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Acid sensing ion channels in rat cerebral arteries: Probing the expression pattern and vasomotor activity

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ABSTRACT

Aims: The recent identification of acid sensing ion channels (ASICs) in vascular beds suggests their possible involvement in modulating vasomotor tone. Therefore, we investigated the gene expression profiles of ASIC subtypes in the middle cerebral artery (MCA) of *Wistar* rats and the functional implication of ASICs in acidosis-induced relaxation as well as maintenance of resting tension.

Main methods: Real time PCR was employed to study the pattern of ASIC mRNA expression in the MCA wall in comparison with (i) matching brain tissue samples and (ii) arteries cultured for 24 h and 48 h. The functional implication regarding vasomotor response to acidosis and maintenance of resting tension was assessed using in vitro myography.

Key findings: A robust mRNA expression of ASIC-1, -2 and -4 was found in brain tissue samples and to a lower extent in freshly isolated MCA. In the MCA wall, short term culture induced a down-regulation of ASIC-1 and -2 expression without any remarkable change in ASIC-4 expression. Acidosis induced a pH-related relaxation of freshly isolated MCA ring segments, being more pronounced after short term culture. Incubation with the ASIC blocker amiloride moderately enhanced acidosis-induced relaxation, in cultured MCAs somewhat stronger than in freshly isolated vessels. In addition, amiloride resulted in a decrease of resting tension, albeit only in freshly isolated MCA.

Significance: Our results comprehensively describe ASIC subtype composition in the rat MCA in physiological and pathological conditions and strongly suggest the involvement of ASICs in the modulation of vasomotor responses under conditions of normal or decreased pH values.

1. Introduction

Acid sensing ion channels (ASICs) are members of the voltage-independent, amiloride-sensitive epithelial Na^+ channel (ENaC)/degenerin family of cation channels first described as selective H^+ binding proteins and, thus, sensors of extracellular acidosis [1]. Activation of ASICs is observed during extracellular acidosis, getting them involved in pain sensation by stimulation of sensory nerve endings during inflammation or ischaemia [2]. In addition, ASICs have been found to be involved in mechanosensation, synaptic plasticity, and fear conditioning among others as reviewed elsewhere [3,4]. Five different ASIC genes are currently listed in the HUGO data base (https://www.genenames.org/cgi-bin/genefamilies/set/290) encoding for distinct ASIC subunits, and splice variants have been shown for ASIC-1 and -2 [5,6]. Three subunits assemble to form homomeric or heteromeric channels [7,8], and the composition actually determines gating characteristics including extracellular pH sensitivity, activation kinetics, ion permeability in addition to Na^+, and modulation by non-proton stimuli [9].

Although basically found in the peripheral and central nervous system ASIC expression has also been detected in non-neuronal cells in smooth-muscle organs such as the urinary bladder [10,11] and arteries from various vascular beds [12–14]. In the blood vessel wall ASIC activation has been electrophysiologically associated with smooth muscle cells [15,16]. So far, studies on the role of ASICs in regulation of vascular tone have focused on myogenic tone in cerebral and renal arteries [14,17,18] and on hypoxia-induced constriction in the pulmonary artery [19]. To the best of our knowledge, the possible involvement of ASICs in modulating acidosis-induced relaxation has not been investigated so far.

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Slight reduction in extracellular pH has long been recognized as an important metabolic factor in adapting regional blood flow in many organs, most notably the brain. Moreover, profound reduction in extracellular pH is a well described pathophysiological factor occurring in brain pathology, most notably ischaemia. In these conditions cerebral arterial function is markedly altered going along with alterations of gene expression as shown for a couple of vasoactive receptors, ion channels, and enzymes among others [20–23]. Many aspects of ischaemia-associated vascular alterations can be found after short term culture of arteries [24]. Therefore, we employed this in vitro model to study cerebral ischaemia-induced vascular pathology with respect to ASICs.

We used the well-established blocker of ASIC activation, amiloride, to address the involvement of ASICs in relaxation induced by extracellular acidosis as well as in the regulation of spontaneous tone at physiological pH in freshly isolated and cultured rat middle cerebral artery (MCA). We also used semi-quantitative real-time polymerase chain reaction (RT-PCR) methodology to describe in depth the pattern of ASIC mRNA expression in naïve MCA and matching brain tissue samples as well as in the MCA after short term culture. Our results indicate that ASICs potentially contribute to the modulation of acidosis-induced relaxation (both in freshly isolated and cultured arteries) as well as to the regulation of resting tone in the normal pH range (only in freshly isolated arteries).

2. Materials and methods

All experiments were performed with the approval by the responsible institutional authority. Male *Wistar* rats were obtained from Janvier (Isle St-Genest, France) and housed under controlled temperature (20–24 °C) and humidity (50–60%) in a 12-hour light/dark cycle with free access to standard raw chow and water.

2.1. Short-term culture of MCAs

The animals were deeply anesthetized with isoflurane (5%) and sacrificed by exsanguination. The brain was carefully removed, transferred into ice-cold HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)-buffered physiological saline solution (PSS_H; see Supplementary material for exact composition). Both MCAs were meticulously isolated, cleaned from any adhering brain and meningeal tissue under a binocular microscope (GZ6, Zeiss, Oberkochem, Germany), mounted on a glass capillary tube with fine tip and slowly perfused with Dulbecco's modified Eagle's medium (DMEM; Gibco, Invitrogen, Carlsbad, CA, USA) to wash off the blood and dislodge adherent blood clots.

Short-term culture was performed for 24 h or 48 h in serum-free DMEM supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich, Taufkirchen, Germany) at 37 °C in humidified 5% CO_2 in air. For arteries cultured for 48 h, the medium was changed after 24 h as previously described [25].

2.2. In vitro myography

Isometric force measurements were performed in MCA ring segments in three groups: (a) in freshly isolated MCA (MCA_F); (b) in MCA cultured for 24 h (MCA_{24h}); and (c) in MCA cultured for 48 h (MCA_{48h}). Ring segments were carefully mounted on two stainless steel wires (50 µm diameter) in organ baths containing 5 ml of PSS_H, continuously aerated with humidified oxygen (pH ~7.3 at 37 °C). Adaptation of ring segments and standard tests for contractility and endothelial function were conducted according to previously described protocols [23,26,27] and detailed in the Supplementary material. Only segments with a functionally intact endothelium were included in the present study. In preliminary experiments we compared the effect of cumulative application of lactic acid and HCl on the resulting pH value in the bath solution and the vascular response. Acidifying the PSS_H with lactic acid or HCl gave fully comparable pH profiles and relaxation responses across the concentration range tested (up to 4 mM; data not shown). Based on these observations lactic acid was further used because of its physiologic relevance.

Acidosis-induced relaxation was assessed by acidifying PSS_H with lactic acid (0.5–4 mM) upon pharmacologically induced precontraction with 1 µM U46619 (Enzo Life Science, Lausanne, Switzerland). To study the effect of ASIC inhibition on resting tension and acid-induced relaxation, the arteries, with resting tensions adjusted to 3.5 mN, were pre-incubated with 30 µM amiloride (Biomol, Hamburg, Germany) for 30 min. In time-matched solvent control experiments, double distilled water was applied instead of amiloride.

2.3. Gene expression studies

For subsequent gene expression studies the MCAs were isolated as described above. Fresh and cultured arteries were transferred into 1.5 ml Eppendorf tubes containing 350 µl of a mixture of RLT buffer (a lysis buffer) and 2-mercapto ethanol (2ME) in the ratio 100:1 and immediately kept at −80 °C until further use. Freshly isolated cerebral hemispheres rid of meningeal tissues and extraparenchymal blood vessels were also frozen.

The MCAs were mechanically homogenized as described previously [23] and total RNA was extracted using the RNeasy®Plus Micro kit (Qiagen, Hilden, Germany) according to the protocol supplied by the manufacturer. The content and integrity of the total RNA extracted from the MCA samples were measured using the RNA 6000Pico LabChip® (Agilent technologies, Boeblingen, Germany). Cryosections (5 µm thick, 20 sections) were prepared from frozen hemispheres and total RNA was extracted as described above. The content and integrity of the total RNA obtained from brain tissue samples were measured with the RNA Nano LabChip® (Agilent technologies). Samples of MCA_{48h} were observed to have remarkably greater total RNA content than MCA_F and MCA_{24h} which were comparable. Overall, we excluded samples with RIN < 8 from gene expression studies. The average RNA amounts and RIN values of the samples used for RT-PCR analyses are listed in Table 1.

Reverse transcription was done using the Sensiscript® kit (Qiagen) in a PCR Express thermal cycler (Hybaid Thermo Scientific) and subsequent RT-PCR was performed in a Stepone® Sequence Detection System using Taqman Real time PCR assays ASICs 1–5 with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beta actin (Actb), and beta 2 microglobulin (B2m) as reference genes (Life Technologies, Darmstadt, Germany). The details on the assays used are presented in the Supplementary material. The analyses were run in 20 µl reaction volumes, and a sample without template served as a control. The PCR protocol file consisted of heating to 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

2.4. Immunohistochemistry and confocal microscopy

We also performed immunofluorescence studies using whole mount staining of freshly isolated and cultured MCAs. The samples were fixed

Table 1
RNA content and RNA integrity numbers (RIN) for the tissue samples obtained in the different experimental groups. Data are given as mean ± SEM ($n = 6$ for each group). MCA_F: freshly isolated middle cerebral artery; MCA_{24h}: MCA taken after short-term culture for 24 h; MCA_{48h}: MCA taken after short-term culture for 48 h.

	RNA content (ng)	RIN
MCA_F	110.7 ± 34.6	9.4 ± 0.2
MCA_{24h}	109.7 ± 24.3	8.4 ± 0.2
MCA_{48h}	221.9 ± 56.0	8.6 ± 0.2

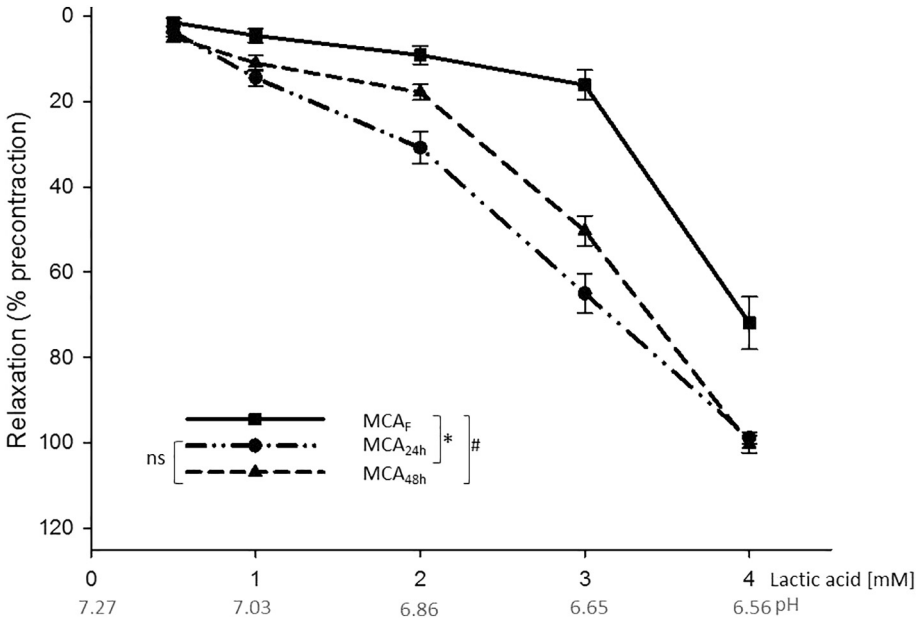

Fig. 1. Concentration effect relationships for vasomotor responses to extracellular acidosis in freshly isolated and cultured rat middle cerebral artery (MCA) ring segments. Depicted on the x-axis are the lactic acid concentrations and corresponding pH values. Compared with freshly isolated MCA (MCA$_F$), there was an enhanced degree of relaxation following culture for 24 h and 48 h. The duration of culture (24 h vs 48 h) did not confer any significant difference on the extent of relaxation. * represents significant difference (fresh vs 24 h); # represents significant difference (fresh vs 48 h); ns indicates no significant difference based on repeated measures ANOVA (n = 18 MCA ring segments in each group).

in 4% paraformaldehyde prepared in phosphate-buffered saline for 10 min. Rabbit anti ASIC-2 (Thermofisher Scientific, Dreieich, Germany) and goat anti-rabbit antibody coupled to Alexa Fluor® 568 (both antibodies from Fisher Scientific, Dreieich, Germany) were used as primary and secondary antibodies respectively (see Supplementary material for staining protocol). The distribution of the immunofluorescence signal was examined using an Axioplan 2 microscope equipped with an Axiocam camera (both from Carl Zeiss Microscopy GmbH, Göttingen, Germany). A more detailed examination was done using confocal microscopy (Eclipse 90i, equipped with a C2fi camera, both from Nikon, Hamburg, Germany).

2.5. Statistics

In each RT-PCR assay the threshold cycle (C_T) when the fluorescence intensity crossed the background intensity was determined. The expression levels for each gene of interest were determined using the ΔC_T approach and the above given reference genes. We also confirmed the efficacy of all expression assays used for reference and target genes by showing a linear amplification over a 10^3 fold dilution range of total RNA. Short-term culture-induced enrichment/depletion of the target genes in the vascular wall were determined by calculating the $\Delta\Delta C_T$ values using the ΔC_T values obtained in MCA$_F$ as reference.

For in vitro myography, contractile responses are measured as milli Newton (mN) force, while relaxation is expressed as a percentage of the level of precontraction.

All values are given as mean ± SEM. Statistical analysis was performed using Student's *t*-test and repeated-measures ANOVA followed by Bonferroni's test, as appropriate (SPSS 20.0 for Windows and SigmaPlot 12.5, Systat Software inc.). A value of $p < 0.05$ was considered statistically significant; n represents the number of animals for gene expression studies and number of MCA ring segments for in vitro myography studies.

3. Results

3.1. Effect of short term culture on acidosis-induced relaxation

Relaxation induced by acidosis was studied in ring segments precontracted with U46619 (1 μM). Short term culture did not remarkably affect contractile response to U46619. The contractile responses to U46619 expressed as a percentage of the maximal contractile response (to 124 mM K^+) are as follows: MCA$_F$: 67.2 ± 4.6; MCA$_{24h}$: 71.7 ± 4.2; MCA$_{48h}$: 82.3 ± 3.5. Following precontraction cumulative application of lactic acid induced a pH-related relaxation in MCA ring segments of all experimental groups as shown in Fig. 1. However, the effect was more pronounced in cultured arteries in which the maximal relaxation was significantly increased and the concentration effect curves clearly shifted to the left.

3.2. Acidosis-induced relaxation in the presence of amiloride

Incubation with amiloride did not remarkably affect the degree of pre-contraction induced by U46619. Following pre-contraction, there was augmentation of acidosis-induced relaxation of the MCA$_F$. This augmentation of relaxation was more pronounced in cultured MCA ring segments (MCA$_{24h}$ > MCA$_{48h}$) compared to control segments (MCA$_F$), and it became obvious even with low concentrations of lactic acid (Fig. 2).

3.3. Effect of amiloride on resting tension

The effect of amiloride (30 μM) on resting tension was assessed during the 30 min of incubation and compared with time-matched solvent-controlled segments. The tension change after incubation in the respective conditions was expressed as a percentage of the initial level of resting tension. Generally, incubation of freshly isolated MCA ring segments with amiloride led to a moderate reduction in resting tension compared with MCA ring segments receiving solvent only. However, following short term culture, incubation with amiloride did not result in a remarkable difference in response compared with ring segments incubated with solvent only (Fig. 3).

3.4. Gene expression studies

In order to improve comparability between parenchymal and arterial tissues the RNA from each brain tissue sample was appropriately diluted with RNAse free water to the average level obtained in MCA$_F$ samples. As a result the C_T values for the three reference genes, GAPDH, Actb, and B2m were found to be similar between brain tissue and MCA$_F$; therefore, the mean of the three was used to calculate the ΔC_T values for comparison between MCA$_F$ and brain tissue. However for comparison between MCA$_F$, MCA$_{24h}$ and MCA$_{48h}$ only the C_T values for Actb were found to be similar across the three groups as GAPDH and

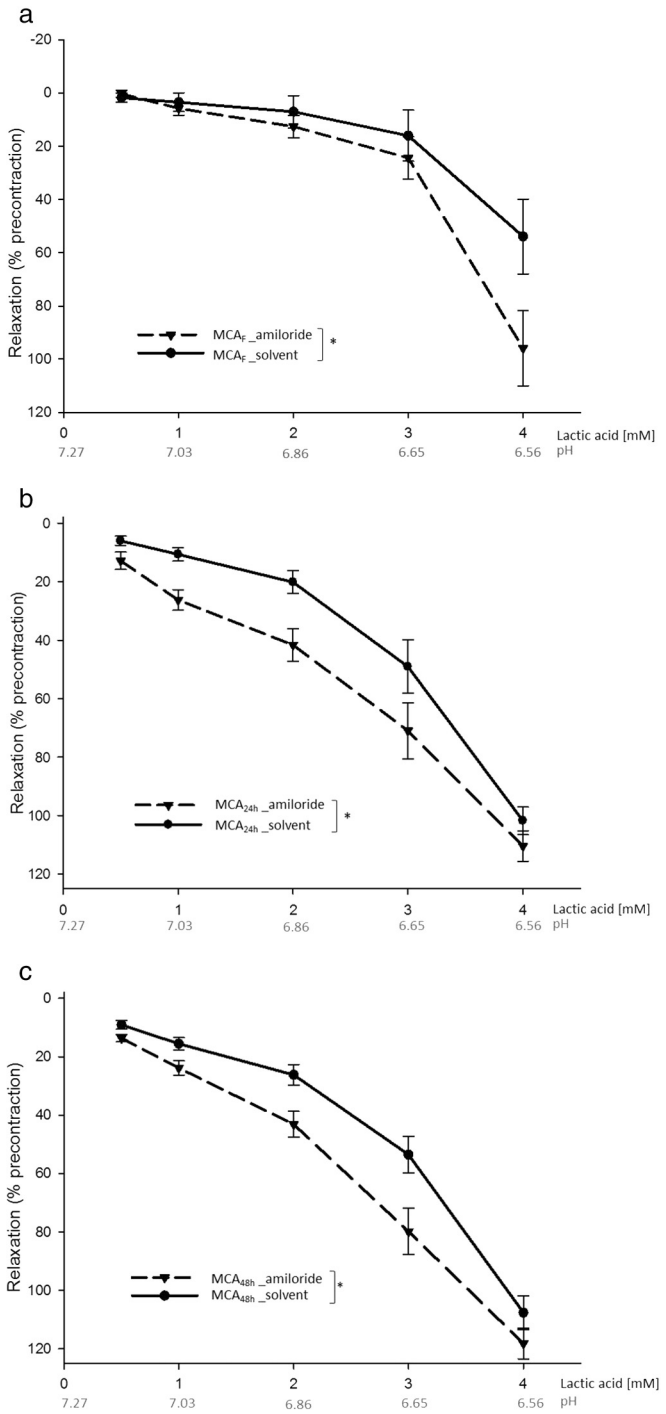

Fig. 2. Effect of 30 μM amiloride on relaxation induced by extracellular acidosis in freshly isolated and cultured rat middle cerebral artery (MCA) ring segments. The presence of amiloride enhanced acidosis-induced relaxation in freshly isolated MCA (MCA_F, $n = 9$; Fig. 2a); in MCA cultured for 24 h (MCA_{24h}, $n = 18$; Fig. 2b); and 48 h (MCA_{48h}, $n = 16$; Fig. 2c). Depicted on the x-axis are the lactic acid concentrations and corresponding pH values. Values are plotted as mean ± SEM. Asterisk indicates significant difference based on repeated measures ANOVA.

B2m expression was upregulated following short-term culture. Therefore, for further analysis, Actb was selected as the reference gene.

3.5. ASIC expression in brain tissue and freshly isolated MCA

A robust expression of ASIC-1, -2, and -4 mRNA was detectable in all parenchymal tissue samples studied. The average expression levels were largely comparable. In contrast, ASIC-3 and -5 expression was never detected. In the vascular tissue samples, ASIC-1, -2 and -4 were consistently expressed; although at markedly lower levels than in brain tissue samples (Fig. 4). Of these three subtypes, ASIC-2 showed the least difference in expression levels between parenchymal tissue and MCA_F. We never found ASIC-3 and -5 mRNA in any cerebroarterial sample.

3.6. Effect of short term culture on ASIC expression

Short-term culture of the MCA for 24 h or 48 h resulted in a downregulation of ASIC-1 and -2 while ASIC-4 was virtually unchanged (Fig. 5). These alterations were quantified using the $\Delta\Delta C_T$ approach (Table 2).

The changes in expression of ASIC-1, -2, and -4 in cultured arteries relative to freshly isolated arteries are summarised in Table 2. Calculation of these fold changes are based on the $2^{-\Delta\Delta CT}$ approach relative to the respective freshly isolated arteries. Downregulation of ASIC-2 amounted to approximately 40% while that of ASIC-1 was approximately 30% following short term culture.

3.7. Immunohistochemistry

To determine if the ASIC mRNA expression described above translates to ASIC protein expression in the vessel wall, whole-mount staining of MCAs was performed. We chose ASIC-2 which showed the most remarkable mRNA expression in freshly isolated and cultured cerebral arteries. Distribution of immunoreactivity (IR) is exemplified in MCA_F and MCA_{24h} as shown in Fig. 6a-d and b. By conventional microscopy, no remarkable difference in the strength of ASIC-2 IR was observed between these two groups (Figs. 6a and b). Using confocal microscopy, ASIC-2 IR was localized to the adventitial layer (6c) of the MCA with very minimal IR in the media (6d) and no IR in the endothelial layer.

4. Discussion

The main findings of the present study are (i) a robust expression of ASIC-1, -2 and -4 mRNA in freshly isolated MCA in the decreasing order: ASIC-2 > ASIC-1 > > ASIC4, (ii) downregulation of ASIC-1 and -2 mRNA expression after short term culture, and (iii) augmentation of acidosis-induced relaxation in isolated MCA ring segments following ASIC blockade by amiloride. Thus, it can be deduced that ASICs are regularly expressed members of the ion channel repertoire in cerebral arteries where they play a role in modulating acidosis-induced relaxation under physiological and pathological conditions as modelled by short-term arterial culture.

4.1. Requirements for RT-PCR analysis

For studies on ASIC gene expression, we included only brain tissue and MCA samples displaying well preserved total RNA at the end of the extraction procedure as judged by an electrophoresis-based kit assay. We only used samples with a RIN value > 8 (on a scale of 0–10), which are considered highly suitable templates for RT-PCR studies [28]. Based on these results, the concentration of total RNA in brain tissue samples was adjusted to the level obtained in MCA samples by appropriate dilution. This approach resulted in highly comparable C_T values for the reference genes Actb, B2m, and GAPDH in freshly isolated MCA and brain tissue samples.

4.2. ASICs expression pattern in brain tissue and MCA

In parenchymal tissue samples there was a robust expression of ASIC-1, -2, and -4 mRNA while ASIC-3 and -5 were hardly or not at all detectable. The presence of ASIC-1 and -2 is not surprising because

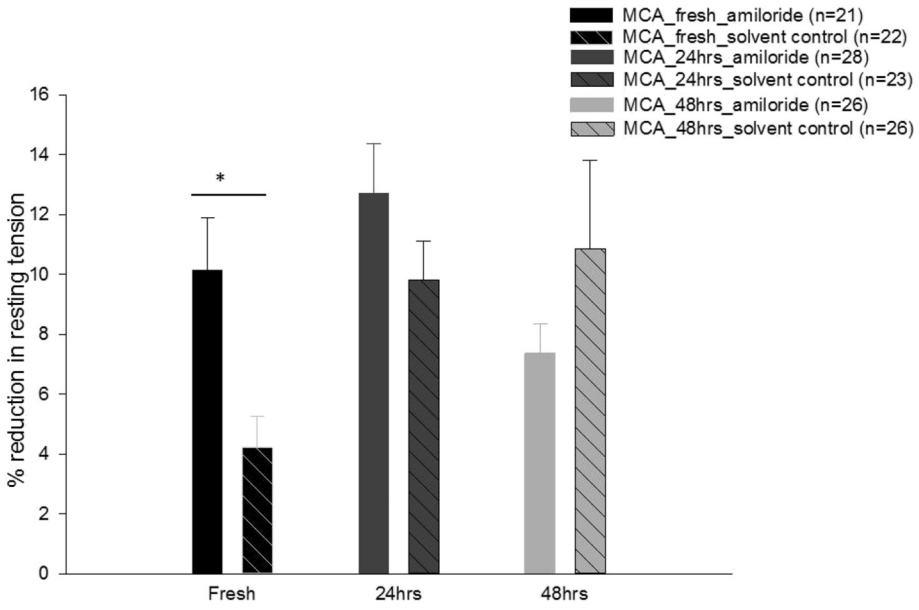

Fig. 3. Effect of amiloride (30 μM, 30 min) on resting tension in rat middle cerebral artery (MCA) ring segments. The reduction in resting tension after 30 min incubation with amiloride or double distilled water (as solvent control) is given in % of starting level. Indicated are mean ± SEM. *$p < 0.005$. n represents the number of MCA ring segments in each group.

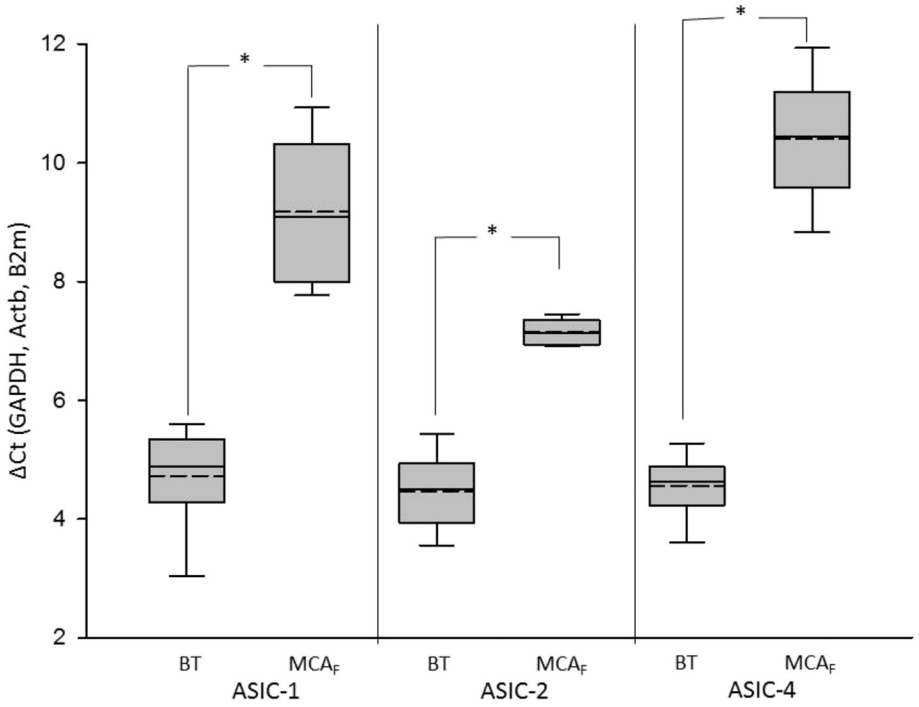

Fig. 4. Comparison of the expression profiles of ASIC subtypes between freshly isolated MCA and brain tissue. The average C_T values of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beta actin (Actb), and beta 2 microglobulin (B2m) served as reference. The horizontal black and broken line in the boxes represent the median and mean value, respectively, while the upper and lower edges of each box represent the 75th and 25th percentile. The error bars represent the range of each data set. Expression of ASIC-1, -2 and -4 is lower in the arterial wall than in brain tissue. *$p < 0.005$ with $n = 6$ animals for each group. MCA$_F$: freshly isolated middle cerebral artery; BT: brain tissue.

these subtypes are known to be widely expressed in the central nervous system of many species as previously reviewed [4,29]. The presence of ASIC-4 mRNA may be considered somewhat unexpected, in particular with respect to the expression level being comparable to those of ASIC-1 and -2. Our results extend a previous study showing ASIC-4 expression in the rat brain by means of in situ-*hybridization* [30]. They also confirm data on brain tissue expression of ASIC-4 from other species such as mouse, naked mole rat, and man [31–33]. We also selectively studied the MCA and found a pattern of ASIC mRNA expression largely comparable to that in brain tissue, although at a considerably lower level. However, this level is well comparable to that of other vasoactive compounds in the MCA wall such as nitric oxide [34]. Our data confirm previous reports on ASIC mRNA expression in mouse brain, pulmonary, and renal arteries [14,15,18], and they are the first to provide a systematic overview of the ASIC pattern present in the rat cerebral arterial system. Furthermore, the expression of ASIC mRNA goes along with the presence of the corresponding gene products. This has previously been shown for ASIC-1 in rat and human cerebral arteries by Lin and coworkers [13], who found ASIC-1 IR in the media suggesting expression in the smooth muscle cells. Therefore, in the present study, we focused on ASIC-2 and detected the fluorescence signal mainly in the adventitial layer forming a meshwork-like pattern with some extension into the abluminal medial layer. This distribution may be taken to indicate expression in perivascular nerve fibers. Our findings are somewhat in contrast to previous studies reporting on the expression of ASIC-2 IR in freshly digested smooth muscle cells from mouse renal and cerebral arteries [15,17,18], and also from rat pulmonary arteries [35]. This discrepancy cannot easily be resolved; however, it might well be related to the fact that we studied the entire vessel wall by whole mount staining instead of individual smooth muscle cells only. We did not

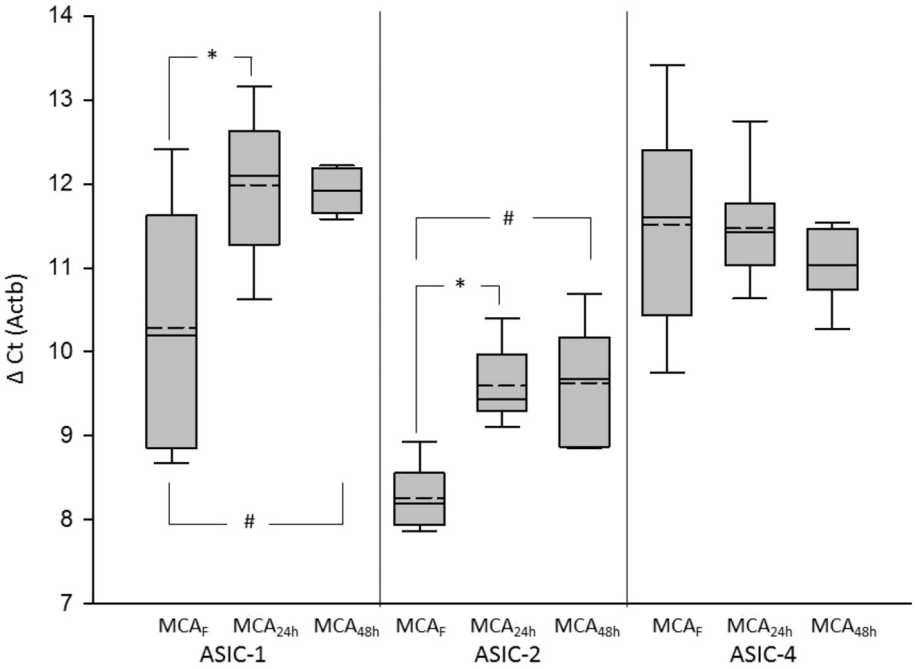

Fig. 5. Box plots indicating the expression (ΔC_T) profiles of ASIC subtypes in middle cerebral artery (MCA) freshly isolated (MCA_F) or after short-term culture for 24 h (MCA_{24h}) or 48 h (MCA_{48h}). The C_T values obtained for beta actin (Actb) served as reference. The horizontal black and broken line in the boxes represent the median and mean value, respectively, while the upper and lower edges of each box represent the 75th and 25th percentile. The error bars represent the range of each data set. $^*p < 0.05$; $^{**}p < 0.005$. $n = 6$ animals for each group.

Table 2
Alterations in ASIC subtype expression after short-term culture of middle cerebral artery (MCA) for 24 h (MCA_{24h}) or 48 h (MCA_{48h}). Expression changes are given in relative terms, i.e. in % of the values obtained in freshly isolated MCA based on the $2^{-\Delta\Delta CT}$ approach. In cultured arteries the expression level for ASIC-1 and -2 was approximately a third of that in freshly isolated arteries while ASIC-4 expression is minimally upregulated. Data are presented as mean and 95% confidence interval.

	MCA_{24h}	MCA_{48h}
ASIC-1	30.9 (18.3–43.5)	32.4 (21.9–2.9)
ASIC-2	39.8 (28.0–50.6)	38.8 (26.8–51.0)
ASIC-4	103.2 (69.8–136.6)	139.9 (103.6–176.1)

consider ASIC-4 a worthwhile target since (i) we found a very low level of mRNA expression for this channel subtype only, (ii) it is thought to be essentially insensitive to extracellular acidosis, and (iii) we, thus, considered this channel subtype of negligible importance for mediation of the acidosis-induced vasomotor response.

4.3. Involvement of ASICs in acidosis-induced relaxation

The presence of arterial wall-specific expression of several ASIC mRNA species raises the question of functional importance of these channels. We therefore studied acidosis-induced vasorelaxation employing isometric tension measurement in MCA ring segments.

Activation of ASICs under these conditions was addressed by the use of amiloride, a well-established blocker of all ASIC subtypes as well as other members of the ENaC/degenerin family. The group of ENaCs needs to be considered here because of experimental evidence in favor of the expression of these channels in the walls of mouse and (normotensive and hypertensive) rat cerebral arteries [36–38]. Activation of ASICs and ENaCs results in influx of Na^+ ions along with membrane depolarization and eventually smooth muscle contraction. Thus, amiloride by blocking these channels would be expected to augment acidosis-induced relaxation. Several aspects strongly suggest that it is in fact ASIC and not ENaC activation that modulates acidosis-induced relaxation: (i) regulation of ENaCs is not pH-sensitive in the rat [39], and (ii) the degree of augmentation by amiloride increased with decreasing pH value in the range of 7.0 to 6.5, which fits well with the pH activation profile of ASIC-1a. Thus, our data are the first to suggest ASICs being activated in acidosis-induced relaxation of cerebral arteries. In this context the exact subtype(s) involved is of minor importance as long as there is currently no panel of subtype-specific agonists and/or antagonists available. From our gene expression studies the presence of mRNA splice variants cannot conclusively be deduced since we did not receive any information from the supplier on the splice variant specificity of the ASIC-1 and -2 assays employed. Moreover, ASIC subunits assemble as homo- or heterotrimers in a varying ratio, and the actual composition determines the resulting channel characteristics including pH-sensitivity, activation profile, and desensitization kinetics the latter being an outstanding feature in many patch clamp studies as discussed in detail previously [40]. Despite this desensitization, ASICs have nevertheless been shown to contribute to the regulation of vasomotor tone in a sustained manner [17], and our own organ bath studies provide further support to this notion. This apparent discrepancy can be explained by several aspects: (i) the fact, that not all ASIC subunits completely desensitize upon exposure to an increased extracellular H^+ concentration, (ii) some ASIC subtypes, most notably ASIC-2b and ASIC4 are considered insensitive to extracellular pH changes, and (iii) a variety of compounds including metabolites, free radicals, neurotransmitters and others have been identified to modulate ASIC behavior (for review, see Kellenberger and Schild [29]), and it appears comprehensible to assume that these factors play a more important role in the intact vessel wall than in patch clamp conditions. Moreover, ASIC activation may also be affected by interaction with the endothelium, especially since the vasomotor response to extracellular acidosis has been suggested to be modulated by release of nitric oxide from the endothelial cells [41]. Therefore, we used ring segments with a functionally intact endothelial lining throughout. With respect to the effect of amiloride on resting tension one cannot exclude involvement of ENaCs since these channels have previously been suggested to be involved in the development of vascular tone and myogenic constriction in cerebral arteries [36–38].

4.4. Effect of short-term culture on ASIC expression and vasomotor action

Tissue acidosis is an important physiological factor in coupling regional perfusion to neuronal activity and the maximum decrease in pH may be up to 0.5 units [42] and at this level of perivascular acidosis,

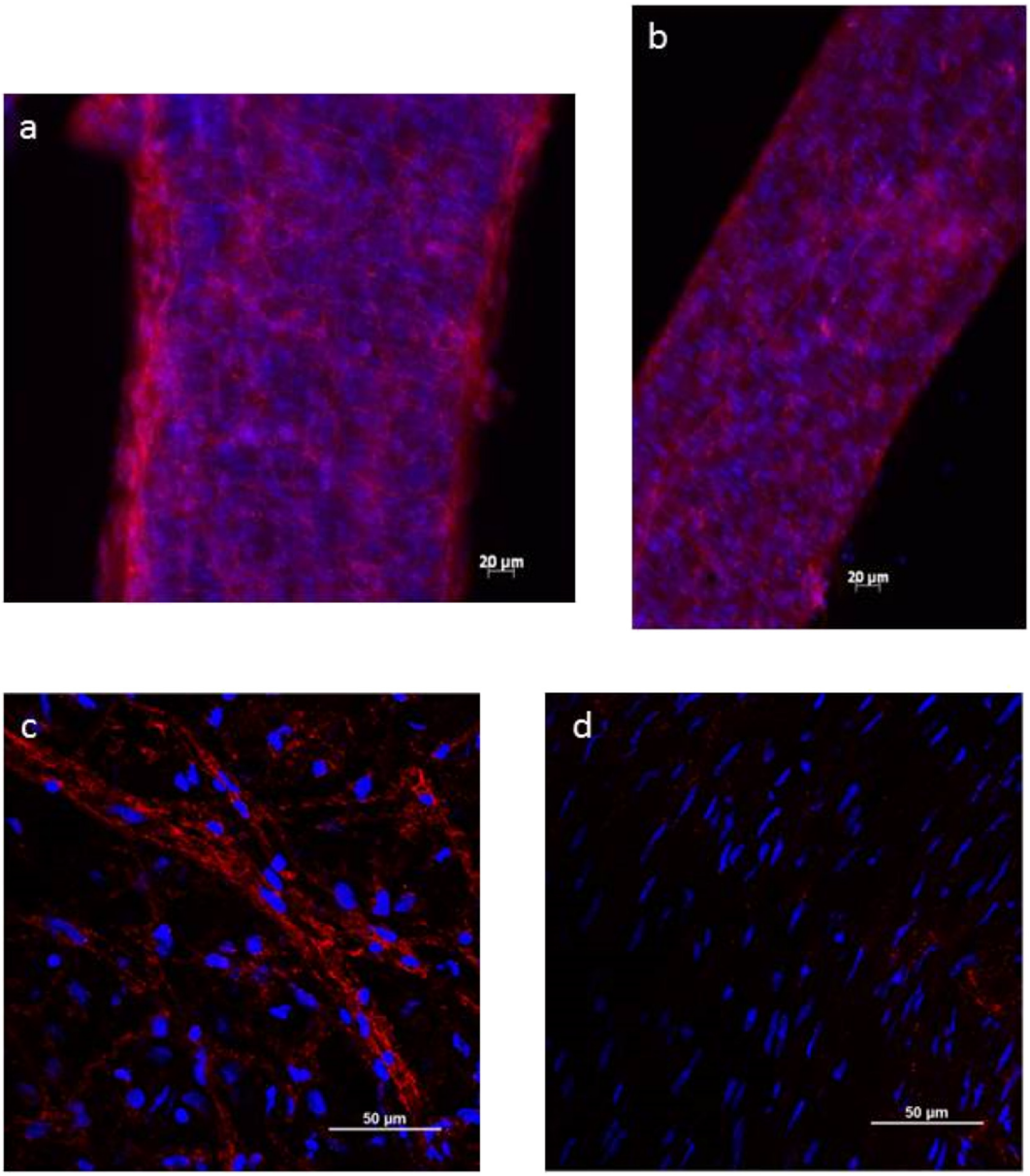

Fig. 6. Free-floating whole-mount immunofluorescent staining of rat middle cerebral artery (MCA) for ASIC-2. Immunofluorescence microscopy showed similar intensities of ASIC-2 immunoreactivity (IR) in freshly isolated (a) and cultured (a) MCA. Confocal microscopy shows that ASIC2 protein is mostly localized in the adventitial layer (c) as there was hardly any immunoreactivity in the media (d).

ASIC activation may well occur. However, a more pronounced degree of tissue acidosis occurs in pathological situations with a decrease of the extracellular pH value in the ischaemic brain to approximately 6.5 or even below as described many years ago [43–45] and often reproduced since then [46]. In this pH range, vasomotor effect of ASIC activation in the cerebroarterial wall has to be expected based on the results of the present study. Moreover, vascular reactivity undergoes significant alterations following brain ischaemia (and other pathological conditions) along with alterations in gene expression as described among others for G-protein coupled receptors (including receptors for bradykinin, endothelin-1, angiotensin-2 [20,23,47] and ion channels such as transient receptor potential cation channels [22]). These alterations can appropriately be mimicked by short-term culturing of arteries as reviewed previously [24]. Therefore, MCAs were challenged by short-term culture for 24 and 48 h in the current study. This resulted in a significant decrease of ASIC-1 and -2 mRNA while the expression level of ASIC-4 was not significantly altered. However, despite the culture-induced decrease in ASIC-2 mRNA, the protein expression did not appear to be substantially altered. On the functional level, the amiloride-sensitive component of acidosis-induced relaxation was significantly enhanced, after 24 h even more than after 48 h, and apparently shifted to less acidotic pH values than in control arteries. These results strongly suggest an increase in the activation characteristics of ASICs after short-term culture.

4.5. Activation of ASICs may have pathophysiological importance

Several studies using cultured neurons and MCA occlusion models in rats and mice have recently suggested that activation of ASICs, presumably those expressed in neurons contributes to the development of ischaemic brain damage [46,48,49]. However, ASICs expressed in the cerebroarterial wall will also be activated during ischaemia, and this might even offer a protective effect due to the limitation of excessive vasodilatation and undue hyperperfusion termed luxury perfusion syndrome [50].

Conflict of interest statement

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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