equilibrium when $[Na^+]_o/[Na^+]_i = [H^+]_o/[H^+]_i$. For a cell with a ten-

fold Na^+ gradient and a pH_o of 7.5, the equilibrium point would occur at a pH_i of 8.5. Presumably due to allosteric regulation by a second intracellular proton binding site, the Na^+/H^+ exchanger usually becomes inactive near the resting pH_i, well below the thermodynamic equilibrium. In our experiments amiloride induced a small acidification from the steady-state pH_i (Fig. 2). If this acidification is due to inhibition of the Na^+/H^+ exchanger, then the glial exchanger is active at the steady-state pH_i measured in the nominal absence of HCO_3^-/CO_2.

In summary, experiments with single and double-barreled pH-sensitive electrodes demonstrate that, in the nominal absence of HCO_3^-, glial cells of *Necturus* optic nerve recover from an acidification by a mechanism which is Na^+-dependent and sensitive to amiloride (1 mM). These results strongly suggest the existence of a Na^+/H^+ exchanger in the glial membrane. The results extend the observations of a glial Na^+/H^+ exchanger to intact vertebrate glial cells. In previous studies we demonstrated that glial cells of *Necturus* possess an electrogenic Na^+/HCO_3^- cotransporter [3, 4], which is also involved in pH_i homeostasis (Astion and Orkand, unpublished observations). The redundancy of these regulatory mechanisms underscores the importance of pH_i in glial cell function.

We are grateful to Dr. Greg Boyarsky and Dr. Nelson Escobales for helpful criticism of an earlier version of this manuscript. Supported by NIH Grants NS07464, GM07170 (MSTP Univ. of Pennsylvania to M.L.A.) and NSF Grant RII8705802.

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