Presence of GABA\(_B\) Receptors Forming Heterodimers With GABA\(_{B1}\) and GABA\(_{B2}\) Subunits in Human Lower Esophageal Sphincter

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Abstract. Baclofen, a GABA\(_B\)-receptor (GABA\(_B\)R) agonist has been proposed to be useful as therapeutic agent for the management of gastro-esophageal reflux disease, but whether the compound acts directly at the lower esophageal sphincter (LES) remains to be elucidated. We performed the present study to assess the presence of GABA\(_B\)R in human LES. Western blot analysis showed that both proteins of GABA\(_B1\)(a)/GABA\(_B1\)(b) and GABA\(_B2\) subunits were present in the muscle layer of LES. Immunohistochemical findings showed that both GABA\(_B1\)- and GABA\(_B2\)-subunit proteins were located on the neurons within the myenteric plexus, and furthermore, both proteins were observed in the same neurons. Reverse transcriptase-polymerase chain reaction analysis also revealed the presence of mRNAs for both subunits of GABA\(_B\)R and also mRNAs for 6 isoforms of GABA\(_B1\) subunits, from GABA\(_B1\)(a) to GABA\(_B1\)(e), except GABA\(_B1\)(d), in human LES. Thus, the functional GABA\(_B\)R-forming heterodimers with subunits of GABA\(_B1\) and GABA\(_B2\) are located on the myenteric neurons in human LES, suggesting that GABA\(_B\)R agonists and antagonists act at least, at the level of the peripheral nervous system.

Keywords: heterodimeric GABA\(_B\) receptor, GABA\(_B1\) subunit, GABA\(_B2\) subunit, human lower esophageal sphincter (LES), gastro-esophageal reflux disease

Introduction

Several reviews have described the presence of \(\gamma\)-aminobutyric acid (GABA)–ergic neurons and two types of GABA receptors, GABA\(_A\) receptor (GABA\(_A\)R) and GABA\(_B\) receptor (GABA\(_B\)R) in the mammalian intestine (1 – 3). In vivo studies indicate that GABA acts more predominantly on the GABA\(_B\)R than the GABA\(_A\)R in intestinal motility (4, 5). Baclofen, a GABA\(_B\)R agonist, has been previously shown to inhibit intestinal motility (4, 5), and baclofen has been detected to inhibit the transient relaxations of the lower esophageal sphincter (LES) in dogs (6), ferrets (7), and humans (8). Furthermore, the compound has been proposed to be useful as a therapeutic agent for the management of reflux disease (9). The mechanism underlying the effect of GABA\(_B\)R agonist has been proposed based on functional studies showing that involvement of GABA\(_B\)R in transient LES relaxations is attributed to the presence of GABA\(_B\)R in the dorsal vagal complex (10, 11), vagal mechanosensitive afferent neurons (11 – 13), and vagal motor neurons innervating the LES (14). However, it is not known if GABA\(_B\)R is present in the LES itself; therefore it remains to be determined whether the GABA\(_B\)R agonists act at the central nervous system and/or the level of the periphery. In the present study, we found for the first time that the functional GABA\(_B\)R forming functional heterodimers with GABA\(_B1\) and GABA\(_B2\) subunits is located on the neurons in the human LES, using Western-blot analysis and immunohistochemical methods. GABA\(_B\)R is the first G protein–coupled receptor discovered to form heterodimers consisting of two different...
GABA\textsubscript{B1} and GABA\textsubscript{B2} subunits (15 – 19). The GABA\textsubscript{B1} subunit is necessary for agonist binding and the GABA\textsubscript{B2} subunit activates the G protein–coupled signaling system (19 – 23), resulting in the expression of functional GABA\textsubscript{A}R. There are seven isoforms of splice variants of GABA\textsubscript{A}R, from GABA\textsubscript{A}R\textsubscript{1} to GABA\textsubscript{A}R\textsubscript{7} (19, 24 – 30); and the localization and function of the GABA\textsubscript{A}R may vary with the different isoforms of GABA\textsubscript{A}R. Therefore, we also attempted to elucidate the subtype of GABA\textsubscript{B1} may vary with the different isoforms of GABA\textsubscript{B1(R)}. There are seven isoforms of splice variants of GABA\textsubscript{B1}, from GABA\textsubscript{B1(a)} to GABA\textsubscript{B1(g)} (19, 24 – 30); and the localization and function of the GABA\textsubscript{B1} may vary with the different isoforms of GABA\textsubscript{B1}. Therefore, we also attempted to elucidate the subtype of GABA\textsubscript{B1} in human LES using reverse transcriptase–polymerase chain reaction (RT-PCR) analysis.

Materials and Methods

Specimens

Specimens of the esophagus and stomach were obtained from patients undergoing surgical resection for esophageal cancer (3 patients) and gastric cancer (4 patients). Each specimen was obtained from an area more than 3 cm from the edge of the cancer. Patients consisted of 6 males and 1 female, aged 61 – 81 years (median, 71 years). Use of the specimens for the present study was approved by The Ethical Committee of Nagasaki University School of Medicine, based on the informed consent of the patients. The tissues of the LES were rapidly separated into muscle and mucosal layer.

Western blot analysis

Tissues were homogenized in 4 ml of protein extraction buffer, included in the protein extraction kit (Qiagen GmbH, Hilden, Germany), followed by centrifugation at 1,700 \( \times \) g for 10 min. The resultant supernatant was centrifuged at 100,000 \( \times \) g for 60 min, and the pellet was resuspended in 50 – 100 ml of the protein extraction buffer. Protein extracts were stored at \(-20^\circ\)C until analysis. The samples of protein extracts (50 – 100 \( \mu \)g) were subjected to electrophoresis in a 7.5% polyacrylamide gel and transferred to nitrocellulose membranes using an electrophoretic gel-transfer apparatus (ATTO, Tokyo). The blots were incubated for 1 h at room temperature in a blocking buffer (Blocking One; Nacalai Tesque, Kyoto), followed by a 2-h incubation with guinea-pig anti-GABA\textsubscript{A}R antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:10,000. After a 1-h exposure to the goat anti-guinea pig secondary antibody conjugated to horseradish peroxidase, blots were developed using chemiluminescence markers following the manufacturer’s protocol (Nacalai Tesque). The blots were apposed to X-ray film, and densitometric images were generated and analyzed using a scanning densitometer with Adobe Photoshop software (Adobe Systems Japan, Tokyo).

Analysis for muscle and mucosal tissues

\( \alpha \)-Smooth muscle actin (SMA) and mucin MUC5AC were employed as a localization marker of smooth muscle and epithelial cells of the digestive tracts, respectively; and the preparations divided into smooth muscle and epithelial cells were assessed by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of mRNAs for SMA and MUC5AC. Total RNA was extracted from the muscle and mucosa of the LES, using a Qiagen Mini Kit (Qiagen GmbH). RT-PCR of SMA and MUC5AC were done according to the methods of Ueda et al. (31) and Guzman et al. (32), respectively. RT-PCR of SMA was carried out with 2 \( \mu \)g of total RNA using the reaction mixture in the presence of 0.2 mg of primer.

Histochemical and immunohistochemical studies

The tissue sections were prepared as follows: Briefly, the tissues were fixed with 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer. After post-fixation with the same fixative for 24 h, the tissues were immersed in 30% sucrose in 0.1 M phosphate buffer for 2 days and embedded in paraffin. Paraffin blocks were cut into 5-\( \mu \)m-thick sections. For histochemical analysis, 5-\( \mu \)m sections were stained with the hematoxylin and eosin reagent, with the general methodology for light microscopy. For immunohistochemistry, slides after deparaffinization were heated in the presence of 0.01 M citric acid (pH 6.0) in a hot water for 30 min. After cooling to room temperature, slides were washed in Tris-buffered saline (TBS) and then incubated overnight with primary antibodies. Primary antibodies used were rabbit polyclonal anti-GABA\textsubscript{A}R antibody (Santa Cruz Biotechnology, diluted 1:50) and anti-GABA\textsubscript{A}R antibody (Santa Cruz Biotechnology, diluted 1:50). For GABA\textsubscript{B1} and GABA\textsubscript{A}R staining, sequential sections were incubated for 40 min with anti-rabbit IgG conjugated with Alexa Fluor 488 (diluted 1:200; Molecular Probes, Tokyo). The sections were observed and photographed under a light microscope (Axioplan II; Carl Zeiss, Jena, Germany). The fluorescence of GABA\textsubscript{B1}-Alexa Fluor 488 and GABA\textsubscript{B2}-Alexa Fluor 488 was observed with a confocal laser scanning fluorescent microscope (LSM 510Meta, Carl Zeiss) at 488-nm excitation and emission at 510 nm.

RT-PCR of mRNAs for GABA\textsubscript{A}R subunits

Total RNA was extracted from the muscle and mucosa of the LES and stomach cardia by using a Qiagen Midi Kit (Qiagen GmbH), and RT-PCR was performed using a thermal cycler (Perkin-Elmer, Norwalk, CT, USA) and an RT-PCR kit (Toyobo, Osaka). Seven independent forward and reverse primers
specific for GABA\textsubscript{B1} subunits and a pair of primers for GABA\textsubscript{B2} subunit were designed on the basis of cloned human or rat GABA\textsubscript{B}R subunits appearing in GenBank and are shown in Table 1. The expected sizes of PCR products using each primer pair are also shown in Table 1. Reverse transcription was performed in a final volume of 20\(\mu\)l using random primers and a reverse transcriptase supplied with the RT-PCR kit. PCR was done in a final volume of 50\(\mu\)l containing 1 mM primers, 1 mM of each DNTP, 2.5 U of recombinant KOD dash DNA polymerase, 10 units of RNase inhibitor, and the RT-PCR buffer supplied with the kit. PCR was done under the following conditions: 30 cycles of 94\(^\circ\)C, 30 s; 60\(^\circ\)C, 30 s; and 72\(^\circ\)C, 90 s. At the end of PCR, samples were kept at 72\(^\circ\)C for 10 min for final extension and then stored at 4\(^\circ\)C. The amplification products were separated by electrophoresis (2.5% agarose gel) and visualized by using SYBR Green nucleic acid gel stain (Molecular Probes) with an FMBIO-II luminescent system (Hitachi, Tokyo).

**Table 1.** Individual GABA\textsubscript{B}-subunit primers for RT-PCR

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sources(^a)</th>
<th>Sequences (5’ to 3’)</th>
<th>Predicted length</th>
</tr>
</thead>
<tbody>
<tr>
<td>GABA\textsubscript{B1(a)} S</td>
<td>AJ01285</td>
<td>AACATGACATCCCAGGAATTTG</td>
<td>387</td>
</tr>
<tr>
<td>Human AS</td>
<td></td>
<td>TCAGGTGGGCTGTGAGTTC</td>
<td></td>
</tr>
<tr>
<td>GABA\textsubscript{B1(b)} S</td>
<td>AJ01286</td>
<td>CACTGCCGCTTCTGGTTGTG</td>
<td>274</td>
</tr>
<tr>
<td>Human AS</td>
<td></td>
<td>TCACACTTGTGCTGTGTTG</td>
<td></td>
</tr>
<tr>
<td>GABA\textsubscript{B1(c)} S</td>
<td>AJ01287</td>
<td>AGTGGAGGAAGACTCTGGAA</td>
<td>470</td>
</tr>
<tr>
<td>Human AS</td>
<td></td>
<td>ATCTTGGGCAAAAGAGCACA</td>
<td></td>
</tr>
<tr>
<td>GABA\textsubscript{B1(d)} S</td>
<td>AB016161</td>
<td>TGAACCGCAGGACACATGA</td>
<td>850</td>
</tr>
<tr>
<td>Rat AS</td>
<td></td>
<td>TCACCTGTTAAAGCAATGCT</td>
<td></td>
</tr>
<tr>
<td>GABA\textsubscript{B1(e)} S</td>
<td>AF301005</td>
<td>AACATGACATCCCAGGAATTTG</td>
<td>420</td>
</tr>
<tr>
<td>Human AS</td>
<td></td>
<td>TCAGGTGGGCTGTGAGTTC</td>
<td></td>
</tr>
<tr>
<td>GABA\textsubscript{B1(f)} S</td>
<td>AF283276</td>
<td>ATGTTACCAGCAGTCCATCTG</td>
<td>119</td>
</tr>
<tr>
<td>Rat AS</td>
<td></td>
<td>GAAACAGCAGCCGGATGTAC</td>
<td></td>
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<tr>
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<td>213</td>
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<tr>
<td>Rat AS</td>
<td></td>
<td>GCTGTCGGTGGTGATGACGGT</td>
<td></td>
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<tr>
<td>GABA\textsubscript{B2} S</td>
<td>AJ012188</td>
<td>AAGTTCCACGGGTACG CCTACGA</td>
<td>287</td>
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<tr>
<td>Human AS</td>
<td></td>
<td>GTGTCGGCCACAGCGTTGACT</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Sources are described by the accession number in GenBank. S, sense; AS, antisense.

Fig. 1. Western blot analysis of subunits of GABA\textsubscript{B1} and GABA\textsubscript{B2} in the preparations from human LES, stomach cardia, and corpus. The blots were incubated for 1 h at room temperature in a blocking buffer (Blocking One), followed by a 2-h incubation with guinea-pig anti-GABA\textsubscript{B1(a)}/GABA\textsubscript{B1(b)} and GABA\textsubscript{B2}. The bands corresponding to GABA\textsubscript{B1(a)} /GABA\textsubscript{B1(b)} and GABA\textsubscript{B2} subunits were detected in the muscle layers of the LES and stomach cardia and corpus.

**Results**

**Western blot analysis of GABA\textsubscript{B}R**

The Western blot analysis was performed using antibodies raised against subunits of GABA\textsubscript{B1(a)}/GABA\textsubscript{B1(b)} and GABA\textsubscript{B2}. The bands corresponding to GABA\textsubscript{B1(a)} /GABA\textsubscript{B1(b)} and GABA\textsubscript{B2} subunits were detected in the muscle layers of the LES and stomach cardia and corpus.

**Statistical analyses**

Statistical significance was tested according to a parametric (Student’s \(t\)) test for paired or unpaired data using a commercial statistical analysis program (Stat-View; Abacus Concepts, Berkeley, CA, USA). All other statistical significances were evaluated using Wilcoxon, Mann-Whitney \(U\) and/or Kruskal-Wallis nonparametric tests and repeated measures ANOVA. Probability (\(P\)) values of <0.05 were considered to be statistically significant.
(Fig. 1), thereby indicating that both GABA_B1 and GABA_B2 subunits are present in the muscle layers of these tissues.

**RT-PCR analysis of mRNAs for SMA and mucin MUC5AC**

SMA and MUC5AC are markers of smooth muscle and epithelial cells of the digestive tract, respectively. Whether the preparations used in the Western blot analysis correspond mainly to the muscle and mucosal tissues was assessed by RT-PCR of mRNAs for SMA and MUC5AC. Expressions of SMA and MUC5AC were detected in the preparations used as the muscle layer and mucosal layer, respectively (Fig. 2).

**Histochemical and immunohistochemical studies of subunits of GABA_B1 and GABA_B2 in human LES and stomach corpus**

As shown in Fig. 3, A and D, neuronal plexus in the human LES and stomach corpus were stained with hematoxylin/eosin staining. Immunostaining with specific antibodies against subunits of GABA_B1 and GABA_B2 was studied in the sections of the human LES and stomach corpus. The fluorescences of GABA_B1–Alexa Fluor 488 and GABA_B2–Alexa Fluor 488 were detected in the neuronal cells within the myenteric plexus of the LES (Fig. 3: B and C) and stomach corpus (Fig. 3: E and F). The fluorescence of GABA_B1 and GABA_B2 subunits was observed in the same neuronal cells in the myenteric plexus of the sequential sections. Intense fluorescence was observed in the cellular membrane (arrow heads in the Fig. 3: B, C, E, and F) and cytoplasm of neurons.

**RT-PCR analysis of mRNAs for subunits of GABA_B1 and GABA_B2**

mRNAs for subunits of GABA_B1 and GABA_B2 in the muscle and mucosa of LES were analyzed using RT-PCR and compared with those for stomach cardia and corpus, brain tissue, and individual cloned receptor. mRNAs for 2 isoforms of GABA_B1, GABA_B1(a) and GABA_B1(b), and GABA_B2, which are considered to form functional GABA_R in the heterologous expression system (15–18), were detected in the muscle layer of the LES (Fig. 4A). When mRNAs for other 5 isoforms of GABA_B1 were analyzed in these tissues, mRNAs for 4 isoforms of GABA_B1, GABA_B1(c), GABA_B1(e), GABA_B1(f), GABA_B1(g), and GABA_B1(h), GABA_B2, and GABA_B2 were detected in the preparations used as the muscle layer and mucosal layer, respectively (Fig. 2).

**Fig. 2.** RT-PCR analysis of mRNAs for SMA and mucin MUC5AC (MUC5) in the preparations from the human LES and stomach cardia. To identify the SMA and MUC5AC mRNAs, total RNA from the tissues was reverse-transcribed following DNAase treatment and then amplified by PCR with each primer pair described in Methods. Amplification products were electrophoresed on 2.5% agarose gel and visualized by SYBR Green staining.

**Fig. 3.** Localization of subunits of GABA_B1 and GABA_B2 in human LES and stomach corpus. Histochemistry and immunostaining of subunits of GABA_B1 and GABA_B2 were performed in the sequential sections from preparations embedded in paraffin. Hematoxylin and eosin staining of neuronal plexus of human LES (A) and stomach corpus (D). Immunofluorescence of subunits of GABA_B1 and GABA_B2 in the LES (B, C) and stomach corpus (E, F), respectively. GABA_B1-receptor immunoreactivity (green) and GABA_B2-receptor immunoreactivity (orange) were presented with pseudo-color. Intense fluorescence was observed in the cellular membrane (arrowhead) and cytoplasm in the neuron within the neuronal plexus. Bar = 50 µm.
and GABA_{B1(d)} but not GABA_{B1(e)} were detected in the tissue (Fig. 4B). Levels of mRNAs for GABA_{B1(c)}, GABA_{B1(e)}, and GABA_{B1(f)} were greater in the LES (Fig. 4B). The bands for GABA_{B1} and GABA_{B2} receptors were sequenced and found to be identical to the reported human GABA_R in levels of nucleotide sequences (data not shown).

**Discussion**

The present study provided the first evidence for the presence of GABA_R proteins and mRNAs in human LES as well as in human stomach corpus. Western blot analysis showed that both GABA_{B1(a)} and GABA_{B2} subunit proteins were present in the muscle layer of the LES. Separation into the muscle and mucosal layer preparations were confirmed by RT-PCR using SMA and mucin MUC5AC, and thus the result of the Western blot analysis indicates that the GABA_R protein was present in the muscle layer of human LES. Since the muscle layer contains not only smooth muscle cells but also neuronal plexus and nerve terminals of motor and afferent neurons, it should be determined whether the GABA_{B1} is located on the neuronal tissue and/or muscle tissue. Histochemical and immuno-histochemical findings provided an obvious fact that both GABA_{B1} and GABA_{B2} subunit proteins were located on the neurons within myenteric plexus, indicating that the functional GABA_R forming heterodimers with GABA_{B1} and GABA_{B2} subunits is located on the neurons in the myenteric neurons in the LES in addition to stomach corpus. The functional GABA_R has been shown to form heterodimers with GABA_{B1} and GABA_{B2} subunits (15 – 18). Whilst GABA_{B1} is responsible for the binding of agonist, GABA_{B2} is considered to be responsible not only for the correct trafficking of the receptor to the cell surface (21, 23, 33), but also absolutely required for downstream of G protein signaling (20, 23, 34, 35). The GABA_R in human LES forms heterodimers consisted of GABA_{B1} and GABA_{B2} subunits and may express a fully functional GABA_R.

There are some reports in the literature describing the mechanism underlying transient relaxations of LES by functional studies. The involvement of GABA_R in the inhibition of transient LES relaxations is attributed to activation of GABA_R present in the dorsal vagal complex (10, 11), vagal mechanosensitive afferent neurons (11 – 13), and vagal motor neurons innervating LES (14). In ferret LES, baclofen has been shown to reduce vagal output at two peripheral sites, presynapti-
cally on preganglionic neurons and on a not-identified site other than LES smooth muscle and inhibitory enteric neurons innervating smooth muscle (36). These studies did not resolve whether GABA<sub>R</sub> was located on the nerve terminals of motor and afferent neurons in addition to the myenteric neuronal cell body. Thus, the present study elucidated the presence of GABA<sub>R</sub> in the neuronal tissues of human LES. The property of these neurons possessing the GABA<sub>R</sub> seems to be different from those in the intestine, in which the GABA<sub>R</sub>s are located on the enteric cholinergic neurons and their stimulations lead to an inhibition of intestinal motility mediated by inhibition of acetylcholine release (9). In the LES, GABA<sub>R</sub> activated by baclofen could be located on the neurons that directly or indirectly modulate the inhibition of transient relaxation of LES.

The isoforms of the GABA<sub>B1</sub> subunit were examined with RT-PCR. mRNAs for GABA<sub>B1</sub> and GABA<sub>B2</sub> subunits were observed in the LES. The GABA<sub>B1</sub> subunit has been identified to have seven isoforms of splice variants (24 – 30). mRNAs for 6 isoforms of GABA<sub>B1</sub> subunits: GABA<sub>B1(a)</sub>, GABA<sub>B1(b)</sub>, GABA<sub>B1(c)</sub>, GABA<sub>B1(d)</sub>, GABA<sub>B1(e)</sub>, and GABA<sub>B1(g)</sub>, excluding GABA<sub>B1(d)</sub>, were detected in the LES and stomach. Similar results have been obtained in colon of dogs (9) and the humans (37). mRNAs for GABA<sub>B1(c)</sub>, GABA<sub>B1(d)</sub>, and GABA<sub>B1(f)</sub> were detected at high levels in the LES and stomach, as compared with the human brain. The GABA<sub>B1(e)</sub> isoform has been shown to be the primary isoform detected in the peripheral tissues, and it is a minor component in the central nervous system (28). There are some reports on the different localization, presynaptic site and/or postsynaptic site, of GABA<sub>B1</sub> in the brain (38). Other isoforms of splice variants of GABA<sub>B1</sub> (24 – 30) may be expressed with different expression patterns in different tissues; the affinity of each GABA<sub>R</sub> subtype may be dependent on the expression levels of each GABA<sub>B1</sub> isoform. One report has shown the presence of two kinds of GABA<sub>R</sub> in ferret LES, one is sensitive to and the other is insensitive to GABA<sub>R</sub> antagonists (36). This may be attributable to a difference of GABA<sub>B1</sub> isoform, and thus what isoform is predominantly localized at the different tissues or cells remains the subject of our ongoing studies. Furthermore, mRNAs and proteins for GABA<sub>B1</sub> and GABA<sub>B2</sub> subunits were detected in the stomach, so the detailed localization of GABA<sub>B1</sub>R protein in the stomach also remains to be assessed in our ongoing studies.

In conclusion, the GABA<sub>R</sub> forming heterodimers with GABA<sub>B1</sub> and GABA<sub>B2</sub> subunits is present in human LES and is located on the neurons in the myenteric plexus. These findings indicate that GABA<sub>R</sub> agonists act on the level of peripheral tissues and could be available for the treatment of gastro-esophageal reflux diseases, by inhibiting transient relaxation of the LES.

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