Immunohistochemical Distribution of Inwardly Rectifying K⁺ Channels in the Medulla Oblongata of the Rat

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ABSTRACT. The inwardly rectifying K⁺ channels, Kir1.1, Kir2.3 and Kir4.1-Kir5.1, are the candidate chemosensory molecules for CO₂/H⁺. We determined the mRNA expression and immunohistochemical localization of these channels in the medulla oblongata of the rat. RT-PCR analysis revealed mRNAs of Kir1.1, Kir2.3, Kir4.1 and Kir5.1 were detected in the medulla. The immunoreactivities for Kir1.1, Kir2.3, Kir4.1 and Kir5.1 were observed in the medulla, and immunolabeling pattern was varied by the subunit. Immunoreactivities for Kir1.1 and Kir2.3 were observed in the nerve cell bodies and glial cells both in the chemosensory areas [nucleus tractus solitarius (NTS), nucleus raphe obscurus (RO), pre-Bötzinger complex (PreBötC)] and non-chemosensory area [hypoglossal nucleus (XII), inferior olive nucleus (IO)]. Kir4.1 immunoreactivity was observed in the glial cells and neuropil, especially in XII and IO. Kir5.1 immunoreactivity was observed in the nerve cell bodies in the XII, RO, and PreBötC, but not in the NTS or IO. In the NTS, a dense network of varicose nerve fibers showed immunoreactivity for Kir5.1. Our findings suggest that Kir channels may not act specific to the central chemoreception, but regulate the ionic properties of cellular membranes in various neurons and glial cells.

KEY WORDS: chemoreceptor, hypercapnic acidosis, inwardly rectifying K⁺ channel, medulla oblongata.


Materials and methods

Materials: Male and female Wistar rats (n=9) were used in the present study, which was approved by the ethics committee of Iwate University. For RT-PCR analysis, three rats were sacrificed in a chamber filled with diethyl ether gas, and dorsomedial part of the medulla oblongata was dissected out. All tissue samples for correction of total RNA were frozen in liquid N₂. For immunohistochemistry, six rats were anesthetized by pentobarbital (15 mg/kg; intraperitoneal injection) and perfused from ascending aorta first with Ringer’s solution (500 ml), followed by Zamboni’s fixative (4% paraformaldehyde, 0.5% picric acid in 0.1 M phosphate buffer; pH 7.4, 500 ml). The medulla oblongata

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Mammalian CO₂/H⁺ sensing organs are roughly divided into two parts, central and peripheral systems [10]. In the central nervous system, chemosensitive neurons have a widespread distribution [11]. For example, CO₂/H⁺-sensitive neurons are found in the locus ceruleus (LC), nucleus tractus solitarius (NTS), medullary raphe, and rostroventrolateral medulla which include pre-Bötzinger complex (Pre-BötC). In the central chemosensitive neurons, a decrease in intracellular pH (pHi) is the initial response to CO₂/H⁺ stimuli [17]. Furthermore, extracellular pH (pHe), as well as bicarbonate and CO₂ concentrations can also modulate CO₂/H⁺ sensing. During sensing of CO₂/H⁺, K⁺ current inhibition may be important during the membrane depolarization of chemosensory cells and during subsequent events such as Ca²⁺ entry to the cells and neurotransmitter discharge [10]. In LC neurons, the outward conductance of inwardly rectifying K⁺ channels (Kir) decreases in response to hypercapnic acidosis [15]. In contrast, Ca²⁺-activated K⁺ channels are inhibited during hypercapnic hypoxia in fetal medullary neurons [25]. These reports indicate that proton-sensitive K⁺ channels play a significant role in sensing of hypercapnic acidosis in the chemosensitive neurons in the brain.

Kir channels are candidates for central CO₂ sensor molecules, and consist of several subfamilies [7, 12]. These channels are important in the modulation of cell excitability and the maintenance of K⁺ homeostasis [20], and comprise a functional homotetramer or heterotetramer [7, 12, 18]. Kir channels are found throughout the body, but are distributed in an organ- and cell-specific manner. Previous studies have revealed that several Kir subunits are sensitive to hypercapnic acidosis [8]. Within the physiological range, homotetramer consisting of Kir1.1 or Kir2.3 [28, 30] and heterotetramer consisting of Kir4.1-Kir5.1 is sensitive to intra- and/or extracellular protons [2, 3, 13, 29, 31]. In situ hybridization revealed colocalization of Kir4.1 and Kir5.1 mRNAs in the chemosensory neurons of the rat central nervous system [27].

In the present study, we tried to characterize the CO₂/H⁺-sensitive Kir subunits, Kir1.1, Kir2.3, Kir4.1 and Kir5.1 in the medulla oblongata by RT-PCR to profile the mRNA expression and by immunolocalization of these subunits. We discuss the function of Kir as the primary sensor molecule for CO₂/H⁺ sensing in central chemosensors.
at the level of the obex were dissected out, and immediately soaked in 30% sucrose in phosphate buffered saline (PBS; pH 7.4) before being frozen. Tissues were semiserially sectioned at a thickness of 20 \( \mu \)m, and mounted on glass slides coated with chrome alum-gelatin.

**RT-PCR amplification:** Total RNAs from the medulla oblongata were isolated using a commercial kit (MELT TM total nucleic acid isolation system, Ambion, Austin, TX). RNA templates were incubated with DNase I (Takara, Tokyo, Japan) for 30 min at 37\( ^\circ \)C before use. RT-PCR was performed with the Qiagen OneStep RT-PCR kit (Qiagen, Tokyo, Japan) with specific primers for Kir1.1, Kir2.3, Kir4.1 and Kir5.1. GAPDH expression was also monitored as a control housekeeping gene. The primers used in the present study are detailed in Table 1. Reverse transcription conditions were as follows: 30 min at 50\( ^\circ \)C for reverse transcription, and 15 min at 95\( ^\circ \)C for initial PCR activation. After the reverse transcription, PCR amplification was performed 40 times as follows: 30 sec at 94\( ^\circ \)C for denaturation, 30 sec at 55\( ^\circ \)C for annealing, and 1 min at 72\( ^\circ \)C for extension. After PCR amplification, samples were applied for 10 min at 72\( ^\circ \)C for final extension. PCR end products were visualized on 2% agarose gels with ethidium bromide. For negative-control experiments, the mRNA template was omitted.

**Immunohistochemistry:** Sections were stained for immunohistochemistry by the avidin-biotin-peroxidase complex (ABC) method. The sections were treated in 0.3% H\(_2\)O\(_2\) in methanol to block endogenous peroxidase activity and incubated for 60 min with non-immune donkey serum (1:50). They were then rinsed in PBS and incubated overnight at 4\( ^\circ \)C with antisera against Kir1.1, Kir2.3, Kir4.1 or Kir5.1 (Table 2). After incubation, the sections were washed again in PBS, and then treated with biotinylated donkey secondary antibody for 30 min at room temperature. Finally, the sections were incubated with the ABC kit reagent (Elite ABC kit, Vector, Burlingame, CA) for 30 min at room temperature. The immunoreaction sites were visualized by incubation with Tris-HCl buffer containing 3,3'-diaminobenzidine tetrahydrochloride (0.2 mg/ml) and 0.003% H\(_2\)O\(_2\). For negative controls, sections were incubated with preabsorbed antibodies. Details of antibodies and concentrations of antigen for preabsorption testing are summarized in Table 2.

### Table 1. Primers for RT-PCR

<table>
<thead>
<tr>
<th>mRNA (Accession #)</th>
<th>Primer sequences</th>
<th>Position</th>
<th>Product length(bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kir1.1 (X7234)</td>
<td>5'-CAGAAGTTGATGAAACGGGACG-3' (sense)</td>
<td>1291–1310</td>
<td>148</td>
</tr>
<tr>
<td></td>
<td>5'-GGACACACGTTACGGCTGC-3' (antisense)</td>
<td>1419–1438</td>
<td></td>
</tr>
<tr>
<td>Kir2.3 (U27582)</td>
<td>5'-GAACCCATAACCGTGTTTCG-3' (antisense)</td>
<td>507–525</td>
<td>280</td>
</tr>
<tr>
<td>Kir4.1 (X86818)</td>
<td>5'-GGAGGTTGAACCTCGTACG-3' (antisense)</td>
<td>767–786</td>
<td>225</td>
</tr>
<tr>
<td>Kir5.1 (AF249676)</td>
<td>5'-GGATGGTTAACTTTGGTGCG-3' (antisense)</td>
<td>1041–1060</td>
<td>330</td>
</tr>
<tr>
<td>GAPDH (AF106860)</td>
<td>5'-AGTGATCGTTCGTCGGCCAG-3' (antisense)</td>
<td>1139–1148</td>
<td>401</td>
</tr>
</tbody>
</table>

### Table 2. Antibodies used in the present study

<table>
<thead>
<tr>
<th>Code</th>
<th>Host species</th>
<th>Dilution</th>
<th>Preabsorption (peptide/antibody)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC-001</td>
<td>rabbit</td>
<td>1:50</td>
<td>3 ( \mu )g/1 ( \mu )gA</td>
<td>A</td>
</tr>
<tr>
<td>AB5376</td>
<td>rabbit</td>
<td>1:100</td>
<td>1 ( \mu )g/1 ( \mu )gB</td>
<td>B</td>
</tr>
<tr>
<td>APC-035</td>
<td>rabbit</td>
<td>1:200</td>
<td>1 ( \mu )g/1 ( \mu )gA</td>
<td>A</td>
</tr>
<tr>
<td>SC-22434</td>
<td>goat</td>
<td>1:50</td>
<td>1 ( \mu )g/1 ( \mu )gC</td>
<td>C</td>
</tr>
<tr>
<td>Clone 11-5B</td>
<td>mouse</td>
<td>1:200</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>Clone GA-5</td>
<td>mouse</td>
<td>1:200</td>
<td>D</td>
<td></td>
</tr>
</tbody>
</table>

| Biotinylated anti-rabbit IgG (H+L) | 711–065–152 | donkey | 1:500 | E |
| Biotinylated anti-goat IgG (H+L) | 705–065–147 | donkey | 1:500 | E |
| FITC-conjugated anti-rabbit IgG (H+L) | 711–095–152 | donkey | 1:100 | E |
| FITC-conjugated anti-goat IgG (H+L) | 705–095–147 | donkey | 1:100 | E |
| TRITC-conjugated anti-mouse IgG (H+L) | 715–025–151 | donkey | 1:100 | E |

a) 2',3'-Cyclic nucleotide 3'-phosphodiesterase; b) Glial fibrillary acidic protein. A, Alomone, Jerusalem, Israel; B, Chemicon, Temecula, CA; C, Santa Cruz Biotechnology, Santa Cruz, CA; D, Lab Vision, Fremont,CA; E, Jackson Immunoresearch, West Grove, PA.
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between nuclei in the chemosensory area [containing the nucleus tractus solitarius (NTS), nucleus raphe obscurus (RO), and pre-Bötzinger complex (PreBötC)] and the nonchemosensory area [containing the hypoglossal nucleus (XII) and inferior olive nucleus (IO)]. Because the PreBötC is functional unit and is the part of ventrolateral medulla, its location was decided by the description of review by Won-Riley and Liu [26].

Double immunofluorescence: Cryostat sections were also used for double immunofluorescence for Kir channel subunits with 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) and glial fibrillary acidic protein (GFAP) in order to identify the Kir immunoreactive glial cells in the oblongata. CNPase and GFAP have been used as immunocytochemical marker for mature oligodendrocytes [24] and for a large part of astrocytes [9]. After incubation with normal donkey serum, sections were incubated with mouse monoclonal antibodies against CNPase or GFAP together with antibody against Kir1.1, Kir2.3, Kir4.1 or Kir5.1 for overnight at 4°C. Then, the sections were incubated with a mixture of TRITC-labeled donkey anti-mouse IgG with FITC-labeled donkey anti-rabbit IgG or FITC-labeled donkey anti-goat IgG for 2 hr at 25°C. The sections were cover-slipped with glycerol-PBS, and examined under an epifluorescence microscope. Details of antibodies were tabulated in Table 2.

RESULTS

RT-PCR: PCR products for Kir1.1, Kir2.3, Kir4.1 and Kir5.1 mRNAs were detected in the medulla oblongata (Fig. 1). Appropriately sized transcripts of the housekeeping gene, GAPDH, were also detected. No signal was detected in the negative controls without RNA template (data not shown).

Immunohistochemistry: Immunoreactivities for Kir1.1, Kir2.3, Kir4.1 and Kir5.1 were observed, and immunolabeling pattern was varied in the subunit. The immunolabeling patterns of Kir1.1 and Kir2.3 were similar each other (Figs. 2A-F). Immunoreactivities for Kir1.1 and Kir2.3 were observed in the nerve cell bodies and glial cells both in the chemosensory [NTS (Figs. 3A, D), RO (Fig. 3E), PreBötC (Fig. 3B)] and non-chemosensory area [XII (Fig. 3C), IO (Fig. 3F)]. The nerve cell bodies in the nucleus ambiguus (AMB) were also immunoreactive for Kir1.1 and Kir2.3 (Figs. 2C, F). Kir4.1 immunoreactivity was observed in the neuropil. The neuropil in the XII and IO were intensely reacted by the Kir 4.1 antibody in comparison with the NTS, RO and PreBötC (Figs. 2G-I, 3G-I). Furthermore, Kir4.1 immunoreactivity was observed in the glial cells in all region examined (Figs. 3G, H). In the XII and RO, some nerve cell bodies also showed immunoreactivity for Kir4.1 (Figs. 3H, I). Many nerve cell bodies were also immunoreactive for Kir4.1 in the dorsal motor nucleus of vagus (DMX; Fig. 3I), but not in the AMB. Kir5.1 immunoreactivity was observed in the nerve cell bodies in the XII, RO, PreBötC and AMB, but not in the NTS or IO (Figs. 3J-L).

In the NTS, a dense network of varicose nerve fibers showed immunoreactivity for Kir5.1 (Fig. 2J, 3J), and a large number of Kir5.1-positive nerve fibers were also observed in the spinal tract of the trigeminal nerve (SpV; Fig. 2L). The RO and PreBötC also contained small numbers of Kir5.1-immunopositive varicose nerve fibers.

Immunoreactivity was decreased or diminished in the negative control sections incubated with preabsorbed antibodies.

Double immunofluorescence: Immunoreactivity for CNPase was not observed in the cells immunoreactive for Kir channel subunits (Figs. 4A-D). Immunoreactivity for GFAP was observed in some cell bodies with thick cytoplasmic processes that immunoreactive for Kir channel subunits except for Kir 5.1 (Figs. 4E-H).

DISCUSSION

A Kir1 inhibitor was reported to reduce the CO2 response of the rostroventrolateral medulla in cats [21]. It has also been shown that pH, in the chemosensitive NTS and ventrolateral medulla was decreased by hypercapnic acidosis and maintained at a lower pH under hypercapnic conditions, whereas nonchemosensitive neurons in the XII and IO exhibited pH recovery in response to hypercapnic acidosis [19]. Because Kir1.1 and Kir2.3 were ubiquitously distributed in the medulla oblongata in the present study, it is possible that these two channels in the medullary neurons including the chemosensory area, NTS, RO, and PreBötC, are closed by the prolonged low pH under hypercapnic acidosis.

The distribution of Kir4.1 and Kir5.1 proteins in the present study contrasts with the reported co-expression of
Kir4.1 and Kir5.1 mRNAs in chemosensitive neurons; this report proposed heterotetramer of Kir4.1-Kir5.1 as the primary CO₂/H⁺ sensors in central chemosensitive neurons [27]. In the present study, neuronal distribution of Kir4.1 was restricted to XII, RO and DMX. On the other hand, that of Kir5.1 was observed in XII, RO, PreBötC and AMB. Thus, the immunohistochemical distribution of Kir4.1 and that of Kir5.1 were different each other except for the motor neurons in the XII and RO. Kir4.1-Kir5.1 heteromeric channels do not seem to be present in a large part of medullary cells. This postulation is supported by earlier data showing no heteromeric Kir4.1-Kir5.1 channels in the rat brain [23]. In the neurons of XII and RO, it is possible that they contain heteromeric Kir4.1-Kir5.1 channels. The expression patterns of Kir4.1 and Kir5.1 may be region-specific in the medullary neurons.

In the present study, Kir5.1 immunoreactivity was observed in the nerve fibers and/or endings. Kir5.1 homomeric channels in the brain were reported to bind postsynaptic density protein 95 (PSD-95) in vivo, and may induce hyperpolarization at postsynaptic sites [22]. Homomeric Kir5.1 channels may therefore regulate K⁺ homeostasis in the synaptic membrane, and the demonstration here of Kir5.1-immunoreactive nerve fibers in the NTS and PreBötC supports a possible role for Kir5.1 in regulating synaptic contact in these regions. Some nerve fibers with Kir5.1 immunoreactivity in NTS and SpV may be central terminals of the sensory neurons of cranial nerves from juglar, nodose and petrosal ganglia.

It has been reported that Kir4.1 were expressed in brain astrocytes [5, 6, 16] and/or oligodendrocytes [1], and Kir4.1-Kir5.1 heteromeric channel was identified in the astrocytes in neocortex and olfactory bulb of mouse [5]. Furthermore, Kir2.3 has been identified in the astrocytes from adult rat brain [14]. In the present study, glial cells that immunoreactive for Kir1.1, Kir2.3 and Kir4.1 were also immunoreactive for GFAP but not for CNPase. Thus, in the medulla oblongata, the glial cells immunoreactive for Kir

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**Fig. 2.** Immunoreactivities for Kir channels in the dorsomedial (left), ventromedial (center), and ventrolateral parts (right) of the rat medulla. A-C, Immunoreactivity for Kir1.1 is shown in cell components of almost all region of medulla. D-F, Immunoreactivity for Kir2.3 in the medulla is also shown in cell components of almost all region. G-I, Neuronal neuropil stained for Kir4.1. In the hypoglossal nucleus (XII) and the inferior olive nucleus (IO), the neuropil are intensely stained. J-L, Kir5.1 immunoreactive nerve fibers are shown in the nucleus tractus solitarius (NTS) and the spinal nucleus of trigeminal nerve (SpV). RO, nucleus raphe obscurus; PreBötC, pre-Bötzing complex, AMB, ambiguous nucleus.
Kir1.1, Kir2.3 and Kir4.1 in the astrocytes may modulate K⁺ current to regulate glial cell membrane potential. In the rat brain, it has been reported that Kir5.1 mRNA is expressed both in neurons and astrocytes [4]. However, it seems that Kir5.1 is not present in medullary glial cells.

In conclusion, Kir1.1 and Kir2.3 may participate in signal transduction of the medullary chemosensitive neurons. However, Kir channels in the medulla oblongata may not act specific to the central chemoreception, but regulate the ionic properties of cellular membranes in various neurons and glial cells.

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