Vol. 184, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1992 Pages 112-117

Na^+/H^+ EXCHANGE IN PORCINE CEREBRAL CAPILLARY ENDOTHELIAL CELLS IS INHIBITED BY A BENZOYLGUANIDINE DERIVATIVE

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Received February 17, 1992

Na^+/H^+ exchange activity has been examined in endothelial cells isolated from porcine brain capillaries. Intracellular pH (pH_i) changes were monitored using a confocal laser scanning microscope and the pH-sensitive fluorescence indicator 2',7'-bis-(2-carboxyethyl)-5,6-carboxyfluorescein (BCECF). Acid load of the brain capillary endothelial cells was performed with a NH_4Cl (20 mM) prepulse. In bicarbonate-free solutions pH_i recovered within 3 to 10 min. Removal of extracellular Na^+ ions demonstrated that H^+ extrusion after an acid load of the cells was Na^+ dependent. The Na^+/H^+ exchange could be completely blocked by EIPA (5-(N-ethyl-N-isopropyl)amiloride) as well as by the novel inhibitor 3-methylsulfonyl-4-piperidinobenzoyl guanidine hydrochloride (HOE 694) in concentrations of 1 to 10 μM, respectively. EIPA and HOE 694 in a concentration of 0.1 μM caused a partial block of Na^+/H^+ exchange.

Brain capillary endothelial cells form a specialized barrier between blood and brain interstitial fluid. Complex tight junctions connecting the endothelial cells prevent paracellular ion passage. Therefore, exchange of polar solutes between blood and brain must be mediated by specific transport systems which are differently distributed to the luminal and antiluminal membrane of cerebral capillary endothelium (1,2). Based on these particular properties the blood-brain-barrier contributes to fluid and ion homoeostasis in brain.

Studies of sodium transport from blood to brain demonstrated the presence of amiloride-sensitive Na^+ transport systems in both the luminal and the antiluminal membrane of cerebral capillaries (3,4). Luminal Na^+ uptake measured after intracarotid injection of $^{22}Na^+$ in vivo was pH independent, whereas, $^{22}Na^+$ uptake determined in isolated rat brain capillaries was influenced by the pH of the uptake buffer. It was concluded that in isolated capillaries $^{22}Na^+$ uptake mainly occured via a Na^+/H^+ antiporter that probably is located in the antiluminal

0006-291X/92 $1.50

membrane. Na^+/H^+ exchange measured as EIPA sensitive $^{22}Na^+$ uptake was also demonstrated for isolated rat cerebral endothelial cells held in cell culture for up to 15 passages (5).

Since the localization of the Na^+/H^+ antiporter in the antiluminal and/or luminal membrane of brain capillaries is not conclusively investigated yet (2,3,4), it is still a matter of debate whether Na^+/H^+ exchange only participate in regulation of the intracellular pH ("housekeeping" function (6)) or whether it can also support a nettransport of H^+ and Na^+ ions across the blood-brain-barrier.

Methods

Capillary endothelial cells were prepared from porcine brain as described previously (7,8). The cells were grown in Earle's medium M 199 supplemented with 20% fetal calf serum (Biochrom, Germany) for up to 8 days. From the primary cultures clusters of 5 to 50 coherent cells were isolated by short trypsinization (3 to 5 min). Dye loading was performed by incubating the cells with 1 to 5 μM of BCECF acetoxymethyl ester (Molecular Probes, USA) for 20 to 30 min at room temperature. Then the cell clusters were washed twice in a bicarbonate-free NaCl solution, transferred to a measuring chamber coated with poly-D-lysine, and allowed to settle down for 10 to 15 min. The NaCl solution contained (in mmol/l): 140 NaCl, 4.7 KCl, 1 $MgCl_2$, 1.3 $CaCl_2$, 10 HEPES, adjusted to pH 7.4 with NaOH. In Na^+-free solutions NaCl was substituted by equimolar concentrations of tetramethylammonium chloride (TMA-Cl), KCl or N-methyl-D-glucamine chloride (NMDG-Cl), respectively. For a NH_4Cl prepulse 20 mM NaCl of the NaCl solution was replaced by 20 mM NH_4Cl. HOE 694 was added from a aqueous stock solution. EIPA was solved in DMSO. All experiments were performed at room temperature (22-26°C).

A confocal imaging system (MRC-600 from Bio-Rad) was adapted to an inverted microscope (Zeiss Axiovert 35, Plan-Neofluar 20x). Laser beam intensity was cut down with a neutral density filter (1% transmission). For excitation the blue line (488 nm) of the argon laser was selected by a bandpass filter. The fluorescence light passed a dichroic reflector (510 nm LP) and a long pass emission filter (515 nm). Single sweep images of 256 x 384 pixels were taken every 20 seconds. To minimize photobleaching the laser shutter was kept closed in between single pictures. Images were stored on computer hard disk. For image analysis the mean fluorescence of an individual cell cluster was calculated for all frames of the image series. At the same time for each picture the cell area as well as the background area were determined to make sure that changes of fluorescence are not caused by changes of cell volume.

In the diagrams shown, the mean fluorescence of BCECF is plotted versus time without any mathematical correction for dye leakage and photobleaching. Therefore, during most experiments a small decline of the resting fluorescence level could be observed.

Results and Discussion

The pH dependent shift of the BCECF excitation spectrum in principle enables excitation ratio measurements to be made using BCECF. Excitation ratios are usually be formed from measurements at 440 and 500 nm. However, confocal scanning microscopy is restricted to lasers as light source. The setup we used for our experiments was equipped with an argon laser emitting light at 488 nm. Therfore, only one-wavelength-excitation measurements were feasible, and, in consequence, the absolute calibration of the intracellular pH was not possible.

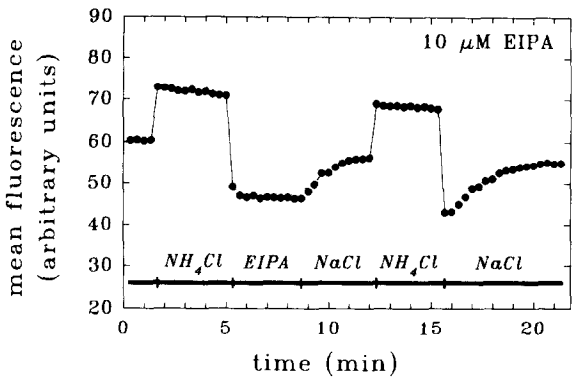

Fig.1. Inhibition of Na^+/H^+ exchange by 10 μM EIPA. Brain capillary endothelial cells were acid-loaded with a NH_4Cl (20 mM) prepulse. In the presence of EIPA pH_i recovery is suppressed. Rapid increase of pH_i by H^+ extrusion could be observed when EIPA was removed from the bath solution. A subsequent second NH_4Cl pulse demonstrates immediate pH_i recovery in the absence of EIPA.

Brain capillary endothelial cells were acid-loaded with a NH_4Cl prepulse of 3 to 4 min. In the initial phase of NH_4Cl application, rapid entry of the membrane permeant base NH_3 into the cell caused a steep rise of intracellular pH. This is reflected by an increase in BCECF fluorescence within a few seconds (Figure 1). During further NH_4Cl incubation slow influx of NH_4^+ ions increased the intracellular NH_4^+ concentration accompanied with a weak decrease of pH_i. When extracellular NH_4Cl was removed, NH_4^+ ions trapped inside the cell released protons and left the cell as uncharged NH_3. The resulting intracellular acidification could be monitored as a sharp decrease in BCECF fluorescence. As long as EIPA (9) in a concentration of 10 μM was present in the bath solution pH_i stayed acidic. Removal of EIPA from the external medium led to recovery of intracellular pH corresponding to an increase of BCECF fluorescence. A second acid load of the same cells (right part of Figure 1) proved that in the absence of EIPA pH_i recovery occurred immediately after removal of external NH_4Cl.

Sodium dependence of pH_i recovery could be demonstrated by replacing external Na^+ ions by K^+ or organic cations like TMA^+ or $NMDG^+$ (Figure 2). After a first NH_4Cl pulse NH_4^+ and Na^+ were removed from the bath solution simultaneously. NH_3 efflux induced a rapid drop of pH_i followed by a prolonged slower acidification. Restitution of an inwardly directed Na^+ gradient by perfusion with NaCl solution restored Na^+ inward and H^+ outward transport recognizable by the recovery of the intracellular pH. Na^+/H^+ exchange activity under control condition is demonstrated in each experiment by a second NH_4Cl pulse. In contrast to TMA^+, $NMDG^+$ and K^+ ions which do not support pH_i recovery after cell acidification, Li^+ ions promoted H^+ extrusion similarly to Na^+ (not shown).

In addition to EIPA the effect of 3-methylsulfonyl-4-piperidinobenzoyl guanidine (HOE 694) on Na^+/H^+ exchange was investigated. This benzoylguanidine derivative also showed to be a

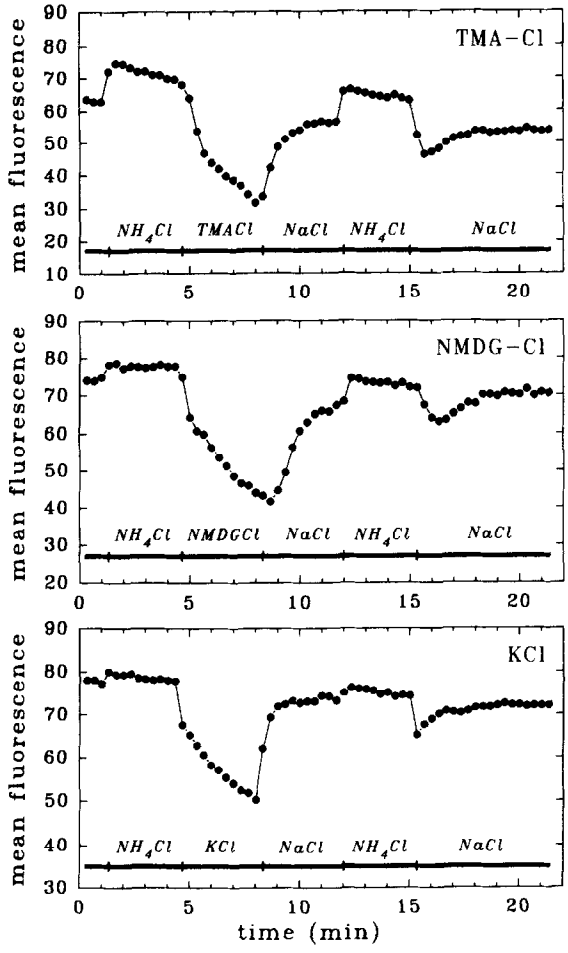

Fig.2. Sodium dependence of pH_i recovery. Replacement of external Na^+ by TMA^+ (upper panel), $NMDG^+$ (middle panel) or K^+ (lower panel) not only prevents pH_i recovery after a NH_4Cl prepulse but, furthermore, induced additional intracellular acidification reflected by a prolonged decrease in the BCECF fluorescence. After reperfusion with NaCl solution pH_i returned to control level. A second NH_4Cl pulse demonstrates Na^+/H^+ exchange activity under control condition.

potent blocker of Na^+/H^+ exchange. The structural formulas of HOE 694 and the amiloride derivative EIPA are given in Figure 3. Structural relationship of these compounds is based on the N-diamino-methylene carboxamide residue. The effectiveness of HOE 694 can be compared to the inhibitory potency of EIPA. As shown in Figure 4 pH_i recovery after an acid load of the cells could be completely suppressed by 10 μM as well as by 1 μM HOE 694. Similarly to 0.1 μM EIPA, HOE 694 in a concentration of 0.1 μM caused a partial block of Na^+/H^+ exchange, discernible as a smaller rise of pH_i recovery in the presence of HOE 694 compared to the control.

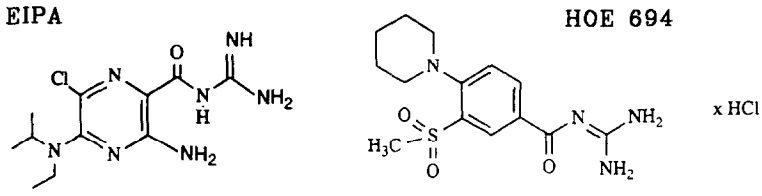

Fig.3. Structural formulas of 3-methylsulfonyl-4-piperidinobenzoyl guanidine (HOE 694) and 5-(N-ethyl-N-isopropyl)amiloride (EIPA).

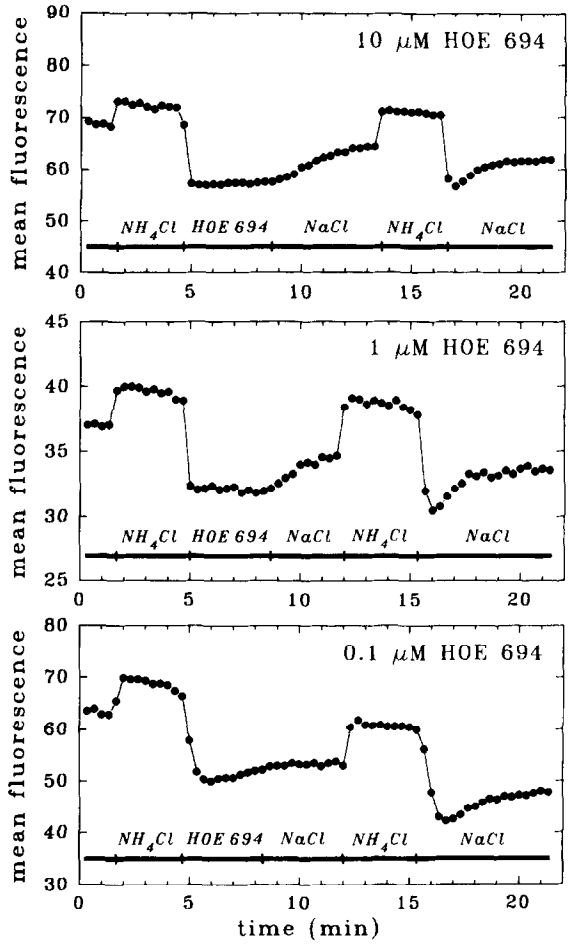

Fig.4. Inhibition of Na^+/H^+ exchange by 3-methylsulfonyl-4-piperidinobenzoyl guanidine. 10 μM (upper panel) as well as 1 μM (middle panel) of this substance completely suppressed pH_i recovery of acid-loaded brain capillary endothelial cells. A concentration of 0.1 μM (lower panel) caused a partial block of pH_i recovery as indicated by a smaller slope of the recovery curve in the presence of HOE 694 compared to the control (second NH_4Cl pulse).

In the present study a confocal laser scanning microscope and the pH-sensitive fluorescent dye BCECF were used to record changes of intracellular pH in cultured brain capillary endothelial cells. Recovery of pH_i after acid load of the cells could be assigned to Na^+/H^+ exchange. It could be demonstrated that 3-methylsulfonyl-4-piperidino-benzoyl guanidine is a potent blocker of Na^+/H^+ exchange.

References

1. Betz, A. L. and Goldstein, G. W. (1986) Ann. Rev. Physiol. 48, 241-250.
2. Risau, W. (1989) NIPS 4, 151-153.
3. Betz, A. L. (1983) J. Neurochem. 41, 1150-1157.
4. Betz, A. L. (1983) J. Neurochem. 41, 1158-1164.
5. Vigne, P., Ladoux, A. and Frelin, C. (1991) J. Biol. Chem. 266, 5925-5928.
6. Casavola, V., Helmle-Kolb, C., Montrose, M.H. and Murer H. (1991) Pflügers Arch 418, 276-283.
7. Mischek, U., Meyer, J. and Galla, HJ. (1989) Cell Tissue Res. 256, 221-226.
8. Hoyer, J., Popp, R., Meyer, J., Galla, H.J. and Gögelein, H. (1991) J. Membrane Biol. 123, 55-62.
9. Vigne, P., Frelin, C., Cragoe, E.J. and Lazdunski, M. (1983) Biochem. Biophys. Res. Com. 116, 86-90.