Intracellular Retention of Recombinant GABA_\text{B} Receptors*

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\textbf{γ-Aminobutyric acid type B (GABA}_B\text{ receptors mediate the transmission of slow and prolonged inhibitory signals in the central nervous system. Two splice variants of GABA}_B\text{ receptors, GABA}_B\text{R1a and GABA}_B\text{R1b, were recently cloned from a mouse cortical and cerebellar cDNA library. As predicted, these receptors belong to the G protein-coupled receptor superfamily. We have used epitope-tagged versions of GABA}_B\text{R1a receptors to study the cellular distribution of these proteins in a variety of non-neuronal and neuronal cell types. Here we report that recombinant GABA}_B\text{ receptors fail to reach the cell surface when expressed in heterologous systems and are retained in the endoplasmic reticulum when introduced into COS cells. In addition, we prove that recombinant GABA}_B\text{ receptors are excluded from the cell surface when overexpressed in ganglion neurons and we further demonstrate that they fail to activate in superior cervical ganglion neurons. Together our observations suggest that recombinant GABA}_B\text{ receptors require additional information for functional targeting to the plasma membrane.}

\gamma\text{-Aminobutyric acid (GABA)}^{1} \text{ is the main inhibitory neurotransmitter in the central nervous system. The fast inhibitory activity of GABA is mediated by the activation of ionotropic GABA}_A\text{ (and GABA}_C\text{) receptors which are members of the ligand gated ion channel superfamily (1). The slower inhibitory actions of GABA are mediated by the activation of GABA}_B\text{ receptors which are G protein-coupled receptors (2, 3).}

GABA}_B\text{ receptors couple to various effector systems mainly through the activation of the specific G proteins G}_o\text{, G}_q\text{, G}_i\text{, and G}_p\text{. Via these signal transduction molecules they inhibit adenyl cyclase, inhibit histamine- and serotonin-induced accumulation of inositol triphosphate, inhibit voltage-gated calcium channels and activate potassium channels (3). In general, activation of GABA}_B\text{ receptors results in modulation of neurotransmitter release from pre-synaptic neurons or hyperpolarization of post-synaptic membranes. These two mechanisms contribute significantly to long-term inhibition of synaptic transmission (3) and their inappropriate function may be implicated in a number of diseases of the central nervous system (3–5).}

Two amino-terminal splice variants of GABA}_B\text{ receptors, GABA}_B\text{R1a and GABA}_B\text{R1b, were recently cloned from a mouse cortical and cerebellar cDNA library (6). As expected, the proteins belong to the seven transmembrane, G protein-coupled receptor superfamily. In particular, the two clones showed significant homology to metabotropic glutamate receptors (7, 6), a Ca}^{2+}\text{ sensing receptor (8), and vromonal receptors (9, 10). Receptors belonging to this family contain an intracellular carboxyl-terminal tail, characteristic seven membrane spanning domains, and a large amino-terminal extracellular domain, which in metabotropic glutamate receptors and GABA}_B\text{ receptors includes a region structurally related to bacterial amino acid-binding proteins (6, 11). Binding properties to antagonists revealed strong similarities between expressed recombinant GABA}_B\text{R1a and endogenous rat cortex GABA}_B\text{ receptors. However, recombinant receptors had a 100-fold reduced affinity for full agonists as compared with native receptors (6). The significance of this discrepancy is still unclear. In terms of coupling to effector systems, GABA}_B\text{R1a mediated the inhibition of adenylyl cyclase in transfected HEK 293 cells. Nevertheless, the receptors failed to couple to K}\text{ channels in Xenopus oocytes (6).}

In order to investigate the possible causes of the inability of recombinant receptors to couple properly to effector systems, we have used epitope-tagged versions of recombinant GABA}_B\text{ receptors to examine for the first time their subcellular distribution in heterologous systems and neuronal cell types. We have found that recombinant GABA}_B\text{ receptors fail to reach the cell surface in several cellular contexts and remain inactive in superior cervical ganglion (SCG) neurons. Our results suggest that intracellular retention constitutes a plausible reason for the inactivity of recombinant receptors in the systems studied so far.}

\textbf{EXPERIMENTAL PROCEDURES}

\textbf{Plasmids and DNA Constructions—pBlueScript based plasmids containing the sequence for GABA}_B\text{R1a receptors were tagged with the 10-amino acid 9E10 epitope (EQKLISEEDL) from c-Myc (12) by site-directed mutagenesis (13). Oligonucleotide GABA}_B\text{R1a-9E10-1 (GGTGGC-GTTGGGGGTAGTGGTCTTGTTATGTTATGGTTCTTGTCGCG-CCGCCCA GAG) was used to tag the receptor between the fourth and fifth amino acid of the mature protein and GABA}_B\text{R1a-9E10-3 (CTCCATCG-C GCCCTATAGGCCCTCCTCCCTGTTATGTTATGGTTCTTGTCGCG-CCGCCCA CAG) was used to tag the receptor at the carboxyl terminus. All mutations and the fidelity of the final expression constructs were verified by DNA sequencing. Sp6-NdeI fragments of these constructs or of untagged sequences were subcloned into a CMV based expression vec-}
Expression of Recombinant GABA<sub>B</sub> Receptors

We sought to constitutive the plasmids AC5-Gbm (amino-terminal 9E10, N-GABA<sub>B</sub>), AC10-Gbm (carboxyterminal 9E10, C-GABA<sub>B</sub>), and AC25-Gb (untagged receptor, GABA<sub>B</sub>h). GABA<sub>B</sub> receptor subunit expression constructs have been previously described (14). A reporter plasmid encoding for the S65T mutant of the jellyfish Aequorea victoria green fluorescent protein (GFP) was used as described previously (16). Prior to incubation with 9E10 antibody, cells were chilled on an ice bed, washed three times with ice-cold PBS, and with iodination medium (0.25% BSA NaHCO<sub>3</sub>-free DMEM, pH 7.4). Cells were then incubated in iodination medium containing 9E10 iodinated to a specific activity of 500 Ci/mmol (Amersham International) and used at 10–15 μg/ml for 90 min on an ice bed. Excess iodine, medium and cells were washed five times with ice-cold PBS. Cells were removed with 1 ml of trypsin, transferred to a bath and counted in a y-counter (Wallac, 1261 Multigamma).

Neuron Primary Cultures, Microinjection, and Recording of Ca<sup>2+</sup> Channel Currents—For electrophysiology, single SCG neurons were dissociated from 15–19-day-old rats and plated on poly-L-lysine-coated glass coverslips bordered by 2-cm plastic rings as described previously (16). After plating, neurons were microinjected with an equal mixture of plasmids carrying cDNA for the N-GABA<sub>B</sub> or C-GABA<sub>B</sub> receptor (final pipette concentration of 0.5 μg/ml dissolved in water) and cDNA for S65T mutant of GFP or GFP cDNA alone. DNA (1.2 μl) was loaded into pre-pulled high resistance (30 megohm) “Pyrex” glass pipettes and injected into the nucleus of single neurons as described previously for injection of antisera or cDNA (17, 18). After injections, cells were incubated for 14–24 h in a humidified incubator (5% CO<sub>2</sub>) at 37 °C. Injected neurons which successfully expressed cDNA were identified as bright fluorescence cells using an inverted microscope (Diaphot 200, Nikon, Japan) equipped with an epifluorescent block N B2E (Nikon). Electrophysiological recordings were made at room temperature 14–24 h after injection. SCG and DRG primary neurons for immunofluorescence, kindly provided by Mariza Dayrell and Kenji Okuse, respectively, were obtained and cultured as described previously (19, 20). Microinjection of neurons was performed as described previously (21) using an Eppendorf Transjector (5246), an Eppendorf Micromanipulator (5171), and a Nikon Diaphot 300 microscope.

Expression of Recombinant GABA<sub>B</sub> Receptors in Heterologous Systems—To achieve this, COS cells were transfected with N-GABA<sub>B</sub> and metabolically labeled for 4 h. After plating, neurons were microinjected with an equal mixture of plasmids carrying cDNA for the N-GABA<sub>B</sub> or C-GABA<sub>B</sub> receptor (final pipette concentration of 0.5 μg/ml dissolved in water) and cDNA for S65T mutant of GFP or GFP cDNA alone. DNA (1.2 μl) was loaded into pre-pulled high resistance (30 megohm) “Pyrex” glass pipettes and injected into the nucleus of single neurons as described previously for injection of antisera or cDNA (17, 18). After injections, cells were incubated for 14–24 h in a humidified incubator (5% CO<sub>2</sub>) at 37 °C. Injected neurons which successfully expressed cDNA were identified as bright fluorescence cells using an inverted microscope (Diaphot 200, Nikon, Japan) equipped with an epifluorescent block N B2E (Nikon). Electrophysiological recordings were made at room temperature 14–24 h after injection. SCG and DRG primary neurons for immunofluorescence, kindly provided by Mariza Dayrell and Kenji Okuse, respectively, were obtained and cultured as described previously (19, 20). Microinjection of neurons was performed as described previously (21) using an Eppendorf Transjector (5246), an Eppendorf Micromanipulator (5171), and a Nikon Diaphot 300 microscope.

Expression of Recombinant GABA<sub>B</sub> Receptors in Heterologous Systems—To achieve this, COS cells were transfected with N-GABA<sub>B</sub> and metabolically labeled with [35S]methionine. GABA<sub>B</sub> receptors were immunoprecipitated using 9E10 monoclonal antibodies directed against the Myc epitope. A predominant protein of approximately 120–130 kDa was specifically immunoprecipitated from...
The expression of recombinant GABA\(_B\) receptors in COS cells was analyzed for surface expression using 9E10 antibody and a different anti-mouse specific secondary antibody (upper panels). Intracellular proteins were detected in intact cells using 9E10 antibody and a different anti-mouse specific secondary antibody (lower panels). Arrows indicate nonpermeabilized and permeabilized cells, respectively. B, cell surface (upper panel) and intracellular (lower panel). N-GABAB receptors in HEK 293 cells. C, surface (upper panel) and intracellular (lower panel) N-GABAB receptors in baby hamster kidney cells.

Expression of Recombinant GABA\(_B\) Receptors

**FIG. 2.** Immunofluorescence of recombinant N-GABA\(_B\) receptors in heterologous systems. A, COS cells were analyzed for expression of GABA\(_A\) \(\beta3\)-9E10 subunit and N-GABA\(_B\) receptors 18 h after transfection. Surface molecules were detected in intact cells using 9E10 antibody and anti-mouse specific secondary antibodies (upper panels). Intracellular proteins were detected in the same cells after permeabilization using 9E10 antibody and a different anti-mouse specific secondary antibody (lower panels). Arrows indicate nonpermeabilized and permeabilized cells, respectively. B, cell surface (upper panel) and intracellular (lower panel). N-GABA\(_B\) receptors in HEK 293 cells. C, surface (upper panel) and intracellular (lower panel) N-GABA\(_B\) receptors in baby hamster kidney cells.
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The possibility that the 9E10 epitope might interfere with
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tain the appropriate conformation (not shown).

Rapid recycling of receptors between an intracellular com-
partment and the plasma membrane, with short permanence
at the cell surface, would result in intracellular accumulation
of most of the protein. To address this possibility, putative cell
surface receptors were labeled with antibodies on live
N-GABAB-transfected COS cells. Cells were exposed to 9E10
monoclonal antibodies and incubated at 37 °C to allow endocy-
tosis of the potential antibody-receptor complexes. Subse-
quently, immunofluorescence was performed to detect these
complexes in vesicular structures underlying the plasma mem-
brane. Although this technique has been applied successfully in
detecting the fast recycling of the GABAᵦ γ 2 subunit,² no
signal was obtained upon examination of N-GABAB transfected
cells that had been fed with 9E10 antibodies (not shown).

The inability of recombinant GABAᵦ receptors to reach the
cell surface under our experimental conditions may result from
a very slow transport machinery. To address this issue the half-life of N-GABAB was determined in COS cells by a pulse-
chase experiment. Briefly, cells were transfected with
N-GABAB and labeled with a pulse of [35S]methionine. At the indicated periods of time cell lysates were prepared
and recombinant GABAB receptors were immunoprecipitated using
9E10 antibodies and protein A-Sepharose. Immune complexes were
separated by SDS-PAGE using 8% gels. Relevant molecular weight
markers are indicated on the left-hand side. Quantitation of the relative
amounts of immunoprecipitated [35S]methionine-labeled GABAB recep-
tors was performed by exposing the polycrylamide gel shown in A to
PhosphorImager. Bands corresponding to N-GABAB were quantitated
and expressed as a percentage of the maximum value. Values were
fitted to an exponential curve and the half-life of the protein was
calculated. This value corresponded to approximately 10 h.

² C. Connolly, unpublished results.

9E10 were very low and were significantly increased by coex-
pression of β2 (Fig. 3A). In agreement with our immunofluo-
rescence results, surface levels of N-GABAB were similar to
those of α1–9E10 (Fig. 3A). More importantly, N-GABAB and
C-GABAB levels did not differ significantly. Since the carboxy-
terminal epitope in C-GABAB is predicted to reside in the
cytoplasm, these results clearly indicate that both tagged re-
ceptors are equally inaccessible to 9E10 antibodies in intact
cells. The same experiment was performed in HEK 293 cells
and similar results were obtained (Fig. 3B). These observations
further suggest that recombinant GABAᵦ receptors fail to
reach the plasma membrane in heterologous systems.

The inability of recombinant GABAᵦ receptors to reach the
plasma membrane in heterologous systems. A, intact mock
transfected, GABAᵦ α1–9E10 subunit, GABAᵦ α1–9E10 β2 subunits, N-GABAᵦ and C-GABAᵦ transfected COS cells were
incubated in the presence of [125I]-conjugated 9E10 antibody at 10–15 μg/ml for 90
min on ice bed. Cells were washed exten-
sively and removed in trypsin. Surface bound [125I]-conjugated 9E10 antibody was
quantitiated in a γ-counter, and expressed as a percentage of the maximum value.
Values for mock transfected control cells were considered background and there-
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expression of N-GABA_B and labeled with [35S]methionine. Immunoprecipitated receptors were separated by SDS-PAGE using 8% gels. Relevant molecular weight markers (High Molecular Weight Markers, Bio-Rad) are indicated on the left-hand side. Arrowheads indicate difference in molecular weight of the majority of the immunoprecipitated receptor complexes were denatured and incubated at 37 °C for 14 h in the absence (-) or presence (+) of 1 milliunit of endoglycosidase H. Samples were separated by SDS-PAGE using 8% gels. Relevant molecular weight markers (High Molecular Weight Markers, Bio-Rad) are indicated on the left-hand side. Arrowheads indicate difference in molecular weight of N-GABA_B before and after Endo H treatment.

Expression of Recombinant GABA_B Receptors

Taking advantage of the presence of N-linked oligosaccharides in GABA_B receptors, the subcellular localization of recombinant receptors was confirmed using an endoglycosidase H sensitivity assay. Glycosylated proteins processed in the ER contain a characteristic high mannose oligosaccharide that is sensitive to digestion by the highly specific enzyme endoglycosidase H (Endo H). In the Golgi stack the oligosaccharide is modified and becomes resistant to Endo H digestion. Sensitivity to Endo H is, therefore, a good indicator of ER localization for N-linked glycosylated proteins (27). To address the sensitivity of recombinant GABA_B receptors to Endo H digestion, COS cells were transfected with N-GABA_B and metabolically labeled with [35S]methionine. Immunoprecipitated receptors were digested with Endo H and analyzed by SDS-PAGE. A predominant form of GABA_B was immunoprecipitated from lysates derived from N-GABA_B transfected cells and was absent from lysates derived from mock transfected controls (Fig. 5B, lanes 1 and 2). Upon Endo H treatment the electrophoretic mobility of the majority of the immunoprecipitated receptor was significantly altered (Fig. 5B, lane 3). This decrease in molecular weight indicates that the N-GABA_B receptor is sensitive to Endo H digestion. Taken together these observations strongly suggest that N-GABA_B enters the secretory pathway and is specifically retained in the ER when expressed in COS cells.

Recombinant GABA_B Receptors Fail to Function when Expressed in Ganglion Neurons—The presence of functional GABA_B receptors in SCG neurons (28, 29, 30) and dorsal root ganglion neurons (DRG) (31) has been reported extensively in recent years (3, 32). To examine the activity of recombinant GABA_B receptors in ganglion neurons, the inhibition of Ca^2+ channel currents in response to the GABA_B receptor agonist l-baclofen was studied electrophysiologically. Primary SCG neurons were injected either with a reporter plasmid carrying the GFP or co-injected with GFP and N-GABA_B. Upon exposure to l-baclofen, a 10.7 ± 3.0% inhibition of Ca^2+ channel currents was observed in GFP injected SCG neurons indicating the presence of endogenous GABA_B receptors coupled to Ca^2+ channels in SCG neurons. The weak inhibition obtained in these experiments suggests that the number of active endogenous receptors in SCG neurons might be small. In contrast, a 42.8 ± 7% inhibition was
Expression of Recombinant GABA$_B$ Receptors

The study of the targeting mechanisms of GABA$_B$ receptors to the plasma membrane is of fundamental importance for the understanding of GABA-mediated signaling and for the development of novel therapeutic agents that modify the function of GABA$_B$ receptors. The results reported in this study suggest that recombinant murine GABA$_B$R1a receptors fail to reach the cell surface when expressed in ganglion neurons, and are specifically retained in the ER when introduced in heterologous systems. Preliminary studies using GABA$_B$R1b, an alternatively spliced receptor that differs from GABA$_B$R1a in the extreme amino-terminal region, have produced similar results (not shown). Thus, exclusion from the plasma membrane does not appear to be restricted to a single class of GABA$_B$ receptor. We suggest that the difficulties in coupling recombinant GABA$_B$ receptors to known effector systems result mainly from inappropriate targeting. Interestingly, a 30% inhibition of forskolin-stimulated adenylyl cyclase activity was reported for one of the splice variants of recombinant receptors (6). However, this effect should be studied in more detail because the assay was performed in cell membranes, and many of them may have had ER origin.

Several explanations for intracellular retention such as interference of the 9E10 epitope, slow transport to the plasma membrane, or rapid endocytosis are unlikely in view of the results obtained in the present study. Nevertheless, other possibilities arise that are in agreement with our results. First, the receptor may be expressed at the plasma membrane at levels below the detection limits of the techniques used in this study. Zhang et al. (34) have used flow cytometry to demonstrate that only 0.3–1.0% of transiently transfected HEK 293 cells express Caenorhabditis elegans ODR-10 odorant receptor at the cell surface. This small fraction of properly targeted receptors appears to be sufficient to mediate a significant response to the appropriate odorant. However, the physiological significance of such small numbers remains to be determined. In the case of recombinant GABA$_B$ receptors, the failure of them to couple to Ca$^{2+}$ or K$^+$ channels argues against the possibility of a small fraction of receptors mediating the responses to GABA or GABA agonists.

Second, the receptor may require accessory molecules for maturation and targeting to the plasma membrane. This pos-

FIG. 7. Immunofluorescence of recombinant GABA$_B$ receptors in ganglion neurons. A, N-GABA$_B$ injected SCG neurons were analyzed for expression of recombinant GABA$_B$ receptors 18 h after injection. Expression of cell surface molecules was examined in intact cell bodies (left-hand panels) and neuronal projections (right-hand panels) using 9E10 antibody and anti-mouse specific secondary antibodies (upper panels). Intracellular protein was detected in the same cells after permeabilization using 9E10 antibody and a different anti-mouse specific secondary antibody (lower panels). B, N-GABA$_B$ injected DRG neurons were analyzed for expression of recombinant GABA$_B$ receptors 18 h after injection. Expression of cell surface molecules was examined in intact cell bodies (left-hand panels) and neuronal projections (right-hand panels) using 9E10 antibody and anti-mouse specific secondary antibodies (upper panels). Intracellular protein was detected in the same cells after permeabilization using 9E10 antibody and a different anti-mouse specific secondary antibody (lower panels). The weak signal present in the upper panels results from bleed-through due to high levels of intracellular receptors.

obtained in the same neurons when Ca$^{2+}$ channel current inhibition was achieved through the activation of endogenous $\alpha_2$ adrenergic receptors upon exposure to noradrenaline. These results are in accordance with previous observations (17) and demonstrate that inhibition of Ca$^{2+}$ channel currents is not saturated at levels affected by L-baclofen. As expected, in cells expressing recombinant N-GABA$_B$ receptors, inhibition of Ca$^{2+}$ channel currents by NA did not differ significantly from the inhibition observed for GFP injected control neurons (Fig. 6B, left). Interestingly, when cells expressing recombinant untagged (not shown) or N-GABA$_B$ receptors were exposed to L-baclofen, Ca$^{2+}$ channel current inhibition was not significantly increased ($2.9 \pm 1.4\%$) (Fig. 6, A and B, left). These observations are considerably different from others reported for activation recombinant G protein-coupled receptors in sympathetic neurons, where 45–65% inhibitions of Ca$^{2+}$ channel currents have been observed above the endogenous response (33). These results clearly indicate that recombinant GABA$_B$ receptors failed to activate when expressed in SCG neurons. The significance of the lower inhibition of Ca$^{2+}$ channel current in N-GABA$_B$ injected cells is still unclear and probably resulted from high cell to cell variation of endogenous receptor activation in response to agonist.

To evaluate the possibility that inactivity of recombinant GABA$_B$ receptors resulted from intracellular retention as in heterologous systems, N-GABA$_B$ was microinjected into SCG and DRG neurons and the subcellular distribution of GABA$_B$ receptors was examined by immunofluorescence. The subcellular distribution in neurons was similar to that obtained for the three cell lines tested, namely, no cell surface receptors were detected in intact neurons (Fig. 7, A and B, upper panels), and large amounts of the protein were detected intracellularly when cells were permeabilized (Fig. 7, A and B, lower panels). This was observed when cell bodies or neuronal projections were examined (Fig. 7). The pattern of expression did not change when cells were examined after 5 h of injection, when small amounts of receptors were detectable by immunofluorescence, or 5 days after microinjection (not shown). These observations demonstrate the absence of cell surface staining in neurons and suggest that retention does not result from intracellular obstruction, due to overexpression of recombinant GABA$_B$ receptors, or to slow transport.
sibility needs to be studied in detail. The fact that the receptor is
excluded from the cell surface in neurons that normally
express GABA₉-mediated responses argues against it. How-
ever, the putative accessory protein could be present in limiting
amounts, could have a very slow turnover, and/or strong asso-
ciation to the receptor, or could regulate the targeting of
GABA₉ receptors in response to activity or developmental cues.
For example, several reports indicate that expression of pre-
synaptic and postsynaptic GABA₉ receptors is extremely well
regulated during postnatal ontogenesis and early stages of
innervation (35–39). Our studies concerning expression of
GABA₉ in different cell lines provide ideal heterologous sys-
tems to attempt to identify potential accessory molecules. Fi-
nally, the recombinant receptor may carry a cis element, such
as a retention signal, that requires highly specific and defined
conditions to allow the transport of the receptor and its release
from the ER to its functional target localization. Alternatively,
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as a retention signal, that requires highly specific and defined
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