Receptor internalization is recognized as an important mechanism for rapidly regulating cell surface numbers of receptors. However, there are conflicting results on the existence of rapid endocytosis of γ-aminobutyric acid, type B (GABA_B) receptors. Therefore, we analyzed internalization of GABA_B receptors expressed in HEK 293 cells qualitatively and quantitatively using immunocytochemical, cell surface enzyme-linked immunosorbent assay, and biotinylation methods. The data indicate the existence of rapid constitutive receptor internalization, with the first endocytosed receptors being observed in proximity of the plasma membrane after 10 min. After 120 min, a loss of about 40–50% of cell surface receptors was detected. Stimulation of GABA_B receptors with GABA or baclofen did not enhance endocytosis of receptors, indicating the lack of agonist-induced internalization. The data suggest that GABA_B receptors were endocytosed via the classical dynamin- and clathrin-dependent pathway and accumulated in an endosomal sorting compartment before being targeted to lysosomes for degradation. No evidence for recycling of receptors back to the cell surface was found. In conclusion, the results indicate the presence of constitutive internalization of GABA_B receptors via clathrin-coated pits, which resulted in lysosomal degradation of the receptors.

GABA_B receptors are G protein-coupled receptors that play an important role in the control of neurotransmission. They are widely expressed in the nervous system and have been implicated as potential targets for neurological diseases, such as epilepsy, pain, spasticity, addiction, schizophrenia, depression, and anxiety (for a review, see Ref. 1). GABA_B receptors mediate slow inhibitory neurotransmission by either activating postsynaptically K⁺ channels or inhibiting presynaptically the release of neurotransmitters by modulation of Ca²⁺ channels. On the structural level, functional GABA_B receptors require the heterodimerization of two distinct seven-transmembrane proteins, termed GABA_B1 and GABA_B2 (2–7). Two main variants of GABA_B1 have been reported (GABA_B1a and GABA_B1b (8)), which are generated by alternative promoter usage (9) and differ solely in their N-terminal domain. Heterodimerization of GABA_B1a or GABA_B1b with GABA_B2 leads to two main GABA_B receptor subtypes, GABA_B1a/GABA_B2 and GABA_B1b/GABA_B2, which are abundantly expressed in all major brain structures (10–13).

An important aspect in the regulation of G protein-coupled receptors is their internalization or endocytosis. To protect cells against receptor overstimulation, the vast majority of G protein-coupled receptors desensitize upon prolonged agonist exposure, followed by rapid internalization. Many G protein-coupled receptors undergo phosphorylation upon agonist exposure by a G protein receptor kinase and subsequently recruit an arrestin protein (14). Arrestins often enhance phosphorylation, sterically interfere with binding of the G protein, and function as a signal for receptor endocytosis (15). Once internalized, receptors are targeted to specialized compartments, where they are dephosphorylated and recycled back to the plasma membrane or targeted to lysosomes for degradation.

The processes of GABA_B receptor desensitization and internalization are currently poorly understood. Initial studies revealed unexpected differences from the common G protein-coupled receptor pathway. Although G protein receptor kinases 4 and 5 directly associate with GABA_B receptors and are indispensable for receptor desensitization in certain cells (16, 17), kinase activity is not required (16). A second mechanism proposed involves the interaction of the GABA_B receptor heterodimer with N-ethylmaleimide-sensitive fusion protein (NSF), which primes the receptor for phosphorylation by protein kinase C upon agonist stimulation and leads to desensitization (18). Thus, although the mechanism of GABA_B receptor desensitization is still poorly understood, it is clearly mediated by processes distinct from the generally accepted model of G protein receptor kinase phosphorylation-induced desensitization and subsequent internalization of the receptors.

Consistent with the atypical mode of desensitization, recent findings suggest that GABA_B receptors are not internalized and do not recruit arrestin in response to agonist exposure (16, 19, 20). Interestingly, Fairfax et al. (19) hypothesized that GABA_B receptors may be targeted directly from the surface to the proteasome for degradation. However, agonist-induced internalization of GABA_B receptors has also been reported (21). In view of these contradictory reports, it remains unclear whether
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GABA<sub>B</sub> receptors undergo noticeable constitutive endocytosis and whether an agonist-induced internalization of GABA<sub>B</sub> receptors exists. In addition, if appreciable internalization of GABA<sub>B</sub> receptors occurs, the underlying pathways are completely unknown.

To clarify this issue, we analyzed endocytosis of GABA<sub>B</sub> receptors expressed in HEK 293 cells and the involved pathways using antibody-based and biotinylation assays. The results suggest that GABA<sub>B</sub> receptors undergo constitutive internalization via the classical dynamin and clathrin-dependent pathway and are targeted to lysosomes for degradation.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—The following primary antibodies were used: rabbit GABA<sub>B1</sub> directed against the N terminus of GABA<sub>B1</sub> (affinity-purified, 1:50 for immunofluorescence (10)), rabbit GABA<sub>B2</sub> directed against the N terminus of GABA<sub>B2</sub> (serum, 1:250–1:5000 for immunofluorescence (22)), rabbit GABA<sub>B1</sub> directed against the C terminus of GABA<sub>B1</sub> (coupled to protein A-agarose for immunoprecipitation (10)), rabbit GABA<sub>B2</sub> directed against the C terminus of GABA<sub>B2</sub> (coupled to protein A-agarose for immunoprecipitation (22)), guinea pig GABA<sub>B2</sub> directed against the C terminus of GABA<sub>B2</sub> (1:1000–1:2000 for immunofluorescence and Western blotting; Chemicon International), mouse Lamp-1 (1:100 for immunofluorescence and Western blotting; Axxora), mouse B1/β2 adaptins (1:250 for immunofluorescence and Western blotting; Sigma), mouse caveolin 1 and mouse caveolin 2 (both 1:250 for immunofluorescence; BD Biosciences).

**Plasmids**—Expression plasmids containing GABA<sub>B1</sub> and GABA<sub>B2</sub> were described previously (23). Dynamin-enhanced green fluorescent protein and dynamin K44A-enhance green fluorescent protein were kindly provided by U. Greber (Institute of Zoology, University of Zurich, Switzerland), and arrestin 3-green fluorescent protein was a gift from H. Hatt (Cell Physiology, University Bochum, Germany).

**Cell Culture**—HEK 293 cells were maintained in MEM containing 10% fetal calf serum, 2 mM glutamine and transfected with appropriate plasmids by calcium phosphate precipitation as described previously (23). Cells were used 2 days after transfection for internalization studies.

**Immunofluorescence-based Internalization Assay**—Living HEK 293 cells transiently transfected with GABA<sub>B1</sub> and GABA<sub>B2</sub> were incubated with the GABA<sub>B1(N)</sub> or GABA<sub>B2(N)</sub> antibody in buffer A (25 mM HEPES, pH 7.4, 119 mM NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 30 mM glucose) containing 10% normal goat serum for 30 min at 4 °C. After washing the cells extensively with ice-cold buffer A, the cells were incubated for 10–120 min at 37 °C in the presence or absence of drugs. Control cultures were kept on ice to prevent internalization of receptors (100% controls). Internalization was stopped by washing the cells with ice-cold buffer A, followed by incubation with horseradish peroxidase-conjugated anti-rabbit antibodies for 60 min at 4 °C. After extensive washes with buffer A, horseradish peroxidase activity was determined using tetramethylbenzidine as substrate (0.24 mg/ml tetramethylbenzidine, 0.2 M sodium citrate, pH 3.95, 0.03% H<sub>2</sub>O<sub>2</sub>). The color reaction was terminated after 2–5 min by the addition of an equal volume of 1 M H<sub>2</sub>SO<sub>4</sub>, and the optical density was recorded at 450 nm in a microplate reader (Synergy HT; Biotek). A nonspecific antibody reaction was determined in parallel cultures of nontransfected HEK 293 cells.

**Biotinylation Assay**—Transfected HEK 293 cells cultured in 6-cm dishes were placed on ice and washed two times with ice-cold buffer A, followed by biotinylation of cell surface proteins with ice-cold sulfoxsuccinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate (Sulfo-NHS-SS-Biotin) (Pierce) in buffer A (0.5 mg/ml) for 15 min in the presence of 100 μM chloroquine (to block lysosomal degradation of proteins). After three washes with buffer A containing 100 μM chloroquine and 1% bovine serum albumin, the cells were incubated for 120 min at 37 °C in the presence of 100 μM chloroquine. Cultures for determination of total cell surface and background labeling were left on ice. In all samples, except for total surface labeling, cell surface biotin was cleaved off with glutathione solution (75 mM glutathione, 75 mM NaCl, 10 mM EDTA, 1% bovine serum albumin) two times for 15 min each on ice. Cells were harvested in buffer A, transferred to Eppendorf tubes, and pelleted by centrifugation. Cells were resuspended in 40 μl of 10 mM Tris, pH 8, 150 mM NaCl containing protease inhibitors (complete Mini; Roche Applied Science), supplemented with 1% SDS and heated for 10 min at 80 °C. After dilution with 400 μl of solubilization buffer (10 mM Tris, pH 8, 150 mM NaCl, complete Mini, 1% Triton X-100), the samples were sonified and centrifuged for 30 min at 100,000 × g and 4 °C. Supernatants containing equal amounts of protein were incubated with 60 μl of streptavidin-Sepharose (GE Healthcare) overnight at 4 °C to precipitate biotinylated proteins. The Sepharose beads were washed two times with solubilization buffer, two times with solubilization buffer containing 0.6 M NaCl and again two times with solubilization buffer. Bound proteins were eluted by incubation in SDS sample buffer for 5 min at 80 °C followed by SDS-polyacrylamide gel electrophoresis and Western blotting using guinea pig GABA<sub>B2</sub> antibodies. Chemiluminescence (Super...
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Signal West Pico Chemoluminescence Substrate (Pierce) signals were captured using a Fuji LAS-1000 plus imaging system (Fujifilm, Tokyo, Japan), and immunoreactive bands were quantified with the AIDA software (version 3.25; Raytest, Pforzheim, Germany).

**Immunoprecipitation**—Immunoprecipitation of GABA<sub>B</sub> receptors from rat brain tissue was performed essentially as described previously (10). HEK 293 cells transiently expressing GABA<sub>B1a</sub> and GABA<sub>B2</sub> were harvested in 10 mM Tris, pH 8, 150 mM NaCl, protease inhibitor mixture (complete Mini; Roche Applied Science), homogenized, and solubilized using 0.5% sodium deoxycholate for 1 h on ice followed by centrifugation at 100,000 × g for 30 min. For immunoprecipitation, GABA<sub>B1a</sub>, or GABA<sub>B2</sub> antibodies covalently coupled to protein A-agarose were added to the supernatant and incubated overnight at 4 °C. Immune complexes were collected by centrifugation and extensively washed with 10 mM Tris, pH 8, 150 mM NaCl, protease inhibitor mixture, 1% Triton X-100. Bound proteins were released by incubation with 2× sample buffer for SDS polyacrylamide gel electrophoresis for 15 min at 65 °C and analyzed by Western blotting.

**RESULTS**

**GABA<sub>B</sub> Receptors Undergo Constitutive but Not Agonist-induced Internalization**—In order to visualize internalization of GABA<sub>B</sub> receptors transiently expressed in HEK 293 cells, surface receptors of living cells were labeled with an antibody directed against the N terminus of GABA<sub>B1a</sub> at 4 °C, a condition that is nonpermissive for internalization. After labeling, the cells were incubated for different time intervals at 37 °C in the presence or absence of GABA<sub>B</sub> receptor agonists. Cell surface and internalized receptors were then differentially visualized. Cell surface receptors were stained with a secondary antibody coupled to a green fluorescent fluorophor and, after fixation and permeabilization of the cells, internalized receptors were labeled with a secondary antibody carrying a red fluorescent fluorophor. Small clusters of internalized receptors, which were predominantly localized in the vicinity of the cell surface, were already detected after 10 min (Fig. 1A). After 30, 60, and 120 min, an increasing intracellular accumulation of GABA<sub>B</sub> receptors in large clusters was observed. Stimulation of receptors with either 100 μM GABA or baclofen did not result in an appreciable increase of receptor internalization (Fig. 1A). Thus, GABA<sub>B</sub> receptors were rapidly constitutively internalized, but there was no evidence for agonist-induced internalization.

The extent of internalization was determined by cell surface ELISA. Surface GABA<sub>B</sub> receptors on living HEK 293 cells cultured in 24-well plates were labeled with GABA<sub>B1a</sub>(N) or GABA<sub>B2(N)</sub> antibodies, respectively, and subsequently incubated for 120 min at 37 °C in the presence or absence of agonists. The amount of cell surface receptors was then quantified with a microplate reader using a secondary antibody coupled to horseradish peroxidase. In line with the results from the immunofluorescence-based internalization assay, a substantial fraction of cell surface staining was lost after 120 min (47 ± 7%), indicating considerable constitutive endocytosis of GABA<sub>B</sub> receptors (Fig. 1B). Activation of GABA<sub>B</sub> receptors with GABA or baclofen did not lead to further loss of cell surface receptors (GABA, 48 ± 4% internalization; baclofen, 48 ± 7% internalization). The loss of cell surface staining was blocked by the addition of hypertonic concentrations of sucrose, a condition that is...
known to inhibit clathrin-mediated endocytosis (Fig. 1B). To further exclude the presence of agonist-induced internalization of GABA\textsubscript{B} receptors, cells were stimulated with GABA or baclofen for 120 min prior to labeling and quantifying cell surface receptors. In case of agonist-induced internalization, a loss of cell surface staining would be expected in the cultures stimulated with agonist. However, no loss of cell surface receptors was observed (Fig. 1C).

Most G protein-coupled receptors displaying agonist-induced internalization recruit an arrestin protein, which serves as a signal for endocytosis (24). However, co-expression of arrestin 3-green fluorescent protein with GABA\textsubscript{B} receptors in HEK 293 cells did not result in a redistribution of arrestin 3-green fluorescent protein from the cytoplasm to the plasma membrane in response to GABA stimulation, being in line with the lack of agonist-promoted internalization (not shown).

Internalization of GABA\textsubscript{B} Receptors Is Not Induced by Antibodies—In an antibody-dependent internalization assay, binding of the antibody to the receptors may induce their endocytosis. To determine whether the antibodies used in the internalization assay affect endocytosis of GABA\textsubscript{B} receptors, we analyzed the effect of antibody concentration on the extent of internalization. If the antibodies used for labeling would induce internalization of GABA\textsubscript{B} receptors, we expected a greater extent of internalization with increasing antibody concentration. Surprisingly, high antibody concentrations appeared to inhibit internalization in the immunofluorescence based assay. At an antibody dilution of 1:250, only a few small clusters of internalized receptors were observed in proximity to the cell membrane after 120 min, whereas at higher antibody dilutions (1:1000 and 1:5000), large clusters of internalized receptors abundantly accumulated within the cells (Fig. 2A). Quantification of this effect by cell surface ELISA revealed an internalization of 21 ± 4% of cell surface receptors after 120 min and at low antibody concentrations an internalization of about 40% (1:1000, 36 ± 6%; 1:5000, 45 ± 9%) (Fig. 2B). Thus, at high concentrations, the antibodies inhibited endocytosis of GABA\textsubscript{B} receptors.

To further exclude that the antibodies used induce internalization of GABA\textsubscript{B} receptors, we analyzed endocytosis of receptors with an antibody-independent cell surface biotinylation assay. For this assay, all surface proteins were biotinylated for 15 min at 4 °C and incubated then for 120 min at 37 °C in the presence or absence of GABA\textsubscript{B} receptor agonists to allow endocytosis. Biotin on cell surface proteins was then cleaved off with glutathione, leaving internalized proteins biotinylated. After solubilization of cells, internalized biotinylated proteins were purified with streptavidin-Sepharose, and the amount of biotinylated GABA\textsubscript{B} receptor was determined by Western blotting with GABA\textsubscript{B} antibodies. As in the antibody-based assays, we observed robust constitutive but no agonist-induced internalization of GABA\textsubscript{B} receptors in the biotinylation assay (Fig. 3A). Quantification of the blots revealed a similar extent of receptor endocytosis as in the antibody-dependent assay (about 40% after 120 min) (Fig. 3B). These results show that a substantial fraction of cell surface GABA\textsubscript{B} receptors expressed in HEK 293 cells constitutively internalize.

GABA\textsubscript{B} Receptors Internalize via the Dynamin- and Clathrin-Dependent Pathway—To determine the mechanism by which GABA\textsubscript{B} receptors internalize, we tested different inhibitors for their ability to block endocytosis. First, we analyzed whether GABA\textsubscript{B} receptors internalized by overexpressing a GTP-binding and hydrolysis-defective dynamin mutant (dynamin K44A) that has been shown to restrain invaginated pits from pinching off (25). Although overexpression of wild type dynamin did not affect internalization of GABA\textsubscript{B} receptors (not shown), dynamin K44A completely blocked endocytosis of the receptors (Fig. 4).

Next, we analyzed whether GABA\textsubscript{B} receptors internalized via a clathrin- or caveolin-dependent mechanism. Hypertonic concentrations of sucrose and chlorpromazine both inhibit the formation of clathrin-coated pits (26, 27) and thus have been widely used to inhibit clathrin-dependent endocytosis. In the presence of sucrose (450 mM) or chlorpromazine (100 μg/ml), internalization was completely blocked (Fig. 4A). However, treatment of cells with nystatin (50 μg/ml) or filipin (5 μg/ml), inhibitors of caveolea/raft-dependent endocytosis (28, 29), did not affect internalization of GABA\textsubscript{B} receptors (Fig. 4A).

![Figure 1](image1.png)

**Figure 1. Constitutive Internalization of GABA\textsubscript{B} Receptors**

**A**—In an antibody-dependent internalization assay, binding of the antibody to the receptors may induce their endocytosis. To determine whether the antibodies used in the internalization assay affect endocytosis of GABA\textsubscript{B} receptors, we analyzed the effect of antibody concentration on the extent of internalization. If the antibodies used for labeling would induce internalization of GABA\textsubscript{B} receptors, we expected a greater extent of internalization with increasing antibody concentration. Surprisingly, high antibody concentrations appeared to inhibit internalization in the immunofluorescence based assay. At an antibody dilution of 1:250, only a few small clusters of internalized receptors were observed in proximity to the cell membrane after 120 min, whereas at higher antibody dilutions (1:1000 and 1:5000), large clusters of internalized receptors abundantly accumulated within the cells (Fig. 2A). Quantification of this effect by cell surface ELISA revealed an internalization of 21 ± 4% of cell surface receptors after 120 min and at low antibody concentrations an internalization of about 40% (1:1000, 36 ± 6%; 1:5000, 45 ± 9%) (Fig. 2B). Thus, at high concentrations, the antibodies inhibited endocytosis of GABA\textsubscript{B} receptors.

**B**—To further exclude that the antibodies used induce internalization of GABA\textsubscript{B} receptors, we analyzed endocytosis of receptors with an antibody-independent cell surface biotinylation assay. For this assay, all surface proteins were biotinylated for 15 min at 4 °C and incubated then for 120 min at 37 °C in the presence or absence of GABA\textsubscript{B} receptor agonists to allow endocytosis. Biotin on cell surface proteins was then cleaved off with glutathione, leaving internalized proteins biotinylated. After solubilization of cells, internalized biotinylated proteins were purified with streptavidin-Sepharose, and the amount of biotinylated GABA\textsubscript{B} receptor was determined by Western blotting with GABA\textsubscript{B} antibodies. As in the antibody-based assays, we observed robust constitutive but no agonist-induced internalization of GABA\textsubscript{B} receptors in the biotinylation assay (Fig. 3A). Quantification of the blots revealed a similar extent of receptor endocytosis as in the antibody-dependent assay (about 40% after 120 min) (Fig. 3B). These results show that a substantial fraction of cell surface GABA\textsubscript{B} receptors expressed in HEK 293 cells constitutively internalize.
To further substantiate the finding that GABA_B receptors may predominantly internalize via the clathrin-dependent pathway, we analyzed their potential co-localization with the AP2 complex, which has been implicated in the recruitment of plasma membrane proteins into clathrin-coated pits (30). The antibody used recognized the β2-adaptin subunit of the AP2 complex located predominantly at the plasma membrane and in addition also the β1-adaptin subunit of the AP1 complex, which is restricted to clathrin-coated membranes of the trans-Golgi network (30). In order to detect co-localization of GABA_B receptors specifically with β2-adaptin, we labeled exclusively cell surface GABA_B receptors with GABA_B2(N) antibodies and let them internalize for 60 min, followed by fixation and permeabilization of the cells and staining with the β-adaptin antibody. This experimental set-up prevented the co-dection of a potential co-localization with β1-adaptin of intracellular GABA_B receptors in the exocytic pathway. Under these experimental conditions, we observed a frequent co-localization of β2-adaptin with cell surface and also with internalized GABA_B receptors (Fig. 4B). Under the same experimental conditions, we found only a rare co-localization of GABA_B receptors with caveolin 1 or caveolin 2, supporting the view that GABA_B receptors in HEK 293 cells predominantly internalize via clathrin-coated pits (Fig. 4B).

The immunofluorescence data suggested the association of GABA_B receptors with the AP2 complex and thus endocytosis via clathrin-coated pits. We therefore tested for a direct interaction of GABA_B receptors with β-adaptin by immunoprecipitation using GABA_B2(C) antibodies coupled to protein A-agarose. The immunoprecipitates were analyzed by Western blotting using anti-β-adaptin antibodies for 120 min at 37 °C in the absence (control) or presence of a dominant-negative mutant of dynamin (dynamin K44A), 450 mM sucrose, 100 μM chlorpromazine, 50 μM/ml nystatin, or 5 μg/ml filipin. Although nystatin and filipin, inhibitors of the caveolin-dependent pathway, did not affect internalization of GABA_B receptors, dynamin K44A, sucrose, and chlorpromazine completely blocked endocytosis, indicating that receptors were internalized via the classical dynamin and clathrin-dependent pathway. For the sake of clarity, expression of dynamin K44A is not depicted in the cells shown. Bar, 10 μM B, GABA_B receptors co-localize with β-adaptin of the AP2 complex. Cell surface GABA_B receptors were labeled with GABA_B2(N) antibodies for 120 min at 37 °C and incubated thereafter for 120 min at 37 °C in the presence or absence of 100 μM GABA or 100 μM GABA + 10 μM CGP 55999A. Subsequently, biotin on cell surface proteins was cleaved off with glutathione, and biotinylated internalized proteins were purified with streptavidin-Sepharose. The presence and abundance of GABA_B receptors in the streptavidin-Sepharose eluate was determined by Western blotting with GABA_B2 antibodys. Each lane represents the signal from one individual culture plate. Total surface receptors, cells were left on ice to prevent internalization and were not subjected to cleavage with glutathione; nonspecific, cells were left on ice to prevent internalization and were subsequently to cleavage with glutathione; internalized receptors, cells were incubated for 120 min at 37 °C to allow endocytosis, followed by stripping off cell surface biotin with glutathione. Robust constitutive but no agonist-induced internalization of GABA_B receptors was observed. B, quantification of the Western blots shown in A. Chemoluminescence signals captured using a Fuji LAS-1000 plus imaging system were quantified with the AIDA software. Signals of “total surface receptors” (control) were set to 100%. Signals for “nonspecific” (i.e. cell surface biotin that was not cleaved off by glutathione) were subtracted from signals of “internalized receptors.” The data represent the mean ± S.D. of four independent experiments performed in duplicate.

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FIGURE 3. Demonstration of GABA_B receptor internalization using a cell surface biotinylation assay. A, surface proteins of HEK 293 cells expressing GABA_B1b and GABA_B2 were biotinylated for 15 min at 4 °C and incubated thereafter for 120 min at 37 °C in the presence or absence of 100 μM GABA or 100 μM GABA + 10 μM CGP 55999A. Subsequently, biotin on cell surface proteins was cleaved off with glutathione, and biotinylated internalized proteins were purified with streptavidin-Sepharose. The presence and abundance of GABA_B receptors (Fig. 4A) eluate was determined by Western blotting with GABA_B2 antibodies. Each lane represents the signal from one individual culture plate. Total surface receptors, cells were left on ice to prevent internalization and were not subjected to cleavage with glutathione; nonspecific, cells were left on ice to prevent internalization and were subsequently to cleavage with glutathione; internalized receptors, cells were incubated for 120 min at 37 °C to allow endocytosis, followed by stripping off cell surface biotin with glutathione. Robust constitutive but no agonist-induced internalization of GABA_B receptors was observed. B, quantification of the Western blots shown in A. Chemoluminescence signals captured using a Fuji LAS-1000 plus imaging system were quantified with the AIDA software. Signals of “total surface receptors” (control) were set to 100%. Signals for “nonspecific” (i.e. cell surface biotin that was not cleaved off by glutathione) were subtracted from signals of “internalized receptors.” The data represent the mean ± S.D. of four independent experiments performed in duplicate.

FIGURE 4. GABA_B receptors are internalized by the dynamin and clathrin-dependent pathway. A, HEK 293 cells expressing GABA_B1b and GABA_B2 were subjected to the immunofluorescence-based internalization assay using GABA_B2(N) antibodies for 120 min at 37 °C in the absence (control) or presence of a dominant-negative mutant of dynamin (dynamin K44A), 450 mM sucrose, 100 μM chlorpromazine, 50 μM/ml nystatin, or 5 μg/ml filipin. Although nystatin and filipin, inhibitors of the caveolin-dependent pathway, did not affect internalization of GABA_B receptors, dynamin K44A, sucrose, and chlorpromazine completely blocked endocytosis, indicating that receptors were internalized via the classical dynamin and clathrin-dependent pathway. For the sake of clarity, expression of dynamin K44A is not depicted in the cells shown. Bar, 10 μM B, GABA_B receptors co-localize with β-adaptin of the AP2 complex. Cell surface GABA_B receptors were labeled with GABA_B2(N) antibodies for 120 min at 37 °C and incubated thereafter for 120 min at 37 °C in the presence or absence of 100 μM GABA or 100 μM GABA + 10 μM CGP 55999A. Subsequently, biotin on cell surface proteins was cleaved off with glutathione, and biotinylated internalized proteins were purified with streptavidin-Sepharose. The presence and abundance of GABA_B receptors (Fig. 4A) eluate was determined by Western blotting with GABA_B2 antibodies. Each lane represents the signal from one individual culture plate. Total surface receptors, cells were left on ice to prevent internalization and were not subjected to cleavage with glutathione; nonspecific, cells were left on ice to prevent internalization and were subsequently to cleavage with glutathione; internalized receptors, cells were incubated for 120 min at 37 °C to allow endocytosis, followed by stripping off cell surface biotin with glutathione. Robust constitutive but no agonist-induced internalization of GABA_B receptors was observed. B, quantification of the Western blots shown in A. Chemoluminescence signals captured using a Fuji LAS-1000 plus imaging system were quantified with the AIDA software. Signals of “total surface receptors” (control) were set to 100%. Signals for “nonspecific” (i.e. cell surface biotin that was not cleaved off by glutathione) were subtracted from signals of “internalized receptors.” The data represent the mean ± S.D. of four independent experiments performed in duplicate.
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**FIGURE 5. Fate of endocytosed GABA<sub>B</sub> receptors.** A, internalized GABA<sub>B</sub> receptors are not recycled back to the cell surface. Cell surface GABA<sub>B</sub> receptors expressed on HEK 293 cells were labeled with GABA<sub>B2/29</sub> antibodies for 90 min at 37°C, a condition that permits endocytosis of receptors during the labeling period. Thereafter, antibodies bound to cell surface GABA<sub>B</sub> receptors were removed with two washes of 0.2 M glycine, pH 2.5, 0.5 M NaCl for 3 min, followed by further incubation of the cells at 37°C for 10–30 min. Potential cell surface receptors (recycled) were stained at 4°C with a green fluorescent secondary antibody, followed by fixation, permeabilization, and staining of internalized receptors with a red fluorescent secondary antibody. No recycling of GABA<sub>B</sub> receptors (green) was detected. Bar, 10 μM. B, GABA<sub>B</sub> receptors are targeted to lysosomes for degradation. Cell surface receptors on HEK 293 cells were labeled with GABA<sub>B2/29</sub> antibodies for 30 min at 4°C and allowed to endocytose for 60 min at 37°C in the presence or absence of 100 μM chloroquine (a blocker of lysosomal function). Cell surface receptors were then stained with a green fluorescent secondary antibody for 60 min at 4°C, and cells were incubated again at 37°C for 30–120 min (chase). Subsequently, the cells were fixed, permeabilized, and stained for internalized receptors with a red fluorescent secondary antibody. Cell surface receptors (chase: 0 min, green) were progressively internalized at 30, 60, and 120 min of incubation at 37°C and were found to be largely co-localized (yellow) with formerly internalized receptors (red). In the absence of chloroquine, GABA<sub>B</sub> receptor staining strongly decreased after 60 and 120 min, indicating degradation of GABA<sub>B</sub> receptors. The presence of chloroquine prevented the loss of GABA<sub>B</sub> receptor protein A-agarose. As expected, β-adaptin immunoreactivity was detected in both GABA<sub>B</sub> receptor immunoprecipitates (Fig. 4C). Although we cannot exclude a possible association with β1-adaptin, the immunoprecipitation experiment in combination with the co-localization study indicates a direct association of GABA<sub>B</sub> receptors with the AP2 complex and thus an endocytosis via clathrin-coated pits.

**GABA<sub>B</sub> Receptors Are Targeted to Lysosomes for Degradation—** Internalized receptors can be principally directed to two distinct destinations: to lysosomes for degradation or recycled back to the cell surface. To analyze the fate of internalized GABA<sub>B</sub> receptors, we first tested whether they are able to recycle back to the cell surface. Cell surface receptors were labeled with antibodies and allowed to internalize for 90 min at 37°C. Subsequently, antibodies bound to cell surface GABA<sub>B</sub> receptors were stripped off with two consecutive washes with 0.2 M glycine, pH 2.5, 0.5 M NaCl for 3 min. The stripped cells were then further incubated at 37°C for 10–30 min to allow receptors to recycle back to the cell surface. Putative recycled receptors were stained with a green fluorescent secondary antibody, whereas internalized receptors were stained, after fixation and permeabilization of the cells, with a red fluorescent secondary antibody. Under these conditions, no recycling of GABA<sub>B</sub> receptors was observed (Fig. 5A). To ensure that the antibody stripping procedure did not interfere with recycling of receptors, a second series of experiments was done, where the stripping step was omitted and bound cell surface antibodies were instead saturated with secondary antibody. Again, no recycling of receptors back to the cell surface was observed (not shown).

Since internalized GABA<sub>B</sub> receptors appear not to recycle back to the cell surface, they are expected to be targeted to lysosomes and eventually degraded. To test for lysosomal degradation, cell surface receptors were labeled with antibodies and allowed to internalize for 60 min at 37°C. After staining of the remaining cell surface receptors with a green fluorescent secondary antibody, cells were incubated again at 37°C for 30–120 min and were subsequently fixed, permeabilized, and stained for internalized receptors with a red fluorescent secondary antibody. Under these conditions, cell surface labeled receptors were progressively endocytosed with time and co-localized with formerly internalized receptors (Fig. 5B, top). In staining and resulted in a pronounced intracellular accumulation of the receptors. Bar, 10 μM. C, inhibition of lysosomal proteases but not of proteosomal activity leads to an increased intracellular accumulation of endocytosed GABA<sub>B</sub> receptors. HEK 293 cells expressing GABA<sub>B1b</sub> and GABA<sub>B2</sub> were subjected to the internalization assay for 2 h in the absence (control) or presence of inhibitors of lysosomal function (100 μM chloroquine, 100 μM leupeptin, or 1 μM pepstatin A) or inhibitors of proteosomal activity (10 μM MG132 or 50 μM lactacystin). Cell surface receptors were stained with a green fluorescent secondary antibody, and internalized receptors were stained with a red fluorescent secondary antibody. Bar, 10 μM. D, internalized GABA<sub>B</sub> receptors do not accumulate in lysosomes. HEK 293 cells expressing GABA<sub>B1b</sub> and GABA<sub>B2</sub> were subjected to the immunofluorescence internalization assay for 120 min at 37°C using GABA<sub>B2/29</sub> antibodies in the absence or presence of 100 μM chloroquine (to inhibit lysosomal activity). Cell surface receptors were stained with a blue fluorescent secondary antibody, internalized receptors were stained with a red fluorescent secondary antibody, and lysosomes were labeled using a monoclonal antibody directed against Lamp 1 (green). No co-localization (yellow) of internalized receptors (red) with the lysosomal marker Lamp 1 (green) was observed, irrespective of blocking lysosomal activity with chloroquine. Thus, internalized GABA<sub>B</sub> receptors appear to accumulate in an endosomal compartment.
addition, staining of cell surface and internalized receptors strongly diminished after 60 and 120 min, indicating degradation of the receptors (Fig. 5B, upper panel). Pretreatment of cells with the lysosomotropic drug chloroquine, which increases endosomal and lysosomal pH and impairs lysosomal function (31, 32), completely prevented the loss of staining and led to the co-localization of signals in large intracellular clusters (Fig. 5B, bottom).

Furthermore, blocking directly lysosomal proteases with either 100 μM leupeptin or 1 μM pepstatin A likewise resulted in an increased intracellular accumulation of internalized GABA<sub>B</sub> receptors, supporting the view that endocytosed GABA<sub>B</sub> receptors are degraded in lysosomes (Fig. 5C). In contrast, blocking proteasomal activity with 10 μM MG 132 or 50 μM lactacystin did not affect intracellular accumulation of GABA<sub>B</sub> receptors (Fig. 5C). These experiments indicate that internalized GABA<sub>B</sub> receptors are predominantly degraded by lysosomes with no appreciable contribution of proteasomal activity.

Since inhibition of lysosomal activity resulted in a pronounced formation of intracellular clusters, we expected that the receptors accumulate in lysosomes. Surprisingly, co-localization experiments of internalized GABA<sub>B</sub> receptors with the lysosomal marker Lamp 1 did not reveal any overlap in localization, even after blocking lysosomal activity with chloroquine (Fig. 5D). Therefore, internalized GABA<sub>B</sub> receptors appear to accumulate in an endosomal compartment before entering lysosomes for degradation.

DISCUSSION

Endocytosis of G protein-coupled receptors involves a sequence of highly orchestrated molecular events. It serves as a mechanism for regulating cell surface levels of receptors and thereby contributes to signaling strength. Currently, the process of GABA<sub>B</sub> receptor endocytosis is poorly understood. The results of the present study suggest that heterologously expressed GABA<sub>B</sub> receptors are constitutively endocytosed predominantly via the dynamin- and clathrin-dependent pathway and are finally sorted to lysosomes for degradation.

To gain the first insights into the mechanisms involved in endocytosis of GABA<sub>B</sub> receptors, we expressed in HEK 293 cells the GABA<sub>B1b/GABA<sub>B2</sub></sub> combination, which is the most abundant GABA<sub>B</sub> receptor subtype in the adult brain (10, 11). Heterologous expression of receptors was chosen, since it is a robust, easy to manipulate and to evaluate system. Using an immunofluorescence assay, robust fast constitutive internalization was detected. Already after 10 min, the first clusters of internalized receptors were observed in the proximity of the cell membrane, and a strong intracellular accumulation of receptors was found after 2 h. Interestingly, after 2 h, only 50–60% of cells expressing GABA<sub>B</sub> receptors displayed intracellular accumulation of receptors (not shown), and its extent varied among cells as well as experiments. However, after blocking lysosomal activity, we observed in almost the entire population of cells expressing GABA<sub>B</sub> receptors a pronounced accumulation of receptors. This observation indicates that in some cells, targeting of receptors to lysosomes and subsequent degradation may be very efficient, thus preventing the intracellular accumulation of a pool of endocytosed receptors.

Endocytosed GABA<sub>B</sub> receptors were found to accumulate in an intracellular compartment before being degraded. Since internalized receptors did not co-localize with the lysosomal marker protein Lamp 1, accumulation of internalized GABA<sub>B</sub> receptors did not take place in lysosomes but most likely in an endosomal sorting compartment. This observation suggests that sorting of internalized receptors to lysosomes is a relatively slow process in most cells, which leads to the accumulation of endocytosed receptors. Even after blocking lysosomal degradation with chloroquine, endocytosed GABA<sub>B</sub> receptors were not detected in lysosomes. However, chloroquine does not only inhibit proteolytic activity by enhancing the acidic pH of lysosomes; it also interferes with endosomal sorting and recycling (30, 31). Therefore, in the presence of chloroquine also sorting of GABA<sub>B</sub> receptors from an endosomal compartment to lysosomes appeared to be perturbed.

Quantification of GABA<sub>B</sub> receptor internalization by cell surface ELISA indicated that about 40–50% of cell surface receptors were endocytosed within 2 h. A similar amount of internalization was found using a cell surface biotinylation assay, where directly the amount of intracellularly accumulated receptors was determined in the presence of chloroquine to prevent lysosomal degradation. Since both the antibody-based and the biotinylation-based assay yielded a similar extent of GABA<sub>B</sub> receptor endocytosis, it is unlikely that binding of the antibody to GABA<sub>B</sub> receptors triggered endocytosis. In contrast, high concentrations of antibodies seemed to stabilize GABA<sub>B</sub> receptors at the cell surface, as indicated by the lower extent of receptor internalization observed in the immunocytochemical assay and by cell surface ELISA. It may well be that the failure to detect GABA<sub>B</sub> receptor internalization in previous studies is due to the use of high antibody concentrations that inhibited endocytosis of the receptors.

In agreement with previous studies (16, 19, 20), no agonist-induced internalization of GABA<sub>B</sub> receptors was observed upon prolonged agonist treatment (10–120 min), irrespective of the method used. Thus, unlike many other G protein-coupled receptors, GABA<sub>B</sub> receptors display robust fast constitutive but no agonist-promoted internalization. However, with the methods used, we cannot exclude the possibility of very fast agonist-induced endocytosis of GABA<sub>B</sub> receptors coupled with rapid recycling back to the cell membrane. In this respect, it is interesting to note that a recent study analyzing GABA<sub>B</sub> receptor-induced internalization of calcium channels in cultured chick sensory neurons suggests the fast endocytosis of a complex of calcium channels, arrestin, and GABA<sub>B</sub> receptor 20 s after baclofen stimulation (33). Intracellular co-localization of calcium channels and arrestin with GABA<sub>B</sub> receptors strongly diminished already after 1 min, indicating a rapid and transient phenomenon. Further experiments are required to clarify the existence of a fast and transient agonist-induced endocytosis.

Endocytosis of GABA<sub>B</sub> receptors was found to be mediated predominantly via the dynamin- and clathrin-dependent pathway, as indicated by its inhibition after overexpressing a dominant negative mutant of dynamin (dynamin K44A) that has been shown to restrain invaginated pits from pinching off (25) and hypertonic concentrations of sucrose as well as chlorpromazine, both of which inhibit the formation of clathrin-coated
pits (26, 27). In addition, cell surface and internalized GABA_B receptors frequently co-localized with the β-adaptin subunit of the AP2 complex, which recruits plasma membrane proteins into clathrin-coated pits (30). This finding was further substantiated by co-immunoprecipitation experiments, which suggest a direct association of the AP2 complex with GABA_B receptors.

Although GABA_B receptors have been abundantly detected in lipid rafts upon expression in Chinese hamster ovary cells (34), a blocker of raft/caveolin-dependent endocytosis (nystatin and filipin (28, 29)) did not affect internalization of GABA_B receptors. In addition, we found that caveolin 1 and caveolin 2 only rarely co-localized with GABA_B receptors expressed in HEK 293 cells. Thus, caveolin-dependent endocytosis appears not to play a significant role in constitutive internalization of GABA_B receptors expressed in HEK 293 cells. Even an abundant localization of receptors in lipid rafts does not necessarily imply their internalization via a raft/caveolin-dependent mechanism, as recently demonstrated for the apolipoprotein E receptor 2. Although apolipoprotein E receptor 2 has been shown to be associated with lipid rafts and caveolin 1, the receptor was endocytosed via the clathrin-mediated pathway (35). In line with this observation, G protein-coupled receptors have been found to predominantly use the clathrin-dependent endocytic pathway, whereas caveolin and clathrin/caveolin-independent pathways appear to be used to a minor extent (24, 36).

Once internalized, GABA_B receptors accumulate in a Lamp 1-negative endosomal sorting compartment (see above) before being degraded. Degradation of internalized GABA_B receptors was detected as a strong decrease of antibody labeling 1 h after internalization, which was completely prevented by the addition of chloroquine to inhibit lysosomal degradation. The view of lysosomal degradation of internalized GABA_B receptors was further supported by a direct inhibition of lysosomal proteases with either leupeptin or pepstatin A, which led to an increased intracellular accumulation of endocytosed GABA_B receptors. Further experiments are required to demonstrate whether GABA_B receptors indeed accumulate in lysosomes upon inhibition of lysosomal proteases. In contrast, the proteasome appears not to significantly contribute to the degradation of GABA_B receptors, since inhibition of proteasomal activity with MG 132 or lactacystin did not result in an enhanced intracellular accumulation of endocytosed GABA_B receptors. Thus, the data suggest that GABA_B receptors expressed in HEK 293 cells are endocytosed via the classical dynamin and clathrin-dependent pathway, accumulate in an endosomal sorting compartment, and are finally degraded in lysosomes.

It is currently unclear whether the mechanism of GABA_B receptor endocytosis and degradation detected in the present study using heterologously expressed GABA_B receptors also applies for neuronal GABA_B receptors. So far, data on primary cultured neurons indicate the lack of agonist-induced internalization (16, 19), which is well in line with the findings of the present study. However, using a biotinylation assay, Fairfax et al. (19) were unable to detect the build-up of an intracellular pool of endocytosed GABA_B receptors even after blocking lysosomal activity with leupeptin. Thus, they hypothesized that GABA_B receptors may be targeted directly from the surface to the proteasome for degradation. In contrast, the presence of classical GABA_B receptor internalization and degradation in lysosomes is well supported by recent immunohistochemical data. In neurons of the supraoptic nucleus, Richards et al. (37) detected GABA_B receptors in early endosomes and in lysosomes. Therefore, it is likely that neuronal GABA_B receptors follow similar mechanisms of internalization and possibly degradation, as revealed in the present study.

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