

Increased expression of TRPV1 receptor in dorsal root ganglia by acid insult of the rat gastric mucosa

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Abstract

It is still unknown which receptors of peripheral sensory pathways encode and integrate an acid-induced nociceptive event in the gastric mucosa. The transient receptor potential vanilloid receptor 1 (TRPV1) and the acid-sensing ion channel 3 (ASIC3) are two nociception-related receptors. Here we investigated (i) to what extent these receptors are distributed in stomach-innervating neurons of dorsal root and nodose ganglia, using immunohistochemistry and retrograde tracing, and (ii) whether their expression is altered in response to a noxious acid challenge of the stomach. We also explored the presence of TRPV1 in the gastric enteric nervous system because of its possible expression by intrinsic sensory neurons. Most stomach-innervating neurons in nodose ganglia were immunoreactive for TRPV1 (80%) and ASIC3 (75%), these results being similar in the dorsal root ganglia (71 and 82%). RT-PCR and Western blotting were performed up to 6 h after oral application of 0.5 M HCl to conscious rats. TRPV1 protein was increased in dorsal root but not in nodose ganglia whereas TRPV1 and ASIC3 mRNAs remained unchanged. TRPV1 mRNA was detected in longitudinal muscle–myenteric plexus preparations of control stomachs and was not altered by the acid challenge. Combined vagotomy and ganglionectomy abolished expression of TRPV1, indicating that it may derive from an extrinsic source. In summary, noxious acid challenge of the stomach increased TRPV1 protein in spinal but not vagal or intrinsic sensory afferents. The TRPV1 receptor may be a key molecule in the transduction of acid-induced nociception of the gastric mucosa and a mediator of visceral hypersensitivity.

Introduction

It has long been known that information about noxious events in the gut is conveyed to the central nervous system primarily by spinal sensory pathways (Gebhart, 2000; Grundy, 2002). Recent data, however, emphasize that vagal pathways may also be involved in the transmission of gastrointestinal pain (Holzer, 2002; Lamb *et al.*, 2003). While noxious distension of the colon, for instance, is signalled to the brain via the spinal cord (Traub *et al.*, 1993), balloon distension of the stomach is preferentially signalled via the vagal nerve (Traub *et al.*, 1996). It is also known that both vagal and spinal afferent fibres can be sensitised (Gebhart, 2000; Lamb *et al.*, 2003). Mechanisms responsible for the sensitisation of visceral afferents include alteration of channel properties and increased expression of receptors (Kirkup *et al.*, 2001; Bielefeldt *et al.*, 2002).

By using a previously described rat model of gastric injury (Schuligoj *et al.*, 1998) we investigated whether noxious challenge of the stomach with acid, a factor in the generation of gastric pain, may increase the expression of nociception-related receptors in spinal and/or vagal sensory neurons. Two receptors, in particular, may be implicated in gastric chemonociception. The first is the transient receptor potential vanilloid receptor 1, TRPV1 (formerly known as VR1), which has an extracellular binding site for protons (Jordt *et al.*, 2000), is a key molecule for integrating painful stimuli (Tominaga *et al.*, 1998) and plays a major role in the generation of inflammatory thermal hyperalgesia (Caterina *et al.*, 2000; Davis *et al.*, 2000). The

second receptor, ASIC3, a proton-detecting ion channel (Waldmann, 2001), is also a transducer of pain associated with tissue acidosis after injury or inflammation (Voilley *et al.*, 2001; Chen *et al.*, 2002).

We first examined the immunohistochemical distribution of TRPV1 and ASIC3 in stomach-projecting neurons of thoracic dorsal root ganglia (DRGs) and nodose ganglia. Primarily small- and medium-sized DRG somata, which are characteristic of nociceptors, contain TRPV1 (Guo *et al.*, 1999) whereas ASIC3 seems rather to predominate in medium- to large-sized neurons (Alvarez de la Rosa *et al.*, 2002). TRPV1 immunoreactivity is also found in nodose ganglia (Patterson *et al.*, 2003) while ASIC3 has not yet been investigated in the vagal sensory system. In the second part of our experiments, expression of TRPV1 and ASIC3 in thoracic DRGs and nodose ganglia was assessed using RT-PCR. TRPV1 expression was also evaluated by Western blotting.

Another sensory system which could perceive and process noxious events in the gut are neurons of the enteric nervous system (ENS). There are reports that TRPV1 might be expressed in enteric neurons and even be located in intrinsic sensory neurons (Anavi-Goffer *et al.*, 2002; Poonyachoti *et al.*, 2002; Chan *et al.*, 2003), although other studies could not verify these observations (Patterson *et al.*, 2003; Ward *et al.*, 2003). We therefore investigated expression of TRPV1 in the gastric ENS under the assumption that this receptor acts as a nociceptive transducer not only in extrinsic sensory afferents but also in intrinsic sensory neurons of the ENS.

Materials and methods

Experimental design

Experiments were carried out on female Sprague-Dawley rats (180–250 g) which were fasted for 20 h prior to experiments but had free

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TABLE 1. Primary antibodies for immunohistochemistry and Western blots

| Receptor | Species | Antibody | Dilution | Source |
|-------------------|------------|----------|----------|---|
| TRPV1 (VR1) | Rabbit | AB-1 | 1 : 200 | Oncogene, Oncogene, CN Biosciences Inc., Germany |
| TRPV1 (VR1) | Goat | R-18 | 1 : 500 | Santa Cruz, Santa Cruz Biotechnology, Santa Cruz, CA, USA |
| ASIC3 (DRASIC) | Guinea pig | AB 5927 | 1 : 400 | Chemicon, Chemicon International Inc., Temecula, CA, USA |
| Neurofilament 145 | Rabbit | AB 1987 | 1 : 200 | Chemicon, Chemicon International Inc., Temecula, CA, USA |

access to water. Hydrochloric acid (0.5 M) was administered intragastrically (IG) at a volume of 1 mL/100 g by use of a soft paediatric feeding tube (Portex, Hythe, England) (Schuligoi *et al.*, 1998). Depending on the experiment, rats were killed after 2, 5 or 6 h with an overdose of intraperitoneal (IP) pentobarbital. The left and right nodose ganglion and the thoracic DRGs from both sides (T8–12) were removed and submitted to RT-PCR and Western blotting. DRGs T8 to T12 exhibit the largest proportion of sensory afferents projecting to the gastric corpus, as determined by calcitonin gene-related peptide immunohistochemistry and retrograde tracing with True Blue (Green & Dockray, 1988). Stomachs were removed and cut in two halves. Each half was stretched and pinned flat on a Petri dish with a silicon elastomer bottom and covered with ice-cold oxygenated 0.1 M phosphate-buffered saline (PBS). The mucosa and the circular muscle layer were peeled off under a dissecting microscope and only the longitudinal muscle with the adherent myenteric plexus (LMMP preparations) was submitted to RT-PCR. The experimental design of the study was approved by the Austrian Ministry of Education, Science and Culture.

Subdiaphragmatic vagotomy and celiac ganglionectomy

Bilateral subdiaphragmatic vagotomy and celiac ganglionectomy were performed under a dissecting microscope (Holzer & Lippe, 1988). Animals were fasted overnight prior to surgery. After induction of anaesthesia with IP pentobarbital (50 mg/kg), the abdomen was opened with a midline incision. After connective tissue had been removed from the surface of the oesophagus the dorsal and ventral trunks of the vagus nerve attached to the subdiaphragmatic portion of the oesophagus were transected and the cut nerve stumps retracted. Celiac ganglionectomy was performed by gently freeing the celiac–superior mesenteric ganglion complex from the surrounding vasculature and by removing the complex from the adherent nerves. Rats were given 10 days to recover from surgery. To assess the completeness of the celiac ganglionectomy, immunohistochemistry for calcitonin gene-related peptide and tyrosine hydroxylase was carried out on LMMP preparations of the gastric corpus (Schicho *et al.*, 2003). Immunoreactivity for these antigens was virtually absent in the tissues from operated animals.

Retrograde tracing with True Blue

In order to locate DRG and vagal neurons that project to the stomach, the retrograde tracer True Blue (Molecular Probes, Leiden, the Netherlands) was used (Green & Dockray, 1988). Rats ($n = 6$) were anaesthetized with intramuscular ketamine (80 mg/kg) and xylazine (8 mg/kg) after a prior application of 1 mg/kg IP atropine sulphate to suppress cholinergic activity. The abdomen was opened with a midline incision and the stomach was exposed. A total volume of 10 μ L of a 4% True Blue suspension was injected into the ventral and dorsal stomach wall (two injections of 2.5 μ L for each aspect) using a 10- μ L Hamilton syringe. Following each injection, the needle was kept in place for at least 1 min to avoid leakage of the dye. The injection sites were then carefully swabbed with saline prior to suturing. After 1 week, animals were anaesthetized with IP sodium pentobarbital (50 mg/kg) and transcardially perfusion-fixed with 0.1 M PBS and 4% paraformaldehyde.

Nodose ganglia and thoracic DRGs (T8–12) from both sides were removed, postfixed in 4% paraformaldehyde at 4 °C for 24 h, and cryoprotected in phosphate-buffered 20% sucrose at 4 °C for another 24 h. Afterwards, ganglia were embedded in artificial medium (Microm, Walldorf, Germany), frozen on dry ice and cut into 14- μ m sections. The sections were thaw-mounted on poly-L-lysine-coated slides and kept at –70 °C until use.

Immunohistochemistry

To determine whether TRPV1 and ASIC3 are present in stomach-projecting neurons of thoracic DRGs and nodose ganglia, immunofluorescence was performed for each receptor and examined for colocalization with True Blue. After an initial wash in 0.1 M PBS–azide (pH 7.4; 0.1% NaN₃; Sigma) tissues were preincubated in a solution of 4% donkey serum, 0.5% Triton X-100 and PBS–azide for 1 h to block nonspecific binding. Antibodies (Table 1) were diluted in a solution containing the same substances. Sections were then incubated overnight at 4 °C. After washing in PBS–azide (3 \times 10 min), tissue was exposed for 1.5 h to the species-specific fluorophore-conjugated secondary antibodies (donkey Cy2 for TRPV1 and Cy3 for ASIC3; Jackson ImmunoResearch, West Grove, PA, USA). Following a final wash in 0.1 M PBS–azide, slides were coverslipped with PBS–glycerol. Negative controls were performed by leaving out the primary antibodies during the staining procedure. TRPV1 antibodies were also incubated with the respective control peptide prior to staining procedures to prove their specificity.

For detection of TRPV1 in the gastric myenteric plexus rats were killed with an overdose of IP pentobarbital. The stomachs were immediately removed, washed in ice-cold oxygenated 0.1 M PBS containing 1 μ M nifedipine (Sigma) and cut open along the lesser and greater curvature. Each stomach half was stretched and pinned flat on a Petri dish with a silicon elastomer bottom. Stomachs were fixed overnight in Zamboni's fixative at 4 °C, rinsed in PBS (3 \times 30 min) and transferred into PBS–azide. LMMP preparations were made from gastric corpus prior to immunohistochemistry in the same way as for RT-PCR. Immunohistochemical procedures followed the same protocol as for sections.

Slides were examined under a fluorescence microscope (Olympus IX 70) equipped with separate filtercubes (wide-band cube for True Blue: dichromatic mirror (DM)400, excitation filter 330–385, barrier filter 420 nm; narrow band cube for Cy2: DM505, excitation filter 470–490, barrier filter 515–550 nm; Cy3 cube: DM568, excitation filter 540–560, barrier filter 575–645 nm). No cross-fluorescence was observed when fluorophores were examined through a filtercube unsuitable for the emitting wavelength of the fluorophore. Images were taken with a high resolution digital camera (Olympus DP 50; 2776 \times 2074 pixels) and processed with image analysis software (Soft Imaging System, Münster, Germany).

Western blots

Both nodose ganglia and thoracic DRGs (T8–12 bilaterally) were collected from each rat ($n = 12$) and homogenised in extraction buffer containing a proteinase inhibitor cocktail (phenylmethylsulphonyl

fluoride, 1 mM; pepstatin A, leupeptin, aprotinin, phenanthroline, each 10 $\mu\text{g}/\text{mL}$; and benzamidine HCl, 16 $\mu\text{g}/\text{mL}$). Homogenates were then spun down at 12 000 *g.* for 30 min and supernatants were kept as aliquots at -70°C . One aliquot of each sample was used for evaluation of total protein content by using a Bio-Rad protein standard assay. Protein samples (20 μg) were loaded onto an 8% SDS-polyacrylamide gel and electrophoresed. The gel was transferred to a polyvinylidene difluoride (PVDF) membrane by use of a wet tank blotter (Bio-Rad, Hercules, CA, USA) at 150 mA for 1 h. Blotting membranes were afterwards blocked with 5% dry milk for 20 min and incubated with TRPV1 antibody (1 : 200; Oncogene) overnight at 4°C . After washing, blots were incubated in a horseradish peroxidase-conjugated secondary antibody (1 : 10 000; goat anti-rabbit IgG) for 2 h at room temperature. Immunoreactivity was developed with a Western blotting detection kit (Amersham Biosciences, Piscataway, NJ, USA) and visualised by exposing the membrane to an X-ray film. Membranes were then incubated for 20 min in stripping buffer (glycine, 0.05 M; EDTA, 1 mg/mL; and NaN_3 , 1 mg/mL, pH 2.5) at room temperature and reincubated overnight at 4°C with an antibody to neurofilament 145 which served as a loading control. Band density \times area were measured by a computerised image analysis system (M2-MCID; Imaging Research, Ontario, Canada) and normalised with the loading control.

RT-PCR

The RT-PCR procedure has been described previously (Beubler *et al.*, 2001). Briefly, total RNA was extracted from both nodose ganglia, thoracic DRGs T8–12 (bilaterally) and LMMP preparations with Trizol reagent (Invitrogen, Lofer, Austria) following the manufacturer's instruction. RNA preparations were then treated with DNase inactivation reagent (DNA-freeTM; Ambion, Austin, TX, USA) and 0.8 μg RNA was reverse transcribed into cDNA with either avian myoblastosis virus reverse transcriptase (Promega, Madison, WI, USA) or Omniscript reverse transcriptase (Quiagen, Valencia, CA, USA) and oligo (dT)15 primers (Promega). The following specific primers were used for PCR (MWG Biotech, Ebersberg, Germany): TRPV1 forward 5'-GACATGC-CACCCAGCAGG-3' and reverse 5'-TCAATTCACACACCTCCC-3' (Mezey *et al.*, 2000), ASIC3 forward 5'-GCTATGCTGCGAAAG-GACAC-3' and reverse 5'-TACGGTGGGAGGCAGAGAGT-3' (Liu & Simon, 2001). A commercially available primer (Clontech, Palo Alto, CA, USA) was used for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which served as an internal reference to determine the relative quantity of RNA. PCR amplification was performed in 2.5 mM MgCl_2 , 10 pmol primers, 0.25 U Taq DNA polymerase (Promega) and 0.2 mM deoxynucleotide mix (Sigma) on a RoboCycler (Stratagene, La Jolla, CA, USA) as follows: for TRPV1, samples were denatured for 2 min at 95°C followed by 1 min annealing at 55°C and 35 extension cycles at 72°C (42 cycles for LMMP preparations). An annealing temperature of 57°C and 34 cycles was used for ASIC3. Amplification was tested for each primer to verify a linear range and not to be at the maximum. PCR products were separated by electrophoresis on a 1.5% agarose gel containing 1 mg/mL ethidium bromide and visualized in a UV transilluminator (Bio-Rad). Band density was analysed with Quantity One Software (Gel Doc 2000 System; Bio-Rad).

Statistical analysis and cell counts

Six animals were used for quantification of receptor- and True Blue-labelled neuronal profiles in nodose ganglia (both sides) and DRGs (T8–12 bilaterally). Every sixth from a consecutive series of sections of 14- μm thickness (three sections from each ganglion) were counted. For each animal, a mean was calculated for thoracic DRGs and for nodose ganglia. True Blue treatment revealed a high enough

background to perform a total count of neuronal profiles in DRGs and nodose ganglia without the need for specific staining with a pan-neuronal marker. Data are expressed as means \pm SEM and evaluated using one-way ANOVA and Tukey's multiple comparison test run on SigmaStat software. Probability values of $P < 0.05$ were regarded as significant.

Results

Retrograde tracing with True Blue and immunohistochemistry for TRPV1 and ASIC3

True Blue was observed in $33.2 \pm 4.1\%$ (left) and in $31.6 \pm 1.6\%$ (right) of neuronal profiles in the nodose ganglion. In left thoracic DRGs (T8–12) $12.7 \pm 1.3\%$ of neuronal profiles were positive for True Blue. The same number was also counted for right thoracic DRGs ($12.3 \pm 0.5\%$). TRPV1 was present in $82.1 \pm 3.5\%$ (left) and $85.6 \pm 2.4\%$ (right) of True Blue-labelled neuronal profiles in the nodose ganglion (Fig. 1A and B) whereas ASIC3 was slightly less present ($75.4 \pm 6.6\%$ in left and $73.2 \pm 3.6\%$ in right nodose ganglion; Fig. 1A and C). In thoracic DRGs (T8–12; bilaterally), TRPV1 was observed in 71.2 ± 5.5 and ASIC3 in $82.2 \pm 3.4\%$ of True Blue-labelled neuronal profiles (Fig. 2). In LMMP preparations of the stomach, TRPV1 was present in nerve fibres of the myenteric plexus while cell bodies of myenteric ganglia were not labelled (Fig. 1D). The faint immunoreactivity sometimes seen in cell bodies was not abolished by the control peptide. Immunoreactivity in fibres, however, was readily blocked in preabsorption experiments and was absent in ganglionectomized + vagotomized animals ($n = 5$). ASIC3 immunoreactivity was absent from intrinsic neuronal cell bodies (data not shown).

RT-PCR in nodose ganglia, thoracic DRGs and LMMP preparations of the stomach

Expression of TRPV1 and ASIC3 mRNAs were not altered in nodose ganglia and thoracic DRGs 2 h (data not shown) and 5 h after IG acid administration as compared to saline treatment (Fig. 3A and B). Transcripts for TRPV1 were also found in LMMP preparations of the stomach and their amount was not significantly altered 5 h after application of the acid stimulus (Fig. 3A). No TRPV1 mRNA was detected in LMMP samples from animals that had undergone vagotomy and ganglionectomy (Fig. 3C), even after the number of cycles was raised to 60 (data not shown). ASIC3 transcripts were absent from LMMP samples of the stomach, confirming the findings by Chen *et al.* (1998).

TRPV1 Western blots in nodose ganglia and thoracic DRGs

TRPV1 protein was significantly elevated (60.6%) in thoracic DRGs 6 h after IG acid administration as compared to saline treatment (Fig. 3D and E) whereas no increase was seen in the nodose ganglia (Fig. 3D).

Discussion

The aim of this study was to find out whether TRPV1 and/or ASIC3, two nociception-related and acid-sensing receptors of vagal and spinal sensory neurons, are up-regulated by a noxious acid challenge of the stomach, a treatment that leads to mucosal injury, local tissue acidosis and pain behaviour (Schuligoi *et al.*, 1998). These two receptors have been shown to react to peripheral inflammation with an increased expression (Carlton & Coggeshall, 2001; Voilley *et al.*, 2001; Amaya *et al.*, 2003). We also wanted to examine TRPV1 in the ENS because of

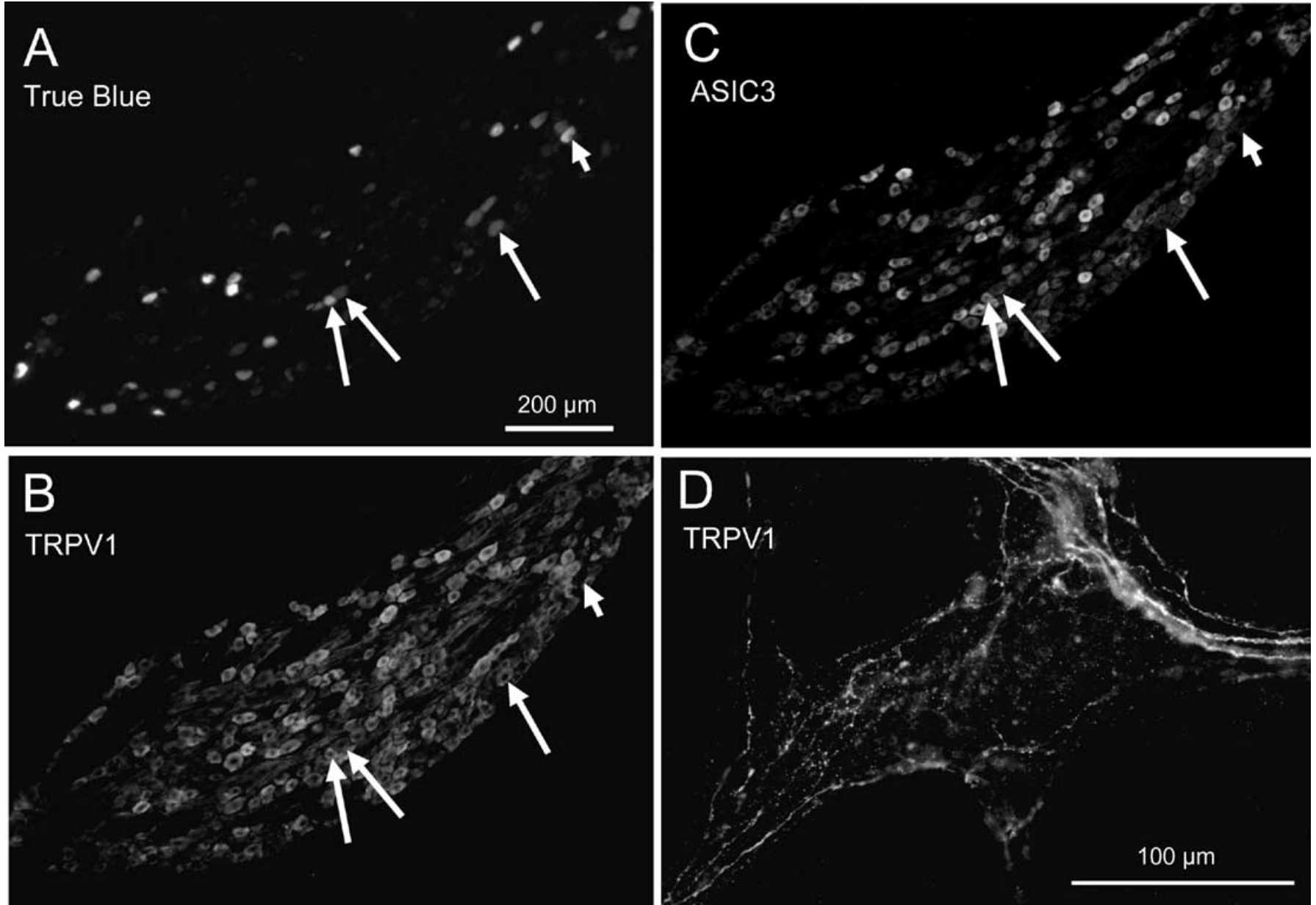


FIG. 1. (A–C) Triple labelling of True Blue with TRPV1 and ASIC3 in the left nodose ganglion of the rat. Long arrows denote True Blue-labelled cells that colocalize with the respective receptor; short arrows denote True Blue-labelled cells without receptor colocalization except for C where faint ASIC3 immunoreactivity can be detected. (D) TRPV1 immunoreactivity in the myenteric plexus of the stomach.

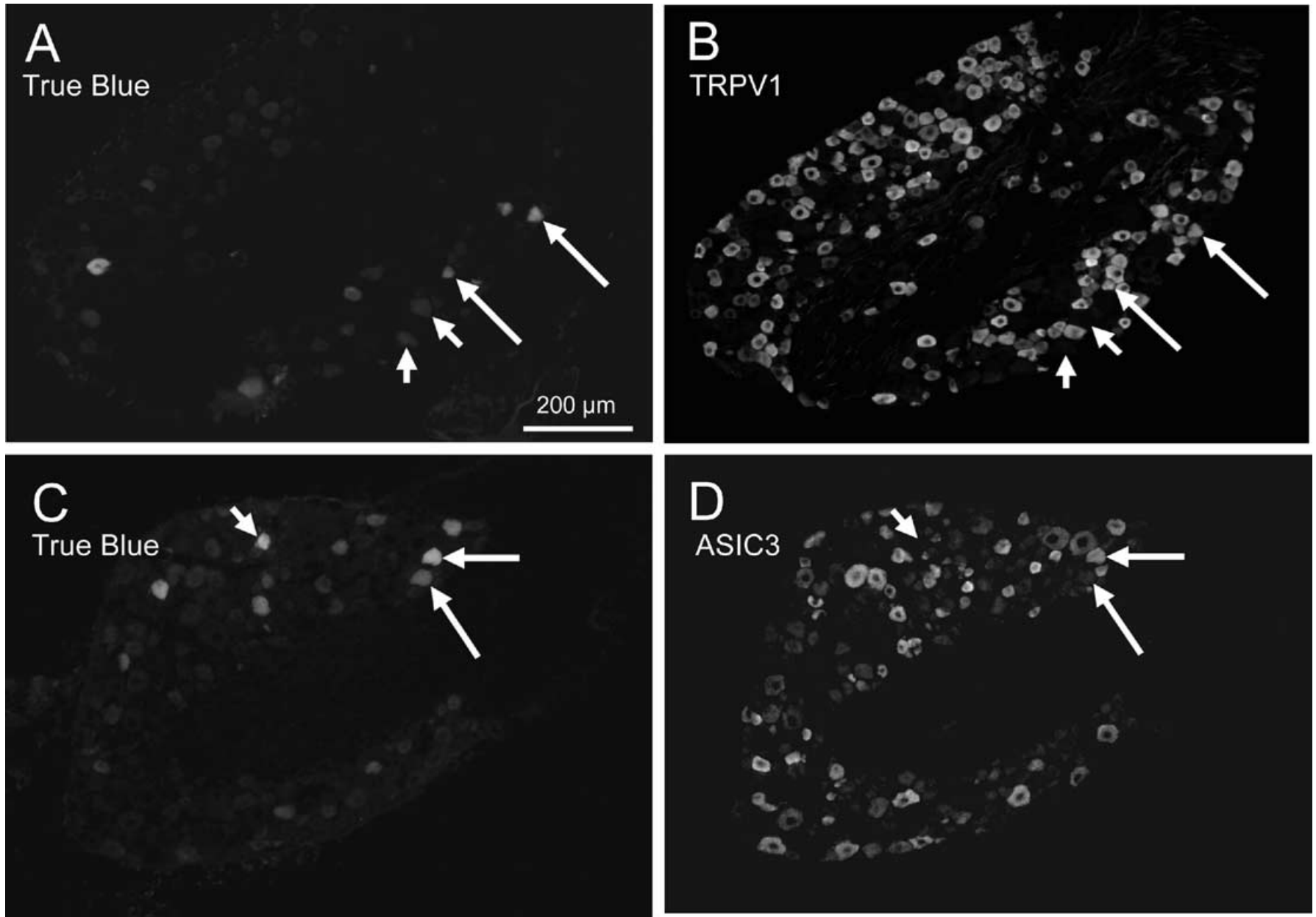


FIG. 2. (A–D) Double labelling of True Blue with (A and B) TRPV1 and (C and D) ASIC3 in sections of a rat thoracic DRG (T10 from right side). Long arrows denote True Blue-labelled cells that colocalize with the respective receptor; short arrows denote True Blue-labelled cells without receptor colocalization.

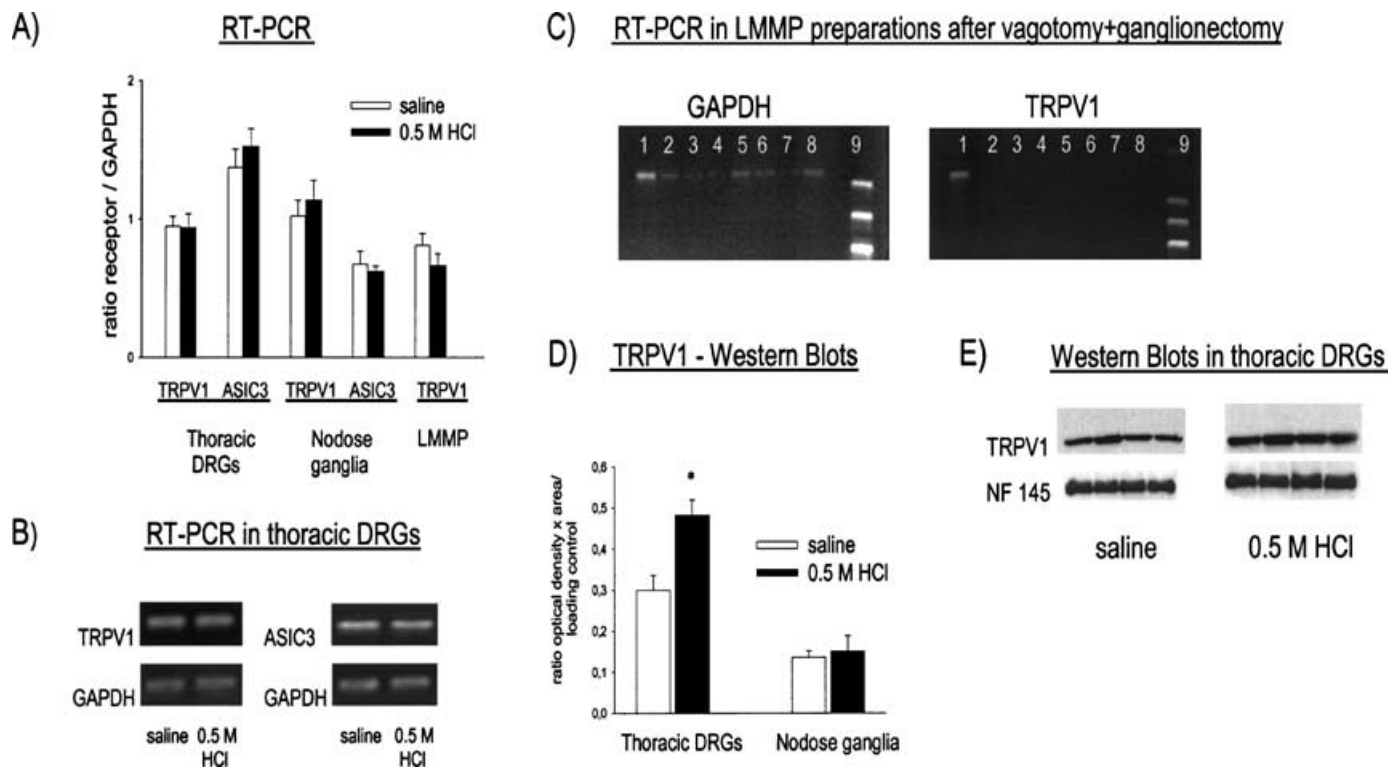


FIG. 3. (A and B) RT-PCR for TRPV1 and ASIC3 mRNAs in thoracic DRGs and nodose ganglia and for TRPV1 mRNA in LMMP preparations of the stomach did not reveal any significant change in expression 5 h after challenge with 0.5 M HCl as compared to saline. Data are expressed as means \pm SEM (ratios of signal intensities of investigated receptor relative to GAPDH); $n = 4-6$. (C) Pictures show gels of RT-PCR for GAPDH and TRPV1 from animals that had undergone vagotomy + ganglionectomy ($n = 7$). Thoracic DRG samples served as a positive control (lane 1). Lanes 2-8 were loaded with LMMP preparations from vagotomized + ganglionectomized animals. TRPV1 transcripts are absent in lanes 2-8 while transcripts for GAPDH are clearly present. Standards were run on lane 9. (D) Western Blots for TRPV1 in thoracic DRGs and nodose ganglia were measured 6 h after IG administration of 0.5 M HCl (or saline). The TRPV1 antibody produced a clear single band at ≈ 90 kDa. In nodose ganglia, no significant difference in TRPV1 expression was seen between acid- and saline-treated animals whereas in thoracic DRGs protein levels rose by $\approx 60\%$ after treatment with 0.5 M HCl; means \pm SEM ($P < 0.05$), $n = 12$. (E) Western blots of thoracic DRGs are shown from four samples of saline-treated animals and from four samples obtained from 0.5 M HCl-treated animals. Protein levels were measured as band density \times area and normalized with neurofilament 145 which served as a loading control.

its possible expression by intrinsic sensory neurons of the gastrointestinal tract (Anavi-Goffer *et al.*, 2002).

By counting neuronal profiles, around 12% of thoracic DRG cells were found to project to the stomach, a finding that fits with a study by Neuhuber *et al.* (1986) who investigated DRG cells of splanchnic afferents with the horseradish peroxidase technique, while in the nodose ganglion >30% of neurons have peripheral territories in the stomach.

The majority of vagal and spinal gastric afferents express TRPV1 and ASIC3; therefore, both sensory systems seem to be equally equipped to respond to changes in the pH of the gastric mucosa. Data on the distribution of TRPV1 mRNA and protein in cells of vagal ganglia, however, vary considerably ranging from failure to detect any signal (Caterina *et al.*, 1997) to their presence in a large majority of neurons (Helliwell *et al.*, 1998; Michael & Priestley, 1999). Our counts of TRPV1-labelled neuronal profiles in the nodose ganglion also differ from other studies (Patterson *et al.*, 2003) most probably because we have used a lower background setting for our counting procedures. ASIC3 immunoreactivity in vagal ganglia has not been investigated before and is reported here for the first time. Considering the large presence of TRPV1 and ASIC3 in stomach-targeting neurons of the afferent vagus and the thoracic DRGs, these receptors are likely to play an important role in monitoring the acidic environment of the stomach.

In accordance with other studies (Patterson *et al.*, 2003; Ward *et al.*, 2003), TRPV1 was only observed in nerve fibres of the myenteric plexus but not in cell bodies of myenteric ganglia. Surgical deaf-

ferentiation abolished TRPV1 transcription signals and immunoreactivity in the rat stomach, suggesting that TRPV1 mRNA may be contained in axons of extrinsic neurons. In keeping with this finding, some studies argue that proteins can be synthesized in nerve fibres (reviewed by Alvarez *et al.*, 2000), and Koenig *et al.* (2000) recently reported that clusters of ribosomes that may represent potential translation sites are distributed along myelinated axons of the rat. It is thus possible that TRPV1 mRNA, which like its protein probably undergoes axonal transport (Szallasi *et al.*, 1995; Tohda *et al.*, 2001), is translated on-site in axons of extrinsic gastric sensory afferents, provided that these axons are equipped with a functional translation machinery. Interestingly, cannabinoid receptors were also shown to be transported in axons and cannabinoid receptor 2 mRNA was detected in a peripheral organ such as the rat spleen (Hohmann & Herkenham, 1999).

We were able to detect an increase in TRPV1 in thoracic DRGs by Western blotting but not RT-PCR. Transcripts for ASIC3 also remained unaltered after IG administration of acid. In line with these findings, quite a few studies have demonstrated an increase of the TRPV1 protein after peripheral inflammation (Carlton & Coggeshall, 2001; Yiangou *et al.*, 2001; Ji *et al.*, 2002; Amaya *et al.*, 2003), but not of its mRNA (Sanchez *et al.*, 2001; Tohda *et al.*, 2001; Voilley *et al.*, 2001; Ji *et al.*, 2002). The relatively early time point for measuring receptor expression was chosen because TRPV1 synthesis seems to increase already within a few hours after induction of peripheral inflammation (Tohda *et al.*, 2001). It is believed that up-regulation of excitatory

receptors leads to enhanced excitability and responsiveness of the nerve cell, thereby representing a possible mechanism for sensitisation (Kirkup *et al.*, 2001). Evidently, TRPV1 is a key molecule in producing hypersensitivity because one of the main characteristics of TRPV1 knockout mice is a deficiency in thermal hyperalgesia (Caterina *et al.*, 2000; Davis *et al.*, 2000). The increased expression of TRPV1 as we see it in our study could therefore represent a novel mechanism of gastric hyperalgesia (Di Marzo *et al.*, 2002).

Another question relates to the neuronal population of thoracic DRGs which up-regulate TRPV1 in response to gastric acid challenge (which will be investigated in more detail in a future study). An increase in TRPV1 after peripheral inflammation occurs primarily in C-fibre nociceptors (Carlton & Coggeshall, 2001; Ji *et al.*, 2002). However, TRPV1 is also coexpressed with neurofilament 200 in DRG cells (Ma, 2002), which is a marker for myelinated A-fibre mechanoreceptors. Likewise, medium-sized A-fibres were shown to respond to peripheral inflammation with an increase in TRPV1 (Amaya *et al.*, 2003). In our experiments, about half of TRPV1–True Blue-labelled neuronal profiles in thoracic DRGs stained for neurofilament 200 (R. Schicho, unpublished observations) indicating that some of the TRPV1-positive fibres that supply the stomach are A fibres in which an up-regulation could have occurred. It is a characteristic of visceral mechanoreceptors that they are polymodal and therefore also sensitive to chemical stimulation (Su & Gebhart, 1998).

Acid is an adequate stimulus to sensitize gastric mechanoreceptors; this has been indirectly demonstrated in a clinical study where symptoms induced by gastric isobaric distensions were modified by HCl infusion (Coffin *et al.*, 2001). With our method of detection (Western blot), an increase in TRPV1 expression was only noticed in thoracic DRGs but not in nodose ganglia. It would thus seem that TRPV1 does not contribute to gastric acid-induced plasticity in vagal afferents. However, there is increasing evidence that both mechano- and chemosensitive vagal afferents can be sensitised. Kang *et al.* (2003) have reported that distension-sensitive afferents in the vagus nerve are sensitised by heat and acid. Furthermore, a study by Lamb *et al.* (2003) has recently shown that hypersensitivity of the gastric mucosa to acid develops in the vagal but not spinal afferent system.

We have interpreted our findings by concluding that up-regulation of TRPV1 could represent a biochemical basis for peripheral hypersensitivity. However, what we cannot deduce from our results is whether TRPV1 and ASIC3 are directly involved in the acute monitoring of acid. Recent data from TRPV1-knockout mice argue against a direct role of TRPV1 in acid sensing in the intestine (Hillsley *et al.*, 2003), but the possible involvement of TRPV1 in acid-induced nociception *in vivo* (Ikeda *et al.*, 2001) needs to be explored in more detail. TRPV1 contributes to the hyperaemic and mucosecretory response to luminal acid in the duodenum (Akiba *et al.*, 2002), while the hyperaemic response to acid back-diffusion in the stomach does not seem to depend on it (Tashima *et al.*, 2002) although it is mediated by capsaicin-sensitive spinal, not vagal, sensory neurons (Raybould *et al.*, 1992). It is therefore possible that acute acid-sensing and acid-induced hypersensitivity are not accomplished by the same receptor or sensory system.

An interesting mechanism of TRPV1 up-regulation has been proposed by Ji *et al.* (2002), who demonstrated that TRPV1 protein, but not transcripts, can be increased by nerve growth factor (NGF)-induced activation of the mitogen-activated protein (MAP) kinase p38. The neurotrophic factor NGF has also been investigated in gastric ulcers and found to contribute to sensitisation of gastric primary afferent neurons (Bielefeldt *et al.*, 2003). The relatively rapid onset of TRPV1 protein production that we see in our experiments (after 6 h) might speak against an involvement of NGF in TRPV1 up-regulation

due to acid challenge. Normally, NGF needs to be retrogradely transported to the cell soma to become involved in a regulatory transduction cascade (Reynolds *et al.*, 2000). New data, however, indicate that NGF internalization and retrograde transport might not be necessary for transducing a long-distance signal (MacInnis & Campenot, 2002).

In conclusion, noxious acid challenge of the stomach causes up-regulation of TRPV1 protein in thoracic DRG neurons. This alteration could lead to an increased responsivity of the nerve cells towards nociceptive and innocuous inputs. TRPV1 and mediators regulating its expression could be primary pharmacological targets for the treatment of bowel diseases that are associated with peripheral hypersensitivity.

Acknowledgements

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Abbreviations

ASIC3, acid-sensing ion channel 3; ENS, enteric nervous system; DRG, dorsal root ganglion; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IG, intra-gastric/intragastrically; IP, intraperitoneal; LMMP, longitudinal muscle myenteric plexus; NGF, nerve growth factor; PBS, phosphate-buffered saline; RT-PCR, reverse transcription–polymerase chain reaction; TRPV1, transient receptor potential vanilloid receptor 1.

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