Abstract  Hypercapnia as well as lowered intracellular pH (pHi) increase the bioelectric activity of CO₂/H⁺-sensitive neurones (VLNcs) of the ventrolateral medulla oblongata. Here we describe that immunoreactive Na⁺/H⁺ exchanger (NHE3) is present in ventrolateral neurones from medullary organotypic cultures (obex level). To test whether VLNcs can be acidified and thereby activated by inhibition of NHE3, we used the novel high-affinity NHE3-inhibitors S1611 and S3226. Both drugs raised the firing rates of VLNcs to at least 150% of the control values, and depolarized membrane potential by up to 15 mV at concentrations (0.5–1 µmol/l) suitable for selective inhibition of NHE3. The changes in bioelectric activity strongly resembled the responses to hypercapnia (P₂CO₂: 60–100 mmHg). In BCECF-AM-loaded cultures a subfraction of ventrolateral VLNcs was found to be intracellularly acidified by 0.05–0.1 pH units following treatment with S1611; the time course of this acidification was similar to that evoked by hypercapnia. All drug effects were sustained and readily reversible upon washing. Non-CO₂/H⁺-responsive medullary neurones as well as hippocampal CA3 neurones were unaffected by up to 20 µmol/l S1611. It is concluded that the selective inhibition of NHE3 acidifies and activates CO₂/H⁺-sensitive neurones within the ventrolateral medulla oblongata.

Key words  Chemosensitivity · Intracellular pH · Na⁺/H⁺ exchanger · Organotypic medullary culture · Respiration

Introduction

Hypercapnia depresses most central neurones [29], but activates CO₂/H⁺-sensitive neurones (VLNcs) of the ventrolateral brainstem [8, 13, 21, 34]. The activation by CO₂/H⁺ appears to be an inherent property of VLNcs and can be studied in vitro using long-term organotypic explants of the brainstem [3]. After weeks in culture these preparations fully retain their recognizable cytoarchitecture in a three-dimensional network composed of neurones within a glial environment with no cell debris. We have used this model system to determine intracellular pH (pHi, by BCECF fluorescence) and its influence on electrophysiological responses. As shown previously, the increase in action potential frequency of VLNcs during hypercapnia may be elicited by a drop in pHi; this may be brought about experimentally either by withdrawal of external bicarbonate or by means of an ammonium prepulse [34]. It should be pointed out that the latter activates VLNcs despite a measurable extracellular alkalosis. These findings show that lowering the pHi of VLNcs is crucial for their activation, a finding consistent with other, indirect evidence [16]. Based on these findings we suggest that the regulation of pHi is ultimately linked to central chemosensitivity. In fact, regulation of pHi in VLNcs or nucleus tractus solitarius neurones differs from that of neurones within non-chemosensitive brain areas [23, 24] as they do not counteract a moderate (ΔpH_i<0.2 units) intracellular acidosis due to hypercapnia (pCO₂ elevated to 80–100 mmHg) or withdrawal of CO₂/HCO₃⁻ from the bath [34]. Excessive acid loading of neurones from the ventrolateral brainstem (ΔpH_i ≡ 0.6 units, value estimated from [23]) is, however, counter-regulated. This acid extrusion can be inhibited by high concentrations of...
amiloride, but not by 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), pointing to a major contribution of Na+/H+ exchange (NHE), while (Na+-dependent) Cl-/HCO₃⁻ antipporter appears to be less involved.

Previous in vivo studies show that inhibition of NHE by 5-(N-ethyl-N-isopropyl)-amiloride (EIPA) fails to activate respiration [14], and amiloride stimulates breathing frequency only at high (>1 mol/l) concentrations [30]. However, the inhibitory properties of amiloride or EIPA strongly depend on the subtype of NHE that is expressed. Recently, mRNA of the Na+/H⁺ exchanger type 3 (NHE3) was detected within the ventrolateral brainstem of new-born rats by in situ hybridization [17]. This finding is of particular importance inasmuch as it can explain the failure of the previous attempts to stimulate respiration by common NHE inhibitors (see above), as inhibition of NHE3 demands high concentrations of amiloride or related compounds (reviewed in [31]).

In the present study we have tried to decrease pHᵢ by inhibiting NHE3 under resting conditions, i.e. without a preceding acid load. To inhibit NHE3 selectively without impairing other NHE-subtypes, especially the dominant NHE1 (see [17]), the novel NHE3 inhibitors S1611 and S3226 [27] were used whose effects on nervous tissue have now been tested by measurements on brainstem and hippocampal neurones. Inasmuch as (1) the electrochemical gradient for H⁺ is inwardly directed [2], (2) metabolism of the cell will further produce H⁺, and (3) VLNCs are activated by decreased pHᵢ [34], inhibition of H⁺ extrusion should decrease pHᵢ and, by this, increase the firing rate of VLNCs. We now present data demonstrating that novel NHE3 inhibitors can selectively activate VLNCs, supporting our hypothesis that control over pHᵢ is crucial for the proper functioning of these neurones.

Materials and methods

Tissue preparation

Transverse medullary slices (360 µm thick) from 1- to 4-day-old rat pups were cut from 300 µm caudal to 600 µm rostral to the obex [3] and cultured on nylon grids for up to 6 weeks in R16 medium as described previously [25]. Acute hippocampal slices were prepared from adult guinea-pigs which were deeply anaesthetized with ether as described [5].

Immunocytochemical detection of NHE3

Cultures were fixed with 40 g/l paraformaldehyde in 0.1 mol/l Na⁺ phosphate buffer (PBS) for 1 h, washed in the same buffer and incubated for 3 days in the presence of 10% (v/v) fetal calf serum with the monoclonal anti-rat NHE3 antibody 2B9 (a generous gift of D. Biemesderfer) which has been characterized previously [35]. The concentration of 2B9 was approximately 1 mg/l in all experiments. Cultures were then washed four times with 0.1 mol/l Na⁺ phosphate buffer for 1 h. A fluorescein- (FITC-) labelled goat anti-mouse IgG antibody (Sigma, dilution 1:40) was applied for 1 day as a secondary antibody. Finally, the tissue was washed in 0.1 mol/l Na⁺ phosphate buffer and stored for up to 1 day until evaluation. In addition to experiments carried out with 2B9, a polyclonal antibody was raised in rabbit: a GST fusion protein of the cytosolic tail of the rat NHE3 (residue 565–690) was used as the immunogen. The immune-serum was preabsorbed with GST. Thereafter, affinity purification was carried out by binding it to the immunogen bound to nitrocellulose. On Western blots the affinity-purified antibody recognized a single band of 83 kDa in cells transfected with NHE3 but not in parental cells or those transfected with NHE1 (data not shown). The polyclonal antibody was diluted 1:1000 and used as described above, with FITC-coupled anti-rabbit antibody (Sigma) being used for detection.

Neurones were additionally labelled with tetanus toxin C fragment coupled to rhodamine (TTC-Rh) to identify NHE3-immunopositive cells as neurones. Therefore 0.2 µg TTC-Rh/ml was applied to fixed cultures for 60 min. Excessive dye was removed by washing with PBS. Fluorescent images were taken with a 40x water-immersion objective mounted on an Olympus microscope, equipped with fluorescence filter sets for FITC and rhodamine.

Characterization of S1611

The IC₅₀ values of S1611 for human NHE1, rabbit NHE2, rat NHE3 and human NHE3 have been reported to be 4.7, 89, 0.69, and 0.046 µmol/l, respectively [27]. However, to estimate the cross-reaction of certain concentrations of S1611 with other NHE subtypes, inhibition curves (not published in [27]) for all aforementioned NHE subtypes were determined. Measurements were carried out on LAP1 cells stably transfected with either human NHE1, rabbit NHE2, rat NHE3 or human NHE3 [9, 26,27] essentially as described [12,27]. In brief, aliquots of approximately 25,000 BCECF-loaded transfected LAP1 cells were acidified by the washout of ammonium (20 mmol/l). Recovery of pHᵢ was measured for 120–300 s (period of the widely linear increase of pHᵢ) using a dual-grating Deltascan single-photon counting fluorimeter (Photon Technology International, South Brunswick, N.J., USA) with excitation wavelengths of 440 nm and 505 nm and an emission wavelength of 535 nm. pHᵢ calibration was achieved with a standard curve obtained from BCECF-AM-loaded LAP1 cells permeabilized with Triton X-100 [27]. Due to the inhibition curve for the rat NHE3 shown in Fig. 1, application of 1 and 0.1 µmol/l S1611 resulted in 60% and 5% inhibition, respectively. In most experiments we used concentrations of S1611 below the IC₅₀ of NHE1, i.e. in the range of 1 µmol/l to preferentially inhibit rat NHE3. Higher concentrations (up to 20 µmol/l), sufficient to partially inhibit NHE1 as well, were used to look for cytotoxic or otherwise unspecific side-effects on non-CO₂/H⁺-responsive neurones.

Electrophysiological experiments

All experiments were carried out in warm (32±1°C) artificial cerebrospinal fluid (ACSF, in mmol/l: NaCl 124, KCl 3, MgSO₄ 1.3, KH₂PO₄ 1.25, glucose 11, CaCl₂ 1.8 and NaHCO₃ 26, equilibrated with 95% O₂ and 5% CO₂, pH 7.4±0.03). The volume of the experimental recording chamber was 2 ml; preparations were superfused at a rate of 3–4 ml/min. PCO₂ was elevated to 80–100 mmHg by gassing the ACSF with CO₂ until a pH of 7.0–7.1 was reached. Stock solution of HOE642, S3226 and S1611 were prepared in dry dimethylsulfoxide (DMSO) and added to the ACSF immediately before the experiment. None of these additives changed the pH of the bathing fluid. Final concentrations of DMSO were 0.0001–0.001% (v/v). Electrophysiological recordings were carried out with conventional sharp electrodes (140–200 MΩ) filled with K⁺ methyl sulfate (2 mol/l). Although more difficult to handle, sharp electrodes were preferred to patch electrodes because the latter tend to progressively destroy the CO₂/H⁺ sensitivity [10,13]. Signals were stored using a computerized system [33]. Counting of action potentials (AP) was carried out at 1 s intervals using a trigger level of approximately 20 mV more positive than the resting membrane potential (MP).

Measurement of pHᵢ in organotypic cultures

Medullary cultures were stained with 0.2 mg/l TTC-Rh for 60 min to identify neurones within the cultured tissue [20]. At the end of this labelling period 5 µmol/l BCEO-F-AM was added for 3 min.