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ORIGINAL ARTICLE

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Characterisation of a human acid-sensing ion channel (hASIC1a) endogenously expressed in HEK293 cells

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Abstract Acid-sensing ion channels (ASICs) are a new and expanding family of proton-gated cation (Na^+/Ca^{2+}) channels that are widely expressed in sensory neurons and the central nervous system. Their distribution suggests that they may play a critical role in the sensation of the pain that accompanies tissue acidosis and may also be important in detecting the subtle pH variations that occur during neuronal signalling. Here, using whole-cell patch-clamp electrophysiology and reverse transcriptase-polymerase chain reaction (RT-PCR), we show that HEK293 cells, a commonly used cell line for the expression and characterisation of many ion channels, functionally express an endogenous proton-gated conductance attributable to the activity of human ASIC1a. These data therefore represent the first functional characterisation of hASIC1 and have many important implications for the use of HEK293 cells as a host cell system for the study of ASICs, vanilloid receptor-1 and any other proton-gated channel. With this latter point in mind we have devised a simple desensitisation strategy to selectively remove the contribution of hASIC1a from proton-gated currents recorded from HEK293 cells expressing vanilloid receptor-1.

Keywords Acidosis · Capsaicin · Desensitisation · Nociceptor · Pain · Patch clamp · Proton-gated ion channel · Vanilloid

Introduction

Proton-gated ion channels have long been known to exist on sensory neurones, where they have been implicated in the sensation of pain occurring as a result of ischaemia, tissue damage and inflammation [18]. Recently, distinct acid-gated currents have also been recorded from central neurones [8, 21]; this, together with evidence suggesting the widespread occurrence of such proton-gated channels in the brain and periphery (see below) suggests that they may have many important functions in vivo.

Recently, the cloning of two different types of ion channel that are directly gated by protons has greatly advanced this field. These are vanilloid receptor-1 (VR1) [5, 13] and acid-sensing ion channels (ASICs) [22]. The latter is a gene family which, for humans, encompasses ASIC1a (also named ASIC or BNaC2), ASIC2a (also named MDEG1, BNC1 or BNaC1) and ASIC3 (DRASIC) [4, 9, 17, 22, 23]. Another family member, ASIC4, has also been described recently; however, this does not appear to form proton-gated channels, at least when expressed alone [1, 10]. All of the human ASICs appear to be widely expressed throughout the central nervous system and periphery, with particularly high levels of ASIC1a and ASIC3 in the sensory neurones of dorsal root and trigeminal ganglia. In contrast to its rat homologue, which was initially named DRASIC due to its dorsal-root-ganglion-specific expression, hASIC3 is also widely expressed in many peripheral tissues [4]. The largely overlapping expression patterns, together with evidence from in vitro experiments showing that ASIC receptors may consist of heteromeric as well as homomeric combinations of subunits [3], suggests that a large functional repertoire of ASICs may occur in vivo. However, at present the subunit compositions and likely roles of these receptors are far from clear.

The study of the functional properties of homomeric or heteromeric combinations of these ion channels routinely involves heterologous expression of the recombinant receptor in suitable cell lines. Human embryonic kidney (HEK) 293 cells have long been a system of choice for the expression and study of many ion channels and recently have been used to characterise VR1 [5, 13, 20] and ASICs [2]. During such studies on recombinant hVR1 [13] we noted the presence of an endogenous proton-gated conductance in the HEK293 cells. Since the occurrence of such an endogenous proton-gated channel has implications for the study of

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vanilloid receptors, ASICs, and in fact any ion channel or protein that is modulated or gated by protons, we went on to characterise the origin of this conductance further. Here we report electrophysiological, pharmacological and molecular evidence to support the conclusion that HEK293 cells endogenously express the ASIC1a receptor. A preliminary report of this work was presented at the Federation of European Neuroscience Societies 2000 meeting [12].

Materials and methods

Cell culture and generation of a hVR1.HEK293 cell line

HEK293 cells were obtained from the American Type Culture Collection (Virginia, USA), and were cultured in modified Eagles medium with Earle's salts, supplemented with 10% fetal calf serum, non-essential amino acids and glutamine as described previously [13]. A stable cell line expressing human VR1 (hVR1.HEK293 cells) was also developed and maintained as described previously [13].

Electrophysiological recordings from HEK293 cells and hVR1.HEK293 cells

Cells were plated onto glass coverslips coated with poly-D-lysine at a density of 13,000 cells cm^{-2} and used after 16–48 h in culture. Whole-cell patch-clamp experiments were carried out at room temperature (20–24°C) in voltage-clamp mode using an Axopatch 200B amplifier controlled using the pClamp7 or 8 software suite as described previously [11]. In the majority of experiments the extracellular solution used consisted of 130 mM NaCl, 5 mM KCl, 2 mM $BaCl_2$, 1 mM $MgCl_2$, 30 mM glucose, 25 mM HEPES, adjusted to pH 7.3 (standard) or alternative pH values by the addition of either NaOH or HCl, and electrodes (2–6 MΩ) were filled with 140 mM CsCl, 4 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES adjusted to pH 7.3 with CsOH. In further experiments, designed to allow the accurate determination of the permeability ratios P_K/P_{Na} and P_{Ca}/P_{Na}, we used a simplified sodium-based intracellular solution consisting of 140 mM NaCl, 0.1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM HEPES, 1.1 mM EGTA, pH 7.3 with NaOH, and a sodium- (140 mM NaCl, 0.1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM HEPES, 10 mM glucose, pH 7.3 with NaOH), potassium- (same, except all NaCl replaced with 140 mM KCl) or calcium- (same, except NaCl replaced with 70 mM $CaCl_2$, and 35 mM choline chloride) based extracellular solution. Drug applications and changes in extracellular pH were performed using a commercially available automated fast solution exchange system (Warner Instruments SF-77B). This device can perform individual solution changes in ≅30 ms and was interfaced with our acquisition software so as to generate precisely timed alterations in extracellular solution. In all, recordings were made from a total of 56 wild-type HEK293 cells with a mean membrane capacitance of 13.0±0.4 pF. In these experiments series resistance averaged 6.4±0.4 MΩ and series resistance compensation, used where appropriate, averaged 74±1% (n=53). All data were acquired to a personal computer for subsequent analysis. Concentration–response data were fitted with a logistic function according to the equation $I=I_{max}/\{1+(EC_{50}/[L])^n\}$, where I_{max} is the maximal response, EC_{50} is the concentration of agonist required for half-maximal effect, $[L]$ is the concentration of agonist, and n is the Hill coefficient. Permeability ratios were determined from the interpolated current reversal potentials (E_{rev}) according to the equations $E_{rev}=(RT/F)\ln(P_K[K^+]_o/P_{Na}[Na^+]_i)$ and $E_{rev}=(RT/2F)\ln(4P_{Ca}[Ca^{2+}]_o/P_{Na}[Na^+]_i)$ for P_K/P_{Na} and P_{Ca}/P_{Na}, respectively. Curve fitting was carried out with either the Clampfit (Axon Instruments) or Origin (Microcal) software packages. In each case the goodness of fit was defined by the least-squares method and all data are presented as the mean ±SEM.

Reverse transcriptase-polymerase chain reaction of human ASIC mRNA

We used RT-PCR to test for the expression of hASIC genes in HEK293 cells. Total RNA was isolated from HEK293 cells using Trizol reagent (Life Technologies) and RT-PCR reactions were primed with the following antisense gene-specific primers: hASIC1a, 5' AATGACCTCGTAGGCGTAGTC; hASIC2a, 5' CATTGTGTCACAAGTACTCAC; hASIC3, 5' GCTGTCGGTTCCAGAAATATC and reverse-transcribed using Superscript II reverse transcriptase according to the manufacturer's instructions (Life Technologies). PCR amplification (*Taq* DNA polymerase, Life Technologies) was carried out using the appropriate sense gene-specific primer: hASIC1a, 5' GCAGATCCTGCTCTGGACTTCC, hASIC2a, 5' TCCTCAGAGATGGGCCTCGAC and hASIC3, 5' ACTATGAGCCAGAGCCCTCTG and antisense gene-specific primer as stated above. The PCR cycling parameters used were 30 cycles at 95°C for 30 s, 50°C for 30 s and 72°C for 45 s. Negative controls were treated in the same way except that no reverse transcriptase was added. Subsequently, 1/10 of the PCR reaction was analysed by electrophoresis on a 1.2% agarose gel and the identity of the PCR products was confirmed by cloning the gel-purified fragments and DNA sequencing.

Results

Endogenous proton-gated currents in HEK293 cells

Application of an acidic extracellular solution (pH 5.3) to HEK293 cells revealed the functional expression of an endogenous proton-gated current in nearly all HEK293 cells studied (98% or 47/48 of cells tested; mean peak amplitude was 334±19 pA in response to challenge with standard extracellular solution at pH 5.3). Even with maintained acidification, the proton-gated current was transient in nature (Fig. 1A). This was due to the rapid activation (10–90% rise time of 501±37 ms, n=13) and subsequent inactivation of the current, the decay of which could be fitted by a monoexponential function with a time constant, τ, of 1064±42 ms (n=13). We investigated the proton sensitivity of this current by measuring the response generated by rapid shifts to a range of proton concentrations between pH 7.0 and pH 5.3 (Fig. 1B). The resulting concentration–response curves revealed a pEC_{50} of 6.45 for proton activation of the ASIC and a high degree of cooperativity exemplified by a Hill coefficient of 8.7 (n=4; Fig. 1C). This suggests that many sites on the ASIC are required to be protonated to elicit channel activation.

Ion selectivity of proton-gated currents in HEK293 cells

To establish the current-voltage relationship of the ASIC current and thus gain insight into the ion selectivity of the endogenous proton-gated current, we used a voltage ramp protocol (–70 to +100 mV in 100 ms). This was applied close to the peak of the proton-gated current (Fig. 2). Control currents, namely a prior ramp protocol recorded at pH 7.3, were subtracted from ramps recorded at pH 5.3 to obtain the net proton-gated current. The resulting current–voltage relationship was approximately

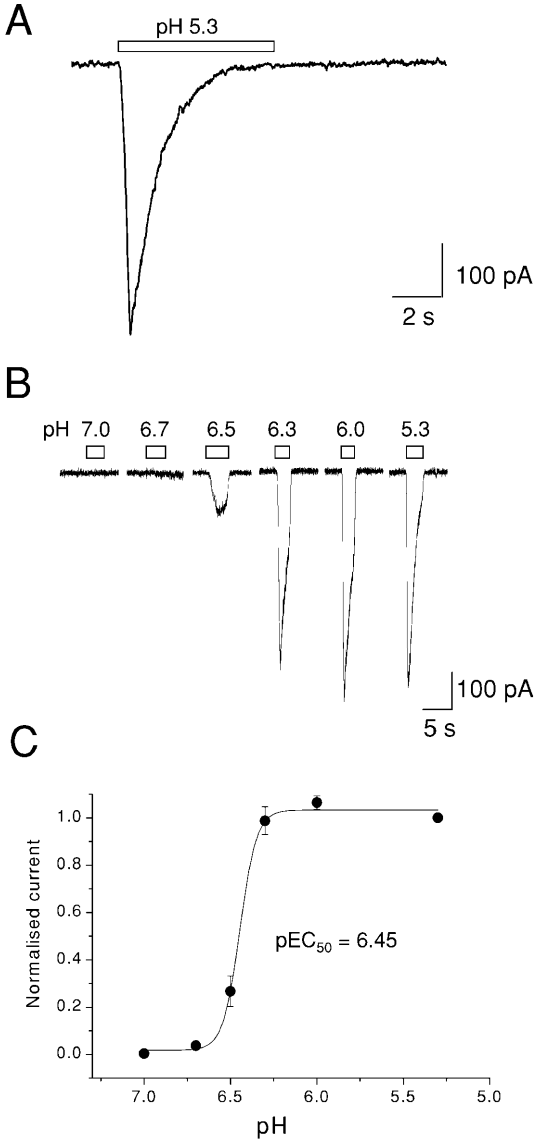

Fig. 1A–C An endogenous proton-gated current in HEK293 cells. **A** A typical whole-cell recording of a transient proton-gated current recorded in response to the application of a solution at pH 5.3 to HEK293 cells for the duration indicated by the *bar*. The proton-gated currents activated rapidly, with a 10–90% rise time of 501±37 ms (n=13). The subsequent inactivation observed in the presence of agonist (desensitisation) resulted in a rapid current decay which could be fitted with a monoexponential function with a time constant, τ, of 1064±42 ms (n=13). **B** Whole-cell recording from a typical cell showing responses to a range of proton concentrations. Activation of the proton-gated conductance was observed at pH<7 and was maximal at pH 5.3. **C** Concentration–response curves for proton activation of the ASIC were constructed from 4 similar experiments to that shown in **B**. Data were normalised to the response at pH 5.3 and fitted with a logistic equation yielding a pEC_{50} of 6.45 and a Hill coefficient of 8.7

linear over the whole voltage range studied with a mean reversal potential of 70±3 mV (n=5), which is consistent with the gating of a highly Na^+-selective channel. To establish if the ASIC was also permeable to other cations, such as K^+ and Ca^{2+}, we conducted ion selectivity experi-

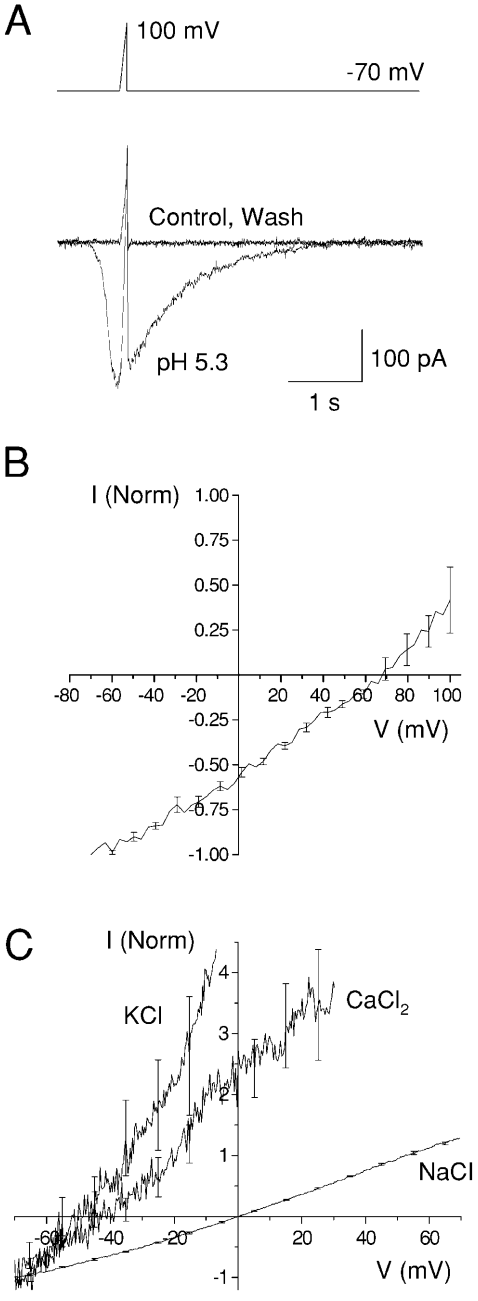

Fig. 2A–C Current–voltage properties of the endogenous acid-sensing ion channel (*ASIC*) current. **A**, **B** The current–voltage relationship of the endogenous ASIC current was defined using a voltage ramp protocol (–70 to +100 mV in 100 ms) coincident with the peak of the proton-gated current. In order to accurately determine the properties of the proton-gated current, we subtracted ramps recorded under control conditions (pH 7.3) from those recorded at pH 5.3. Subsequently data were normalised to the current recorded at –70 mV and averaged across cells (n=5). Occasional *error bars* from the averaging process are shown. The resulting current–voltage relationship is approximately linear over the whole voltage range studied with a reversal potential of 70±3 mV (n=5). **C** Further current–voltage experiments were conducted in symmetrical NaCl solution (see Materials and methods) and in modified solutions in which extracellular sodium (140 mM NaCl) was replaced with either potassium (140 mM KCl) or calcium (70 mM $CaCl_2$). The interpolated reversal potentials from such experiments were used to determine the permeability ratios P_K/P_{Na} and P_{Ca}/P_{Na} for the ASIC (see text)

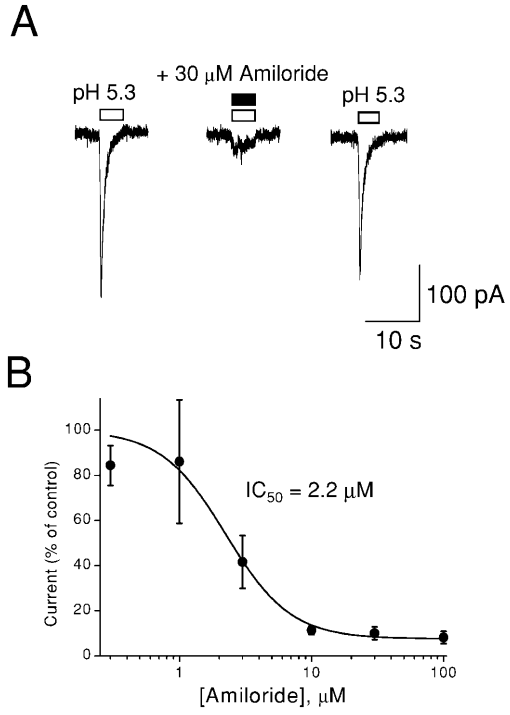

Fig. 3A, B Amiloride inhibition of the endogenous ASIC. **A** Sample traces from a typical experiment used to determine the degree of inhibition of the proton-gated current by 30 μM amiloride. **B** The potency of amiloride was estimated by constructing a dose–response relationship based on data similar to and including those shown in **A** (n=2–6). In all cases, amiloride was pre-applied for >20 s before being co-applied with the agonist (pH 5.3). A logistic curve fit to the data yielded an IC_{50} of 2.2 μM

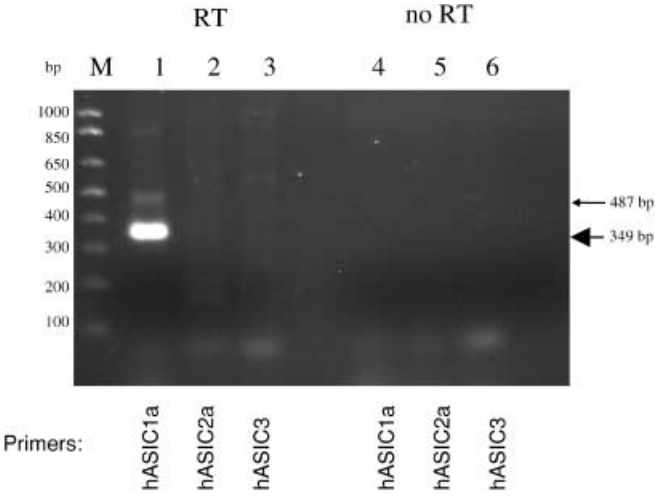

Fig. 4 Expression of ASICs in HEK293 cells determined by RT-PCR. RT-PCR analysis of total RNA isolated from HEK293 cells was used to test for the expression of the human ASIC subunits cloned to date. The primers used were as follows: *lanes 1* and *4*: hASIC1a, *lanes 2* and *5*: hASIC2a, *lanes 3* and *6*: hASIC3. *Lanes 1–3* are from PCR reactions including reverse transcriptase and *lanes 4–6* are negative controls in which reverse transcriptase was omitted. *M* denotes the 1-kb DNA plus ladder used. The band labelled 349 bp represents a specific PCR product amplified with the hASIC1a primers. A second weaker band at 487 bp correlates with the additional amplification of a known splice variant of hASIC1a previously identified by Garcia-Anoveros et al. [9]. Gel purification, cloning and subsequent DNA sequencing of these PCR products confirmed their identity as the expected portions of the hASIC1a gene

ments under essentially biionic conditions. In experiments utilising Na^+ as the predominant cation in both the intracellular and extracellular solutions, the acid-evoked currents exhibited a reversal potential close to 0 mV (0.2±0.5 mV, n=5) as expected. The current–voltage relationship was again approximately linear with a rectification ratio ($I_{+70\ mV}/I_{-70\ mV}$) of 1.28±0.03 (n=5). Substitution of extracellular NaCl with KCl or $CaCl_2$ shifted the reversal potential to –43.2±3.7 mV (n=4) or –40.9±3.7 mV (n=4), respectively (Fig. 2C). These shifts in the reversal potential are consistent with permeability ratios P_K/P_{Na}=0.19 and P_{Ca}/P_{Na}=0.02. The ASIC is therefore highly sodium selective (P_{Na}/P_K=5.26; P_{Na}/P_{Ca}=50) but does allow both K^+ and Ca^{2+} to permeate.

Amiloride inhibition of proton-gated currents in HEK293 cells

A defining feature of the ASICs identified to date is a marked sensitivity to the diuretic agent amiloride. We therefore tested the ability of amiloride to inhibit the endogenous ASIC in the HEK293 cells. We found that 30 μM amiloride caused almost complete block of the ASIC-like conductance in HEK293 cells (Fig. 3A). Further experiments using a range of amiloride concentrations established an IC_{50} for amiloride of 2.2 μM (Fig. 3B).

Endogenous expression of human ASIC1 in HEK293 cells detected by RT-PCR

The results described above provide strong evidence for the expression of an ASIC in HEK293 cells. We therefore used RT-PCR to test directly for the endogenous expression of members of the hASIC family in these cells. We used gene-specific primers against all of the proton-gated hASICs that have been described to date, namely hASIC1a, hASIC2a and hASIC3 (Fig. 4). The results show convincingly that HEK293 cells express the hASIC1a gene (and a splice variant) but not hASIC2a or hASIC3. Subsequent gel purification, cloning, and double-stranded DNA sequencing of the PCR products confirmed their identity as the expected portions of the hASIC1a gene.

Selective inhibition of hASIC1a using a desensitisation strategy

The electrophysiological profile established for the endogenous ASIC (activation threshold at pH ≅6.7 and

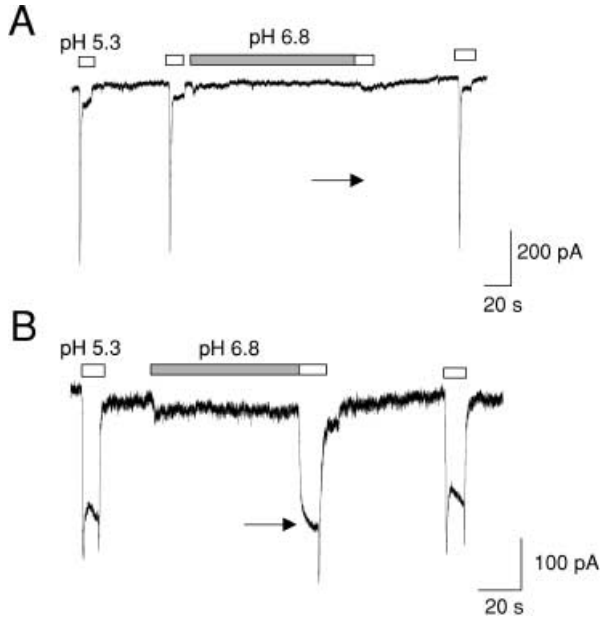

Fig. 5A, B Separation of the ASIC and VR1 conductances using a receptor desensitisation strategy. Proton-gated currents were evoked in hVR1.HEK293 cells which stably express hVR1 together with the endogenous ASIC. In the two examples shown, one from a cell where the ASIC current dominates (**A**), and another from a cell possessing both prominent ASIC and hVR1 conductances (**B**), prolonged exposure to pH 6.8 (for 60 s) resulted in selective desensitisation of the ASIC with little or no effect on hVR1 (*n*=7). The *arrow* indicates the expected location of the ASIC-mediated response to challenge with pH 5.3, which is absent following the pre-exposure to pH 6.8. This desensitising effect on the ASIC current was also reversible upon return to pH 7.3. Therefore, initial pre-treatment with slightly acidic solutions provides a means to effectively isolate VR1-mediated acid responses from those endogenously present in HEK293 cells

rapid inactivation or desensitisation) differs from that of other proton-gated channels such as VR1, which require greater acidification for activation and exhibit a lesser propensity to desensitise [13, 20]. We therefore reasoned that a simple receptor desensitisation protocol may prove to be an effective strategy for removing the influence of the ASIC1a current in a number of experimental systems such as electrophysiology and calcium imaging studies. We examined this in HEK293 cells stably expressing human VR1 (hVR1.HEK293 cells, see [13]). We found that pre-exposure of the cells to pH 6.8 (for 60 s) effectively and completely removed the transient ASIC current from responses generated by subsequent challenge to pH 5.3, whereas the slow activating, non-desensitising, hVR1-mediated responses were apparently unaffected (Fig. 5). Therefore, initial pre-treatment of cells with a solution of reduced pH provides a means to effectively isolate VR1-mediated acid responses from those endogenously present in HEK293 cells. This may prove to be a useful tool since even high concentrations of amiloride do not cause complete inhibition of hASIC1a ([22]; Figure 3) and may even modulate VR1 function [16].

Discussion

Over the last few years the cloning of the ASIC family of receptors and VR1 has added new impetus to the study of proton-gated conductances in sensory neurones and the central nervous system. The intensive study of these ion channels in recombinant cell systems is now sure to follow. HEK293 cells are often a cell system of choice for such studies with homomeric or heteromeric combinations of receptor subunits. Our finding that HEK293 cells functionally express ASIC1a is an important factor in the design, planning or interpretation of such studies.

We first noted the functional expression of an ASIC-like conductance in HEK293 cell during our studies on human VR1 [13] and we have subsequently characterised the properties of this proton-gated conductance in full to determine its molecular identity. Our whole-cell patch-clamp studies showed that the endogenous receptor has a high sensitivity to extracellular protons (pEC_{50} of 6.45), exhibits fast activation and inactivation kinetics, is highly Na^+ selective, permeable to K^+ and Ca^{2+}, and is reasonably potently blocked by amiloride. All of these findings are consistent with this channel being the human orthologue of rat ASIC1a characterized by Waldmann et al. [22] and clearly differentiate this channel from hASIC2a and hASIC3, which both require higher proton concentrations for activation (pEC_{50}s<5) and exhibit slower kinetics of activation and desensitisation [3]. hASIC3 is also characterised by a pronounced, sustained, current phase [3, 4] which was not seen in the HEK293 cells.

Our data concerning the ion selectivity of hASIC1a are largely consistent with data published by Sutherland et al. [19] who quote a P_{Ca}/P_{Na} value of 0.06 for rat ASIC1a but are much lower than the P_{Ca}/P_{Na} ratio of 0.4 estimated by Waldmann et al. [22]. The reason(s) for this difference are not clear but may indicate a degree of channel blockade by the high calcium concentrations used in our study, since increasing the extracellular calcium concentration has been previously shown to reduce ASIC1a currents carried by sodium [22]. Whatever the explanation, it is clear that ASIC1a is capable of conducting Ca^{2+} under physiological recording conditions since clear Ca^{2+} signals, attributable to the activation of the endogenous ASIC, are consistently observed in wild-type HEK293 cells using Ca^{2+} imaging studies [14].

The rapid desensitisation characteristic of ASIC1a is perhaps what has prevented other investigators from noting its presence in previous studies. In these cases, a more slowly generated pH change probably resulted in significant desensitisation prior to activation and prevented detection of the ASIC signal. In fact, the high proton sensitivity and rapid desensitisation of the endogenous ASIC led us to devise a simple desensitisation strategy to separate the proton-gated ASIC- and VR1-mediated conductances in a hVR1.HEK293 cell line. This was driven by the lack of suitable pharmacological tools to eliminate the ASIC activity: amiloride is not

selective amongst ASICs and may even have activity versus VR1 itself [16]. Our desensitisation strategy works because of the different properties of VR1 versus the endogenous ASIC. Specifically, VR1 exhibits much slower desensitisation and a lesser sensitivity to protons [13, 20]. We believe this desensitisation-based ASIC-removing strategy will be useful in a number of experimental systems such as electrophysiology and calcium imaging where it will allow effective prior elimination of the ASIC signal thus permitting the properties of the VR1 channel (or other) to be studied in isolation.

Our RT-PCR studies, to test for the expression of ASIC genes in the HEK293 cells, clearly demonstrate that these cells express the ASIC1a gene, but not the ASIC2a or ASIC3 genes. This is entirely consistent with the interpretation of our electrophysiological data, where the close similarity of the properties of the ASIC to the properties of rASIC1a strongly support the idea that these currents arise primarily from the homomeric assembly of hASIC1a subunits into functional proton-gated channels. Our results are therefore likely to represent the first functional characterisation of the hASIC1a receptor which was originally cloned from a human cDNA library [9]. It is possible, however, that additional ASIC subunits, such as human homologues of rat ASIC1b (ASICβ) [7] and ASIC2b (MDEG2) [15] which have not been reported to date, or the proton-insensitive ASIC4 subunit could contribute to the phenotype of the currents we have recorded; the apparent homogeneity in the currents we have recorded, however, does provide some evidence against this possibility in this instance. Further studies to address which ASIC subunit heteromers are permissible, and what, if any, are their functional roles, are a future requirement. In this respect, the recent report of a tarantula toxin (PcTX1) [8], which is a specific inhibitor of the rat ASIC1a receptor, is an exciting development and may provide the first truly useful pharmacological tool for dissecting the function of the various homomeric and heteromeric ASIC receptors that are likely to exist. PcTX1 appears to inhibit only homomeric rat ASIC1a receptors [8] and its use may therefore allow the endogenous proton-gated conductance that we have described in the HEK293 cells to be unequivocally assigned to hASIC1a.

In conclusion, we have provided molecular, pharmacological and electrophysiological evidence for the expression of functional ASICs in HEK293 cells. Our electrophysiological characterisation of the proton-gated conductance combined with our RT-PCR data demonstrating the endogenous expression of the hASIC1a gene in HEK293 cells strongly support the idea that these currents arise primarily from the homomeric assembly of hASIC1a subunits into functional proton-gated channels. These findings have important implications for the use of HEK293 cells in the study of any ion channels gated or even modulated by protons as well as other proteins that may heterodimerize or interact with ASIC subunits. Preliminary studies by Cesare et al. [6] also suggest that the occurrence of ASICs is not just limited to HEK293 cells but may be a widespread feature of a number of commonly used cell systems including *Xenopus* oocytes and COS-7 cells.

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