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HETEROGENOUS $GABA_B$ RECEPTOR-MEDIATED PATHWAYS ARE INVOLVED IN THE LOCAL GABAERGIC SYSTEM OF THE RAT TRIGEMINAL GANGLION: POSSIBLE INVOLVEMENT OF KCTD PROTEINS

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Abstract—It is well known that Gamma-aminobutyric acid (GABA) plays an important role in signal transduction in the central nervous system. However, the function of GABA in the peripheral nervous system, including sensory ganglions, is still unclear. In this study we have characterized the expression, cellular distribution, and function of $GABA_B$ receptor subunits, and the recently discovered $GABA_B$ auxiliary subunits, K^+ channel tetramerization domain-containing (KCTD) proteins, in rat trigeminal ganglion (TG) neuronal cells, which are devoid of synapses. We found heterogeneous expression of both $GABA_{B1}$ and $GABA_{B2}$ subunits, and a near-plasma membrane localization of KCTD12. In addition, we found that $GABA_{B2}$ subunits correlated with KCTD16. Whole-cell current-clamp recordings showed that responses to the $GABA_B$ receptor agonist, baclofen, were variable and both increases and decreases in excitability were observed. This correlated with observed differences in voltage-dependent K^+ current responses to baclofen in voltage-clamped TG neuronal cells. The functional diversity of the $GABA_B$ergic regulation on the excitability of the TG neuronal cell bodies could be due to the heterogenous expression of KCTD proteins, and subsequent regulation of plasma membrane K^+ channels. Taken together with our previous demonstration of a local $GABA_A$ receptor-mediated system in rat TG, we provide an updated GABAergic model in the rat TG that incorporates both $GABA_A$- and $GABA_B$-receptor systems.

Key words: GABA, sensory ganglion, satellite cell, neuron–glia interaction, K^+ channel tetramerization domain-containing protein.

INTRODUCTION

Gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the mammalian central nervous system (CNS), and this inhibitory effect is due to GABA binding to the GABA receptors (Mohler, 2006; Tabata and Kano, 2006). It is well known that GABA plays an important role in controlling the level of sensory information by modifying primary-afferent-mediated transmission to the dorsal horn or medulla in the CNS (Malcangio and Bowery, 1996). However, the function of GABA in the peripheral nervous system is still unclear (e.g., Magnaghi, 2007).

The cell bodies of pseudounipolar neurons in mammalian sensory ganglia are generally devoid of synaptic contacts and are thought to play only supportive roles in the maintenance of axons which transmit sensory information from the periphery to central terminals (Lieberman, 1976). It is generally accepted that $GABA_A$ receptor subunits synthesized in the cell bodies are transported to the intraspinal terminals where the receptors function. However, our previous data (Hayasaki et al., 2006) suggested that the neuronal cell bodies in rat trigeminal ganglion (TG) express functional $GABA_A$ receptors and that the satellite cells may function to store and release GABA. On the basis of these findings, we proposed a hypothesis that GABA may play some physiological function roles in the TG (Hayasaki et al., 2006; Vit et al., 2009).

The GABA receptors are divided into two main types, $GABA_A$ and the $GABA_B$ receptors. The $GABA_A$ receptor is a ligand-gated chloride channel and is known to mediate rapid neuronal transmission by increasing membrane Cl^- conductance (Verkman and Galietta, 2009). On the other hand, GABA binding to the $GABA_B$ receptor is linked to G-proteins and induces a slow response by regulating Ca^{2+} and/or K^+ conductances in a cAMP-dependent manner (Bowery, 1993; Uezono et al., 1998; MacDermott et al., 1999; see Bettler et al., 2004; Pinard et al., 2010, for review). Some morphological studies

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Abbreviations: 4-AP, 4-aminopyridine; CNS, central nervous system; DRG, dorsal root ganglion; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; GABA, gamma-aminobutyric acid; GAD, glutamate decarboxylase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; KCTD, K^+ channel tetramerization domain-containing; PBS, phosphate-buffered saline; SD, Sprague–Dawley; SSC, saline-sodium citrate buffer; TEA, tetraethylammonium; TG, trigeminal ganglion.

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revealed that $GABA_B$ receptor-like immunoreactivity was detected pre- and post-synaptically on the primary afferent and spinal dorsal horn (Margeta-Mitrovic et al., 1999; Yang et al., 2001). It was reported that in hippocampal neurons, $GABA_B$ receptors caused an increase in voltage-dependent transient K^+ currents, which might contribute to presynaptic inhibition by hyperpolarizing the membrane and/or by shunting the presynaptic action potentials (Gage, 1992). It was reported that dorsal root ganglion (DRG) neurons expressed three distinct classes of K^+ currents in varying quantities; dominant sustained (K-current; I_K), fast inactivating transient (A-current; I_A) and slow inactivating transient (D-current; I_D) (Everill et al., 1998; Everill and Kocsis, 1999). Takeda et al. (2004) also reported that activation of $GABA_B$ receptors inhibited the excitability of rat small-diameter TG neurons presumably by potentiation of voltage-dependent K^+ currents. These reports suggested a possibility that the $GABA_B$ system plays an important physiological role in TG. However, the distribution and the physiological functions of the $GABA_B$ system in TG are thus far unknown.

Another important feature of the $GABA_B$ system is that it shows substantial kinetic and pharmacological diversity, despite the fact that the receptors themselves are structurally simple, consisting of one of two $GABA_{B1}$ subunits ($GABA_{B1a}$ and $GABA_{B1b}$), together with a $GABA_{B2}$ subunit. Thus there are only two structurally distinct receptor subtypes, $GABA_{B1a,2}$ and $GABA_{B1b,2}$ (Bettler et al., 2004; Pinard et al., 2010). This suggests that the diversity of $GABA_B$-mediated responses might come from unknown auxiliary subunit modifying the function of the $GABA_B$ receptors and/or signaling system further down-stream from the receptor and effector proteins.

In this study, we attempted to characterize the $GABA_B$ergic system in the rat TG and clarify the underlying mechanism of its functional diversity. We investigated the expression and distribution of $GABA_B$ receptor subunits and the recently discovered auxiliary subunits, K^+ channel tetramerization domain-containing (KCTD) proteins (Schwenk et al., 2010), using *in situ* hybridization and immunohistochemistry, and the function and mechanism of the $GABA_B$-mediated system on the excitability of TG neuronal cell bodies using whole-cell voltage and current-clamp techniques.

EXPERIMENTAL PROCEDURES

Tissue preparation for *in situ* hybridization and immunohistochemistry

Male Sprague–Dawley (SD) rats (2–3 weeks old) were anesthetized with pentobarbital sodium (Dainippon Pharmaceutical Co., Ltd.) (50 mg/kg, i.p.) and perfused with Ringer's solution then 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.4) (Abe et al., 2005). The TG was removed and immersed in the same fixative overnight at 4 °C. After brief rinsing with phosphate-buffered saline (PBS), the specimens were immersed in 30% sucrose in PBS overnight at 4 °C. Sections (10 μm thick) were cut with a cryostat and air-dried at room temperature.

Isolation of TG neurons for immunocytochemistry and electrophysiology

SD rats (2–3 weeks old) were killed by decapitation after they were anesthetized with isoflurane (Sigma). The TG was removed and minced with a scalpel. The minced ganglia were placed immediately into Hanks' Balanced Salt Solution (GIBCO, Auckland, NZ) with 1 mg/ml trypsin (Sigma) and 2.8 mg/ml collagenase (type I-A, Sigma) (Si et al., 2004; Takeda et al., 2004). TG was incubated in a shaking water bath for 60–70 min at 32 °C. After incubation, TG was washed twice with Leibovitz's L-15 Medium (GIBCO, Auckland, NZ) supplemented with 10% heat-inactivated new born calf serum and 1% antibiotics. The isolated neurons were placed onto poly-D-lysine-coated coverslips and maintained for at least 120 min until used. For immunocytochemistry the coverslips were fixed 4% with paraformaldehyde for 15 min.

In situ hybridization (ISH)

The DNA probe sequences used for *in situ* hybridization were selected with the Genetyx-SV/R software and were as follows: ($GABA_{B1a}$ anti-sense probe, 5′-CAAATAAGACTTGGAGCAGAT TCGGACACAGCGGCTGGGTGTGTCCATAT-3′; sense probe, 5′-ATATGGACACACCCAGCCGCTGTGTCCGAATCTGCTC CAAGTCTTATTTG-3′, GenBank Accession No.: Y10369), ($GABA_{B1b}$ anti-sense probe, 5′-ACCCCAGCCGCCATCACCAG CAGAAGAGGCAGCGGCCA-3′; sense probe, 5′-TGGCCGC TGCCTCTTCTGCTGGTGATGGCGGCTGGGGT-3′, GenBank Accession No.: Y10370), ($GABA_{B2}$ anti-sense probe, 5′-CTGAG ATGGTCTTGATTTGTTTGGAGCTCAGGGGCTCAAA-3′; sense probe, 5′-TTTGAGCCCCTGAGCTCCAAACAAATCAAGACCAT CTCAG′, GenBank Accession No.: AJ011318). Both sense and antisense DNA probes were generated using a digoxigenin (DIG) DNA labeling kit (Roche Diagnostics, Canada) in accordance with the manufacturer's instructions.

The slides were treated with 0.3% hydrogen peroxide in methanol–0.2 N HCl, and digestion was performed with 25 μg/ml proteinase K. After fixation for 5 min with 4% paraformaldehyde in PB, sections were immersed in PBS with 2 mg/ml glycine and kept in 40% deionized formamide in 4× saline–sodium citrate buffer (SSC) (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) until hybridization. Hybridization was performed overnight at 40 °C with 1 μg/ml digoxigenin-labeled antisense oligo-DNA dissolved in the hybridization buffer containing 10 mM Tris–HCl (pH 7.4), 0.6 M NaCl, 1 mM EDTA, 1× Denhardt's solution, 250 μg/ml yeast tRNA, 125 μg/ml salmon testis DNA, 10% dextran sulfate, and 40% deionized formamide. After hybridization, the slides were washed three times with 2× SSC for 3 h and with 0.5× SSC for 2 h at 37 °C. The slides were then blocked for 30 min in normal goat serum and exposed to sheep anti-Dig-Fab fragments (Roche) for 1 h. Following the washout, the alkaline phosphatase reaction was developed with BCIP/NBT solution (Roche) for 18 h.

We classified the nerve cells based on cell diameter according to the detailed method previously described (Hayasaki et al., 2006). Cells greater than 35 μm were classified as "large cells" and those less than 35 μm were classified as "small cells". For *in situ* hybridization images staining intensity was measured with the Image J software (http://www.rsbweb.nih.gov/ij/).

Immunohistochemistry and immunocytochemistry

Specimens were blocked for 30 min in 10% normal goat serum or 10% normal rabbit serum 0.3% Triton X-100 in PBS, pH 7.4, and then incubated overnight in the primary antibodies. Primary antibodies utilized were: rabbit anti-$GABA_{B1}$ (B17) (1:3000; a gift from Dr. Shigemoto (Kulik et al., 2002)), guinea pig anti-$GABA_{B1}$ (1:3000; Chemicon International, Temecula, CA, USA), guinea