Nociceptors of dorsal root ganglion express proton-sensing G-protein-coupled receptors

Chia-Wei Huang, a,1 Jian-Ning Tzeng, a,1 Ying-Ju Chen, a Wei-Fen Tsai, a Chih-Cheng Chen, b and Wei-Hsin Sun a,∗

a Department of Life Sciences, National Central University, JungLi, Taiwan
b Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan

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One major goal in pain research is to identify novel pain targets. Tissue injury, inflammation, and ischemia are usually accompanied by local tissue acidosis, the degree of associated pain or discomfort well correlated with the magnitude of acidification. Proton-sensing ion channels, transient receptor potential/vanilloid receptor subtype 1, and acid-sensing ion channel 3 are involved in acidosis-linked pain. However, whether recently identified proton-sensing G-protein-coupled receptors (GPCRs) also have some contributions is unclear. Proton-sensing GPCRs, including OGR1, GPR4, G2A, and TDAG8, are fully activated at pH 6.4–6.8 in vitro. To understand whether the proton-sensing GPCRs are expressed in nociceptors, we cloned the four mouse genes and examined their tissue distribution and localization in pain-relevant loci, the dorsal root ganglion (DRG). The OGR1 family members were widely expressed in neuronal and non-neuronal tissues. Their transcripts were expressed in the DRG, and most (75–82%) were present in small-diameter neurons responsible for nociceception. Approximately 31–40% of total DRG neurons expressed at least two proton-sensing GPCRs. We have also demonstrated that gene expression of proton-sensing GPCRs is changed in ASIC3 knockout mice. Our finding suggests that proton-sensing GPCRs could have some roles in nociception or in compensation of loss of ASIC3 gene.

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Introduction

Tissue injury, inflammation, ischemia, and cancer often heighten the pain experience by increasing the sensitivity of nociceptors to both thermal and mechanical stimuli. This phenomenon results, in part, from the production and release of chemical mediators (protons, adenosine triphosphate, prostaglandins, bradykinin, serotonin, etc.) from the primary sensory terminal and from non-neural cells in the environment. Protons are one of important effectors for nociceptors. A relatively high proton concentration, called acidosis, has been suggested to be a direct link between disease and pain (Steen et al., 1992; reviewed in Reeh and Steen, 1996).

Transient receptor potential/vanilloid receptor subtype 1 (TRPV1) and the acid-sensing ion channel (ASIC) family (containing at least seven ASIC subtypes—1a, 1b, 2a, 2b, 3, 4, and 5) are proton-sensing ion channels (Caterina et al., 1997; Waldmann, 1997; Chen et al., 1998; reviewed in Caterina and Julius, 2001; Krishtal, 2003). Disruption of the TRPV1 gene in mice reduced responses of dorsal root ganglion (DRG) neurons to acid (Caterina et al., 2000; Davis et al., 2000), which suggests that TRPV1 is involved in acid-induced pain. Surprisingly, mice lacking the ASIC3 gene still respond to acid stimuli (Price et al., 2001; Chen et al., 2002). However, ASIC3 was found to be essential for mechanical hyperalgesia induced by acid injection in skeletal muscle or by muscle inflammation (Price et al., 2001; Sluka et al., 2003, 2007; Mogil et al., 2005). Accordingly, even though proton-sensing ion channels could be critical to acidosis-linked pain, some other pH-sensing receptors could be involved in pain modulation.

In 2003, Ludwig et al. found two G-protein-coupled receptors (GPCRs), ovarian cancer GPR 1 (OGR1) and GPR4, fully responding to protons at pH 6.8 and stimulating inositol triphosphate and cAMP formation, respectively. Later, the other two family members, G2 accumulation (G2A) and T-cell death-associated gene 8 (TDAG8), were identified as proton receptors, with full activation at pH 6.4–6.8 (Murakami et al., 2004; Wang et al., 2004; Ishii et al., 2005). OGR1, GPR4, and G2A were previously identified as receptors for sphingosylphosphorylcholine (SPC) or lysophosphatidylcholine (LPC) (Xu et al., 2000; Zhu et al., 2001; Kabarowski et al., 2001), but the original publications have now been retracted. Whether OGR1, GPR4, and G2A are SPC or LPC receptors remains unclear. In addition to responding to protons, TDAG8 also responds to psychosine (Im et al., 2001; Wang et al., 2004). The primary sequence of G2A is less similar to that of the other three family genes, and four of five critical...
histidine residues that are involved in pH-sensing of OGR1 (Ludwig et al., 2003) are replaced by other amino acids in G2A. Data from Radu et al. (2005) suggested that G2A is less likely to be a pH sensor because it did not generate a significant response after acid stimulation. Recently, Obinata et al. (2005) found that G2A responds to oxidized free fatty acid (9-hydroxyoctadecadienoic acid).

The proton-sensing GPCRs have been found in many tissues. Human OGR1 mRNA is strongly expressed in the brain, lung, and placenta but weakly in the spleen, testes, peripheral blood leukocytes, and heart (Xu and Casey, 1996). Human GPR4 transcripts have high expression in the lung and low expression in the heart, liver, skeletal muscle, kidney, and pancreas (Mahadevan et al., 1995). Murine or human G2A mRNA is abundant in the spleen and thymus and less so in the heart and lung (Weng et al., 1998). The human TDAG8 gene is specifically expressed in the thymus, with some expression in the spleen (Choi et al., 1996).

OGR1 family members have half-maximum activation at pH 7.2–7.5 and full activation at pH 6.4–6.8 (Ludwig et al., 2003; Murakami et al., 2004; Wang et al., 2004). At this range, only ASIC3 can generate a sustained current related to persisting sensation (pH 7.3–6.7) (Yagi et al., 2006). Disruption of the ASIC3 gene increases mouse response to acid stimuli, but no gene expression change is found with other ASIC family members or TRPV1 (Price et al., 2001; Chen et al., 2002). Whether the OGR1 family genes are expressed in pain-relevant anatomic loci, DRG, and whether these genes are expressed differentially in ASIC3−/− mice, is unclear. To address these questions, we cloned four mouse genes and then examined their expression in mouse DRG. All transcripts of OGR1 family members were expressed in DRG, and most were present in small-diameter DRG neurons (75–82%) responsible for nociception. In small-diameter nociceptors, these four genes were mainly present in isoleucin B(4) (IB4)-positive neurons (61–74%). Of these DRG neurons, approximately 31–40% expressed at least two proton-sensing GPCRs. We have also found that gene expression of proton-sensing GPCRs was changed in ASIC3−/− mice. Our results suggest an involvement of OGR1 family members in nociception.

Results

Expression of proton-sensing GPCRs in the peripheral and central nervous system

To understand the physiological function of proton-sensing GPCRs, mouse proton-sensing GPCR genes were cloned and their expression patterns were examined in different tissues. OGR1, GPR4, G2A and TDAG8 were widely and variably expressed in different tissues (Fig. 1). All four genes were expressed in non-neural tissues. In neural tissues, OGR1, GPR4, G2A and TDAG8 transcripts were present in both the peripheral and central nervous systems (brain, spinal cord, DRG, and trigeminal ganglion [TRG]), although G2A expression was lower than the other three genes.

Expression change of proton-sensing GPCR genes in ASIC3 knockout mice

OGR1 family members are activated at the pH range where only ASIC3 can generate a sustained current related to persisting sensation.
sensation (pH 7.3–6.7) (Ludwig et al., 2003; Murakami et al., 2004; Wang et al., 2004; Yagi et al., 2006). Disruption of the ASIC3 gene increases mouse response to acid stimuli, but no gene expression change was found with other ASIC family members or TRPV1 (Price et al., 2001; Chen et al., 2002). To investigate the effect of loss of ASIC3 on expression of OGR1 family members, we examined the expression of OGR1, GPR4, G2A, and TDAG8 in tissues from ASIC3 knockout mice. Compared with wild-type tissues, most of the knockout tissues showed similar expression levels for the four genes (Fig. 1).

Despite the presence of these proton-sensing GPCR genes in neural tissues, RT-PCR revealed no significant change in gene expression in ASIC3-deficient mice. Thus, we performed quantitative PCR in neuronal tissues involved in sensory processes and found OGR1 family members with expression changes in different tissues in ASIC3 knockout mice (Fig. 2). OGR1 transcripts showed a 3-fold increase in expression in brain, GPR4 a 2-fold decrease in TRG, G2A a 3-fold increase in DRG, and TDAG8 more than 7-fold increase in spinal cord.

Proton-sensing GPCRs expressed in DRG of thoracic and lumbar regions

To understand whether OGR1 family members are differentially expressed in DRG of thoracic and lumbar regions, single DRG isolated from different thoracic or lumbar regions were used to examine expression. Gene expression levels were represented as relative cycle numbers (Ct) to GAPDH expression levels. Low cycle numbers indicate high expression levels. OGR1 family members were present in the pool of all DRG (including cervical, thoracic, lumbar, and sacral regions) (Fig. 3). OGR1 had the lowest ΔCt after normalization by GAPDH (ΔCt = 10.65 ± 0.11), GRP4 the highest number of cycles (ΔCt = 15.21 ± 0.42) and the number of cycles for G2A and TDAG8 were between those of OGR1 and GPR4 (ΔCt = 13.61 ± 0.05 and 13.53 ± 0.43, respectively). OGR1 seems to be the most abundant gene in DRG. In single DRG analysis, despite some variation in expression of gene transcripts among DRGs, all four genes were expressed in thoracic and lumbar DRG (Figs. 3B, C). The mean cycle numbers (ΔCtavg) in the thoracic region for OGR1, GPR4, G2A, and TDAG8 were 10.65 ± 0.17, 13.13 ± 0.34, 13.36 ± 0.49, and 12.55 ± 0.43, respectively. In the lumbar region, the mean cycle numbers were 9.62 ± 0.28, 11.8 ± 0.65, 12.69 ± 0.34, and 10.66 ± 0.49, respectively. Accordingly, OGR1 was expressed at the highest levels in DRG from both the thoracic and lumbar regions, but the expression levels of the other three genes seem similar in both regions. DRG from lumbar 4 expressed the four genes at more equivalent levels (ΔCt for OGR1, GPR4, G2A, and TDAG8 of 9.58 ± 0.13, 10.8 ± 0.42, 11.9 ± 0.42, and 10.6 ± 0.37, respectively).

Proton-sensing GPCRs are present in nociceptors

Given that all OGR1 family members are present in pain-relevant anatomic loci, DRG, we wondered whether these receptors were located in nociceptors. Thus, we examined the localization of OGR1 family members in lumbar DRG neurons using in situ

Fig. 4. Localization of proton-sensing GPCRs in DRG neurons. DRG tissues were sectioned and hybridized with dig-labeled antisense cRNA probes, followed by co-staining with antibodies against peripherin (PERI, green fluorescence) and N52 (red fluorescence). (A) Phase-contrast fields (a, d, g, j) are neurons labeled with cRNA probes. Fluorescence images (b, e, h, k) show neurons labeled with green (PERI only), red (N52 only), and yellow colors (PERI and N52). Phase-contrast images and fluorescence images were combined to obtain merged images (c, f, i, l). Arrows indicate the peripherin-positive neurons labeled with antisense probes. Arrowheads are the N52-positive neurons labeled with antisense probes. The scale bar is 40 μm. (B) The histogram shows the percentage of total neurons that expressed genes in PERI, N52, or overlap subpopulations.

Most proton-sensing GPCRs are present in small and medium DRG neurons

Among total ganglion cells, approximately 35–37% were labeled with N52, 71–73% with peripherin, and ~8% with both N52 and peripherin (Fig. 4A). The proportions of mouse lumbar DRG neurons labeled with N52 and peripherin are similar to those for adult rats: 74% for peripherin, 34% for N52, and 8% an overlap population (Ferri et al., 1990).

OGR1, GPR4, G2A, and TDAG8 were expressed in the lumbar DRG, comprising 26±2%, 29±3%, 32±3%, and 28±3%, respectively, of the total ganglion cell population (Figs. 4A, B, Table 1). Both large- and small-diameter neurons expressed OGR1 family members, but the genes were mainly expressed in small-diameter neurons. Of the ganglion cells that were labeled with OGR1 probes, 82±2% were co-stained with peripherin, 31±3% with N52 and 13±2% with both peripherin and N52 (Fig. 4B). Approximately 81±2% of GPR4, 75±2% of G2A, and 77±4% of TDAG8 co-expressed with peripherin, whereas 26±3% of GPR4, 35±3% of G2A, and 29±5% of TDAG8 co-labeled with N52.

Among peripherin-immunoreactive (IR) afferents, 29±5%, 31±3%, 35±3%, and 31±3% were labeled with OGR1, GPR4, G2A, and TDAG8, respectively. Of the neurons stained with N52, 22±3% were OGR1, 24±3% GPR4, 30±3% G2A, and 22±3% TDAG8. Thus, most of the proton-sensing GPCRs were expressed in small- to medium-diameter neurons.

Localization of proton-sensing GPCRs in both IB4-positive and IB4-negative neurons

To further characterize the distribution of proton-sensing GPCRs in peptidergic or non-peptidergic small-diameter neurons, the four genes were first labeled with antisense probes and then co-stained with anti-peripherin antibodies and IB4–FITC conjugates. Of those neurons labeled with peripherin, 54–56% were stained with IB4 and 44–46% were IB4-negative (Fig. 5A). This finding is consistent with results found in adult mice (54% IB4-positive and 46% IB4-negative) (Dirajalal et al., 2003).

All proton-sensing GPCRs were expressed in both IB4-positive and IB4-negative neurons (Figs. 5A, B, Table 2). Most of the OGR1 and GPR4-expressing neurons were co-stained with IB4 (65±4% of OGR1 and 63±4% of GPR4). However, of neurons labeled with G2A, 48±4% were stained with IB4 and of TDAG8-expressed neurons, 47±4% were IB4-positive. Of the peripherin-IR neuron populations, 74±4% of OGR1-positive neurons were co-stained with IB4 and 61±5% of GPR4-expressing neurons, 64±4% of G2A-expressing neurons, and 64±4% of TDAG8-positive neurons were IB4-positive (Fig. 5B). Accordingly, in peripherin-IR neurons, proton-sensing GPCR genes were primarily expressed in the IB4-positive population.

Co-expression of proton-sensing GPCRs with TRPV1

Co-localization of proton-sensing GPCRs with TRPV1 is shown in Fig. 6. TRPV1 is predominantly expressed in small-diameter DRG neurons in rats and mice (Caterina et al., 1997; Tomimaga et al., 1998; Guo et al., 1999; Zwick et al., 2002; Ugawa et al., 2005). In rats, approximately 35–40% of total DRG neurons expressed TRPV1 on in situ hybridization (Ugawa et al., 2005), but more than 50% of total DRG neurons have TRPV1-IR (Guo et al., 1999). In mouse, only 22% of total neurons have TRPV1-IR (Zwick et al., 2002). We found 40–42% of peripherin-IR neurons with TRPV1-IR (Fig. 6A). Of the TRPV1-positive neurons, 55–59% co-localized with peripherin, and 40–44% were peripherin-negative (Fig. 6A).

In the population of TRPV1-positive neurons, 26±4%, 22±3%, 41±5%, and 40±5% were labeled with OGR1, GPR4, G2A, and TDAG8, respectively. A large population of OGR1-expressing neurons (67±3%) was co-expressed with TRPV1 (Fig. 6A, B, Table 3). Of neurons labeled with GPR4, 46±3% co-localized with TRPV1, and 46±5% of G2A-positive neurons and 38±5% of TDAG8-positive neurons were labeled with TRPV1 (Figs. 6A, B).

Co-expression of proton-sensing GPCRs with ASIC3

To understand whether the OGR1 family genes are co-localized with ASIC3 in DRG neurons, we paired continuous 6-μm semi-thick sections of DRG. Each pair was hybridized with antisense cRNA of two different genes (one was proton-sensing GPCR gene and the other was ASIC3 gene), then co-stained with antibodies against peripherin. In the population of the peripherin-positive neurons, 47±3% were expressed with ASIC3. Of the ASIC3-positive neurons, 70% co-localized with peripherin and 30% were peripherin-negative (Fig. 7A). These results are consistent with previous studies that ASIC3 gene was expressed in small- and large-diameter neurons (Alvarez de la Rosa et al., 2002; Molliver et al., 2005; Ugawa et al., 2005). Among ASIC3-positive neurons,
Fig. 5. Expression of proton-sensing GPCR genes in IB4-positive and -negative neurons. DRG tissues were sectioned and hybridized with dig-labeled antisense cRNA probes, followed by co-staining with anti-peripherin antibodies (PERI, red fluorescence) and IB4–FITC conjugates (IB4, green fluorescence). (A) Phase-contrast fields (a, d, g, j) are neurons labeled with RNA probes. Fluorescence images (b, e, h, k) show neurons labeled with red (PERI only), green (IB4 only), and yellow colors (PERI and IB4). Phase-contrast images and fluorescence images were combined to obtain merged images (c, f, i, l). Arrowheads indicate the peripherin-positive neurons labeled with antisense probes. Arrows are the peripherin and IB4 co-expressed neurons labeled with antisense probes. The scale bar is 50 μm. (B) The histogram shows the percentage of total PERI-positive neurons that expressed genes in IB4-positive or -negative subpopulations.

neurons, 55±2%, 54 ±2%, 53±2%, and 48 ±2% expressed OGR1, stained with antibodies against N52 and peripherin. Of the total DRG antisense cRNA of two different proton-sensing GPCR genes, then co-pairs of continuous 6-

PERI (+) and IB4 (+) 55 (52–58)
IB4 (+) 35 (33–40)
PERI (+) and IB4 (+) 57 (54–60)
PERI (+) and IB4 (+) 43 (40–46)

GPR4

PERI (+) 26 (24–28)
IB4 (+) 31 (28–34)
PERI (+) and IB4 (+) 55 (52–58)
PERI (+) and IB4 (+) 45 (42–48)

G2A

PERI (+) 26 (24–28)
IB4 (+) 31 (28–34)
PERI (+) and IB4 (+) 55 (52–58)
PERI (+) and IB4 (+) 45 (42–48)

TDAG8

PERI (+) 26 (24–28)
IB4 (+) 28 (25–31)
PERI (+) and IB4 (+) 55 (52–58)
PERI (+) and IB4 (+) 45 (42–48)

Table 2
Localization of proton-sensing GPCRs in both IB4-positive and IB4-negative neurons

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Probe-labeled neurons/total subgroup neurons</th>
<th>Subgroup probe-labeled neurons/total probe-labeled neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>OGR1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PERI (+)</td>
<td>27 (25–29)</td>
<td>81 (77–84)</td>
</tr>
<tr>
<td>IB4 (+)</td>
<td>35 (33–40)</td>
<td>65 (61–69)</td>
</tr>
<tr>
<td>PERI (+) and IB4 (+)</td>
<td>55 (52–58)</td>
<td>74 (70–78)</td>
</tr>
<tr>
<td>PERI (+) and IB4 (+)</td>
<td>45 (42–48)</td>
<td>26 (22–30)</td>
</tr>
<tr>
<td>GPR4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PERI (+)</td>
<td>26 (24–28)</td>
<td>78 (75–81)</td>
</tr>
<tr>
<td>IB4 (+)</td>
<td>30 (27–33)</td>
<td>63 (60–67)</td>
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<td>61 (58–64)</td>
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<td>43 (40–46)</td>
<td>39 (36–42)</td>
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<tr>
<td>G2A</td>
<td></td>
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<tr>
<td>PERI (+)</td>
<td>26 (24–28)</td>
<td>62 (58–66)</td>
</tr>
<tr>
<td>IB4 (+)</td>
<td>31 (28–34)</td>
<td>48 (44–52)</td>
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<td>36 (32–40)</td>
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<tr>
<td>TDAG8</td>
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<tr>
<td>PERI (+)</td>
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<td>66 (64–69)</td>
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<tr>
<td>IB4 (+)</td>
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<tr>
<td>PERI (+) and IB4 (+)</td>
<td>45 (42–48)</td>
<td>36 (32–40)</td>
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</table>

The number represents the percentage of neurons that expressed single gene or two genes in total DRG neuron populations. Confidence intervals (95%) for proportions are shown in parentheses. The number of total cells counted was of the order of 1500–2500.

47±5%, 59±5%, 67±8%, 59±4% were co-labeled with OGR1, GPR4, G2A, and TDAG8, respectively (Fig. 7B).

Co-localization of proton-sensing GPCRs in DRG neurons

Co-localization of proton-sensing GPCRs was examined using pairs of continuous 6-μm DRG sections. Each pair was hybridized with antisense cRNA of two different proton-sensing GPCR genes, then co-stained with antibodies against N52 and peripherin. Of the total DRG neurons, 55±2%, 54±2%, 53±2%, and 48±2% expressed OGR1, GPR4, G2A, and TDAG8, respectively (Figs. 8A, B, Table 4). Most of these genes were localized in the small-diameter neurons (66–72%, Fig. 8B). A total of 31%–40% of neurons contained at least two receptors (Fig. 8A, Table 4). Approximately 72±4% of OGR1-positive neurons co-expressed with GPR4, 62±4% with G2A, and 56±4% with TDAG8. Among GPR4-positive neurons, 64±4% co-localized with OGR1, 68±4% with G2A, and 54±3% with TDAG8. Of G2A-positive neurons, 73±3% co-expressed with OGR1, 70±3% with GPR4, and 76±4% with TDAG8. More than 73±4% of TDAG8-expressing neurons co-localized with OGR1, 68±4% with GPR4, and 64±4% with G2A. These four genes showed a high degree of co-localization in DRG neurons.

Discussion

To better understand the molecular mechanism of acidosis-linked pain, it is important to elucidate the roles of proton-sensing GPCRs in nociception. To this end, we first demonstrated that proton-sensing GPCRs, the OGR1 family members, were expressed in DRG, primarily within non-peptidergic nociceptors (61–74%). Of the total ganglion neurons, 31% to 40% expressed at least two proton-sensing GPCR genes. Accordingly, OGR1 family members may play some roles in nociception. We have also found that proton-sensing GPCRs genes had compensatory change in ASIC3 knockout mice, suggesting that these genes could be involved in internal mechanisms to adjust for the loss of ASIC3.

Tissue distribution of proton-sensing GPCRs

RT-PCR revealed mouse OGR1, GPR4, G2A, and TDAG8 widely and variably expressed in different tissues (Fig. 1). We found more tissues expressing OGR1 family genes than did previous studies (Mahadevan et al., 1995; Choi et al., 1996; Xu and Casey, 1996; Weng et al., 1998), possibly because of different methods used to detect gene transcripts. It is not surprising that proton-sensing GPCRs are present in many tissues because pH homeostasis is critical in cell function. OGR1 family members could be pH sensors to maintain cellular pH in physiological conditions. Nevertheless, we cannot exclude that each proton-sensing GPCR may have diverse functions in different types of cells.

Previously, only OGR1 was reported in brain tissue (Xu and Casey, 1996). Our studies reveal that all four OGR1 family members are present in neuronal tissues (brain, spinal cord, TRG, and DRG), which suggests that these receptors may participate in some neuronal functions. Given that proton is one of the important factors in nociception, the presence of OGR1 family members in DRG further implies that proton-sensing GPCRs could be involved in nociception. The expression of each OGR1 family member in a single DRG varies slightly, although all receptor transcripts are expressed in lumbar and thoracic DRG (Fig. 3). Only lumbar 4 DRG expressed more equivalent levels of each gene.

Gene expression of proton-sensing GPCRs in ASIC3 knockout mice

Loss of ASIC3 strikingly increases animal sensitivity to acid stimuli, but no expression changes are found with other ASIC members or TRPV1 (Price et al., 2001; Chen et al., 2002). Given that only ASIC3 generates a sustained current at the pH range at which OGR1 family members are fully activated (Yagi et al., 2006; Ludwig et al., 2003; Murakami et al., 2004; Wang et al., 2004), the expression of OGR1 family genes should change to adjust for the loss of ASIC3. As in wild-type tissues, tissues from ASIC3 knockout mice also showed expression of OGR1 family members, and expression levels seem similar to that in wild-type tissues (Fig. 1). Although RT-PCR did not reveal any difference in the expression of the four genes in neural tissues, quantitative PCR revealed significantly changed expression in brain, TRG, DRG, and spinal cord (Fig. 2). OGR1 family genes were co-localized with ASIC3 gene in ASIC3−/− DRG (Fig. 7). These results suggest that the four genes are involved in different internal machinery to adjust for the loss of ASIC3.

Most of the proton-sensing GPCRs are present in non-peptidergic nociceptors

Of the total ganglion cell population, 26% expressed OGR1, 29% GPR4, 32% G2A, and 28% TDAG8 (Fig. 4, Table 1), for a similar number of neurons expressing these four genes in lumbar...
Fig. 6. Co-localization of proton-sensing GPCR genes and TRPV1 gene. DRG tissues were sectioned and hybridized with dig-labeled antisense cRNA probes, followed by co-staining with anti-peripherin (PERI, red fluorescence) and anti-TRPV1 (green fluorescence) antibodies. (A) Phase-contrast fields (a, d, g, j) are neurons labeled with RNA probes. Fluorescence images (b, e, h, k) show neurons labeled with red (PERI only), green (TRPV1 only), and yellow colors (PERI and TRPV1). Phase-contrast images and fluorescence images were combined to obtain merged images (c, f, i, l). Arrowheads indicate the peripherin-positive neurons labeled with antisense probes. Arrows are the peripherin and TRPV1 co-expressed neurons labeled with antisense probes. The scale bar is 50 μm. (B) The histogram shows the percentage of total PERI-positive neurons that expressed genes in TRPV1-positive or -negative subpopulations.
positive nociceptors in inflammatory pain, likely due to increased TRPV1 expression and function or activation of protein kinase C peptidergic neurons that bind IB4 and are sensitive to glial cell and CGRP and are sensitive to nerve growth factor, and non-peptidergic neurons that express the neuropeptide substances P receptors are primarily associated with light-myelinated and non-nociceptors.

A subset of proton-sensing GPCRs is present in myelinated non-nociceptors

The presence of proton-sensing GPCRs in large myelinated neurons reflects their possible roles in mechanosensation. The peripheral large-diameter neurons have been known to respond to innocuous mechanical stimuli (Lewin and Stucky, 2000). Although proton signaling related to mechanosensation seems puzzling, loss of ASIC3 gene has been shown to alter mechanoreceptor function (Price et al., 2001). ASIC3 influence in mechanosensation may not be due to proton signaling, but proton signaling mediated by OGR1 family could play some roles in the function of some mechanoreceptors. Alternatively, the function of proton-sensing GPCRs in myelinated neurons depends on the other ligands such as lysophospholipids or hydroxyoctadecadienoic acid (Xu et al., 2000; Im et al., 2001; Kabarowski et al., 2001; Zhu et al., 2001; Wang et al., 2004; Obinata et al., 2005).

Co-localization of proton-sensing GPCRs with TRPV1

Of the peripherin-positive neurons, 40% to 42% were TRPV1-IR (Fig. 6, Table 3). According to our estimate that 71–73% of the total neurons were peripherin-positive, approximately 28–31% of total neurons expressed TRPV1, which agrees with results of a previous study in mouse (20%, Zwick et al., 2002). This number is lower than that found in rat (50% in Guo et al., 1999; 35–40% in Ugawa et al., 2005). More surprisingly, only 55–59% of TRPV1-IR neurons co-localized with peripherin, which suggests that a subset of TRPV1 is expressed in large-diameter neurons, a finding inconsistent with previous results that TRPV1 is predominantly in small-diameter neurons (Caterina et al., 1997; Tominaga et al., 1998; Guo et al., 1999; Zwick et al., 2002; Ugawa et al., 2005). This inconsistency could be partly due to the antibody used. The antibody against rat TRPV1 sequence (corresponding to residues 4–21 of the N-terminal sequence) may not recognize mouse TRPV1 specifically. To clarify this, we have used in situ hybridization method to confirm the localization of TRPV1 in DRG neurons and found that only 75% of TRPV1-positive neurons are peripherin-positive (data not shown). These results conclude that a subset of TRPV1 is expressed in large-diameter neurons, although the majority of TRPV1 is in small-diameter neurons.

Among TRPV1-IR neurons, 41% co-expressed with G2A, 40% with TDAG8, 26% with OGR1, and 22% with GPR4. G2A and TDAG8 seem to have a higher degree of co-localization with TRPV1. Because TRPV1 is essential for inflammatory thermal hyperalgesia (Caterina et al., 2000; Davis et al., 2000), G2A and TDAG8 may have some functions in inflammatory pain.

Co-localization of proton-sensing GPCRs with ASIC3

In the population of the peripherin-positive neurons, 44% to 50% were expressed with ASIC3 (Fig. 7). According to our estimate that 71% to 73% of the total neurons were peripherin-positive, approximately 31% to 37% of total neurons expressed ASIC3, which agrees with results of previous studies in rat DRG (Molliver et al., 2005; Ugawa et al., 2005). Although 70% of the ASIC3-expressing neurons were co-localized with peripherin, a considerable number of ASIC3-expressing neurons were in peripherin-negative neurons (30%). This suggests that ASIC3 is expressed in small-, medium-, and large-diameter neurons, consistent with previous findings (Alvarez de la Rosa et al., 2002; Molliver et al., 2005; Ugawa et al., 2005). Of the ASIC3-positive neurons, 47±5%, 59±5%, 67±8%, and 59±4% were co-localized with OGR1, GPR4, G2A, and TDAG8, respectively (Fig. 7). Among the four genes, G2A showed higher.

Table 3

Co-expression of proton-sensing GPCRs with TRPV1

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Probe-labeled neurons/total neurons</th>
<th>Subgroup</th>
<th>Probe-labeled neurons/total neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>OGR1</td>
<td></td>
<td>GPR4</td>
<td></td>
</tr>
<tr>
<td>PERI (+)</td>
<td>26 (22–30) 72 (65–77)</td>
<td>PERI (+)</td>
<td>23 (21–27) 68 (65–70)</td>
</tr>
<tr>
<td>TRPV1 (+)</td>
<td>26 (22–30) 67 (60–73)</td>
<td>TRPV1 (+)</td>
<td>22 (20–25) 46 (43–49)</td>
</tr>
<tr>
<td>PERI (+) and TRPV1 (+)</td>
<td>44 (39–49) 14 (11–18)</td>
<td>PERI (+) and TRPV1 (+)</td>
<td>38 (35–41) 10 (8–12)</td>
</tr>
<tr>
<td>PERI (+) and TRPV1 (−)</td>
<td>56 (52–62) 12 (9–15)</td>
<td>PERI (+) and TRPV1 (−)</td>
<td>62 (59–65) 13 (12–16)</td>
</tr>
<tr>
<td>G2A</td>
<td></td>
<td>TDAG8</td>
<td></td>
</tr>
<tr>
<td>PERI (+)</td>
<td>27 (24–30) 52 (47–57)</td>
<td>PERI (+)</td>
<td>31 (26–35) 63 (57–68)</td>
</tr>
<tr>
<td>TRPV1 (+)</td>
<td>41 (36–46) 46 (41–51)</td>
<td>TRPV1 (+)</td>
<td>40 (35–45) 38 (33–43)</td>
</tr>
<tr>
<td>PERI (+) and TRPV1 (+)</td>
<td>45 (37–52) 17 (14–20)</td>
<td>PERI (+) and TRPV1 (+)</td>
<td>41 (36–46) 17 (14–20)</td>
</tr>
<tr>
<td>PERI (+) and TRPV1 (−)</td>
<td>55 (50–60) 10 (8–12)</td>
<td>PERI (+) and TRPV1 (−)</td>
<td>60 (55–64) 14 (10–17)</td>
</tr>
</tbody>
</table>

The number represents the percentage of neurons that expressed single gene or two genes in total DRG neuron populations. Confidence intervals (95%) for proportions are shown in parentheses. The number of total cells counted was of the order of 600–2200.

DRG. This finding is consistent with that from an analysis of a single lumbar DRG by quantitative PCR (Fig. 3). Most of the proton-sensing GPCR-expressing neurons (75–82%) co-localized with peripherin (Fig. 4, Table 1), which suggests that the receptors are primarily associated with light-myelinated and unmyelinated nociceptors. Nociceptors can be subclassified into two populations: peptidergic neurons that express the neuropeptide substances P and CGRP and are sensitive to nerve growth factor, and non-peptidergic neurons that bind IB4 and are sensitive to glial cell line-derived neurotrophic factor. Peptidergic neurons are considered important to inflammatory pain and non-peptidergic neurons to neuropathic pain (reviewed in Snider and McMahon, 1998).

Recent findings have suggested a novel role for IB4-positive nociceptors in inflammatory pain, likely due to increased TRPV1 expression and function or activation of protein kinase C ε (PKCε) (Breese et al., 2005; Hucho et al., 2005). OGR1 family members are mainly expressed in IB4-positive nociceptors (61–74%, Fig. 5, Table 2). It is possible that OGR1 family members have some roles in neuropathic and inflammatory pain.

A subset of proton-sensing GPCRs is present in myelinated non-nociceptors

Approximately 27–29% of proton-sensing GPCR-expressing neurons were labeled only with N52 (Fig. 4, Table 1), which suggests that proton-sensing GPCRs are expressed in myelinated non-nociceptors. The presence of proton-sensing GPCRs in large myelinated neurons reflects their possible roles in mechanosensation.

higher accessibility of probes to 6- (Fig. 8, Table 4). This proportion is more than previous estimates accounted for 48% to 55% of the total ganglion cell population.

Co-localization of proton-sensing GPCRs in nociceptors

..degree of co-localization with ASIC3. This may partly explain the results that only G2A showed expression change in ASIC3−/− DRG tissue (Fig. 2).

Co-localization of proton-sensing GPCRs in nociceptors

The neurons that contained a single proton-sensing GPCR gene accounted for 48% to 55% of the total ganglion cell population (Fig. 8, Table 4). This proportion is more than previous estimates with 12-μm DRG sections (26–32%, Fig. 4) and may reflect a higher accessibility of probes to 6-μm DRG sections as compared with 12-μm sections. In agreement with the 12-μm results, receptor transcripts were primarily present in small-diameter neurons (Figs. 4B and 8B).

Of the total ganglion neurons, 31% to 40% expressed at least two OGR1 family genes (Fig. 8). Previous studies have reported that OGR1 family members mediate proton signaling through two different cascades of second messengers. OGR1 and G2A elicit cyclic AMP (cAMP) formation (Ludwig et al., 2003; Murakami et al., 2004). GPR4 and TDAG8 induce cyclic AMP (cAMP) formation between OGR1 family members is necessary for their function.

Zaslavsky et al. (2006) recently found that GPR4 can show the difficulty in G2A generating a proton response (Radu et al., 2005). If dimerization is required for the function of proton-sensing GPCRs, it could explain the results of previous studies showing the difficulty in G2A generating a proton response (Radu et al., 2005). G2A may form a heterodimer with the other genes to be functional.

Experimental methods

Cloning of proton-sensing GPCRs

Proton-sensing GPCRs were cloned into pBluescript II by PCR. The reaction mix (50 μl) included 0.2 mM of dNTP (Invitrogen, Carlsbad, CA), 1.5 mM of MgCl2 (Invitrogen), 1 U of Taq polymerase (Invitrogen), and 500 nM each of primers. A specific primer set for each gene was designed by use of the primer design software Primer3 (http://frodo.wi.mit.edu/CGI-bin/primer3/primer3-www.cgi). The primer sets for each gene were as follows: OGR1 (1534 bp), 5′-tcgtaattgcaacagctgaagag-3′ (forward) and 5′-acatcaggaactgtgctttgatc-3′ (reverse); GPR4 (1886 bp), 5′-aacagtgccacgtcgctgcag-3′ (forward) and 5′-caacagctgacgtgactgcc-3′ (reverse); G2A (1213 bp), 5′-cactagtaaactacagctgacaaca-3′ (forward) and 5′-caacagctgacgtgactgcc-3′ (reverse); and TDAG8 (1469 bp), 5′-ggagatcgcacactcacta-3′ (forward) and 5′-aacagtgccacgtcgctgcag-3′ (reverse). The PCR condition was 1 cycle of 3 min at 95 °C, 36 cycles of 45 s at 95 °C, 1.5 min at 55–65 °C, 2–3 min at 72 °C, and 1 cycle of 3 min at 72 °C. All clones were sequenced and the sequences were matched with the accession numbers of the GenBank database. The accession numbers are as follows: NM_175493 for OGR1, NM_175668 for GPR4, NM_019925 for G2A, and NM_008152 for TDAG8.

Tissue collection and RNA preparation

Tissues from 8- to 12-week-old wild-type or ASIC3 knockout male mice (CD-1 strain) were removed by use of a fine forceps and immediately frozen in dry ice. Total RNA from different tissues was extracted by use of TRIzol reagent (Invitrogen) according to the manufacturer’s instructions.

Mouse genotyping involved PCR. The primer sets were as follows: ASIC3−/−, 5′-atctgggcaactgtgcttc-3′ and 5′-gtttgctcggaggtagta-3′; and ASIC3+/−, 5′-caacagctgacgtgactgcc-3′ and 5′-ccctggtcgcagactggag-3′. The animals were bred and cared for in accordance with the current Guide for the Use of Laboratory Animals (National Academy Press, Washington, DC). Experimental protocols were approved by the local animal use committee (IACUC, Academia Sinica).

Synthesis of cDNA and RT-PCR

Total RNA of 1 to 5 μg was reverse transcribed by use of Superscript II RT (Invitrogen) with oligo dT (Invitrogen) as used for a primer. Derived cDNA was used as a template for PCR experiments. The reaction mix (10 μl) included 0.2 mM of dNTP, 1.5 mM of MgCl2, 0.5 U of Taq polymerase, 1× PCR buffer, and 100 nM each of primers. The primer sets for each specific gene were designed by use of the primer design software PrimerQuest (http://biotools.idtdna.com/biotools/primerquest/primerquest.asp). The primer sets for each gene were as follows: OGR1 (151 bp), 5′-gaagatcagc-
carried out in the ABI Prism 7300 detection system. The amplified product was detected by measurement of SYBR green I, which was added to the initial experiment mixtures. The threshold cycle (Ct) values obtained through the experiments indicate the fractional cycle numbers at which the amount of amplified target reached a fixed threshold. The Ct values of both target and internal reference (mGAPDH) were measured from the same samples, and the expression of the target gene relative to that of mGAPDH was calculated by the comparative Ct method. This method normalizes the expression levels and allows calculation of the relative efficiency of the target and reference amplification. The efficiencies of the target and reference amplifications were validated by calculating the absolute value of the slope of log input amount vs. ΔCt. The slopes for OGR1, GPR4, G2A, and TDAG8 were 0.052, 0.045, 0.074, and 0.095, respectively. The slopes (±0.1) indicate that the efficiencies of the target and reference amplifications were approximately equal. All data are presented as mean±SEM. A Student’s *t* test was performed between ASIC3+/− and ASIC3−/− animals and the statistically significant level was set at *P*<0.05.

**In situ hybridization and immunohistochemistry**

Lumbar DRG tissues were taken and immediately put into freezing solution. Serial sections 12 μm thick were cut by use of a cryostat (Leica microsystem 3510S, Germany). For co-localization experiments, several pairs of contiguous sections 6 μm thick were prepared. Sections were attached to slides coated with 3-aminopropyltriethoxysilane (APS, 2%). After fixation with 4% parafomaldehyde (Merck) at 4 °C for 30 min, sections were acetylated for 10 min with 0.12% (v/v) triethanolamide (Merck) and 0.25% (v/v) acetic anhydrides (Merck). After preincubation with hybridization buffer (50% formamide, 4× SSC, 2× Denhardt’s solution, and 50 μg/ml tRNA) for 2 h at room temperature, the digoxigenin-UTP (dig, Roche)-labeled complementary RNA (cRNA) probes diluted in hybridization buffer were denatured and hybridized to the DRG section overnight at 65 °C. The dig-labeled probes were generated by *in vitro* transcription with T7 and T3 polymerases (Roche) from nucleotide sequences as follows: nucleotides 1809–1959 for OGR1 (NM_157493); nucleotides 2554–2753 for GPR4 (NM_175668); nucleotides 993–1282 for G2A (NM_019925); and nucleotides 1183–1599 for TDAG8 (NM_008152), and nucleotides 1764–2139 (NM_183000) for ASIC3. Following the hybridization, the slides underwent high-stringency washing cycles: four times of 2× SSC (20× SSC stock: 3 M NaCl and 0.3 M sodium citrate, pH 7.0) at 72 °C for 10 min, three times of pre-warmed 2× SSC at 72 °C for 30 min, three times of pre-warmed 0.1× SSC at 72 °C for 60 min, and twice of 0.2× SSC at room temperature for 10 min. After washing, the dig-labeled cRNA probes were detected with an alkaline-phosphatase conjugated anti-digoxigenin antibody (Roche) by incubation for 1 h at room temperature. Development of signals involved use of a mix of nitro-blue tetrazolium chloride, 0.45%, and 5-bromo-4-chloro-3′- indoxylophosphate p-toluidine salt, 0.35% (Sigma, St. Louis, MO). The specificity of hybridization signals was confirmed by a control study involving sense cRNA probes for each gene.

After hybridization signal detection, sections were washed with 1× PBS and then co-stained with various combinations of primary antibodies,

**Quantitative PCR**

The reaction mix (25 μl) included 6 μl of 2× master mix (containing SYBR green I and AmpliTaQ Gold DNA polymerase, Applied Biosystems, Foster City, CA), 100 nM each of primers and cDNA. Each assay was run in quadruplicate of one or three independent preparations. The DRG pool had at least 10 ganglia and the TG pool was from two ganglia. The thermal cycling conditions were 95 °C for 5 min, followed by 35 cycles of 15 s at 95 °C, and 1 min at 60 °C (65 °C for G2A). PCR product detection was carried out in 3% agarose gel. The internal control was also measured from the same samples (mGAPDH, NM_001001303, 232 bp, primers: 5′-ggagccaaacggtcatcatc-3′ (forward) and 5′-gaggggccatccac-3′ (reverse)).

**Table 4**

| Co-localization of proton-sensing GPCRs in DRG neurons |
|---------------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| OGR1 | 55 (52–58) | 61 (58–64) | 56 (53–59) | 55 (52–58) | 56 (53–59) | 55 (53–57) |
| G2A | 52 (49–55) | 44 (40–48) | 45 (42–48) | 57 (54–61) | 48 (46–50) |

The number represents the percentage of neurons that expressed single gene or two genes in total DRG neuron populations. Confidence intervals (95%) for proportions are shown in parentheses. The number of total cells counted was of the order of 800–1000.

followed by suitable secondary antibodies. All antibodies were diluted in 1× PBS containing 1% BSA. All antibody incubations were carried out at 4°C overnight. Primary antibodies were against N52 (1:500, Sigma) or peripherin (1:500, Sigma). Secondary antibodies were goat-anti-mouse IgG conjugated to TRITC (1:250) and goat-anti-rabbit-IgG conjugated to FITC (1:250).

The specimens were examined by use of a 20× objective in a fluorescence microscope (Zeiss, Axiovert 200, Germany). The digitized images were captured by AxioVision software. One thousand neurons were usually counted from 8 sections, and confidence intervals (95%) for proportions were estimated.

Acknowledgments

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References


capsaicin receptor integrates multiple pain-producing stimuli. Neuron 21, 531–543.


