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European Journal of Pharmacology 481 (2003) 15–23
www.elsevier.com/locate/ejphar

Agonist-induced desensitization and endocytosis of heterodimeric $GABA_B$ receptors in CHO-K1 cells

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Received 27 March 2003; received in revised form 29 August 2003; accepted 3 September 2003

Abstract

γ-Aminobutyric acid B ($GABA_B$) receptor is the first discovered G protein-coupled receptor that requires two subunits, GB1 and GB2, to form a functional receptor. Whereas the molecular and functional characteristics of $GABA_B$ receptors have been recently extensively studied, the mechanisms underlying receptor desensitization and endocytosis are still poorly understood. We have investigated the effect of continuous agonist exposure on the human $GABA_B$ receptor functional response and redistribution when expressed in Chinese hamster ovary (CHO-K1) cells. The wild-type $GABA_B$ receptor-mediated inhibition of the adenylate cyclase activity appeared desensitized after 2 h in the presence of GABA (100 μM). Fusion proteins were generated by attachment of cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) to GB1 and GB2, respectively, and confocal microscopy experiments in intact living cells semi-stably expressing the constructs were performed. Incubation of co-expressing CFP–GB1 and YFP–GB2 cells in the presence of GABA (100 μM) for 2 h induced a profound receptor internalization, and CFP–GB1 and YFP–GB2 appeared co-localized in the endosome (labelled with Cy3-transferrin). The internalization was blocked by a selective $GABA_B$ receptor antagonist. These results represent the first clear visualization of agonist-induced internalization of the unique heterodimeric $GABA_B$ receptor.

Keywords: Desensitization; Endocytosis; $GABA_B$ receptor; Green fluorescent protein (GFP); Receptor dimerization

1. Introduction

γ-Aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the central nervous system. $GABA_B$ metabotropic receptors mediate slow inhibition by interacting with guanine-nucleotide binding proteins (G proteins) to inhibit adenylate cyclase activity, activate K^+ channels, and inactivate voltage-dependent Ca^{2+} channels (Billinton et al., 2001; Bormann, 2000; Bowery, 1993).

GB1 was initially identified by expression cloning (Kaupmann et al., 1997). However, the agonist affinity for cloned GB1 receptors was significantly lower than that of native receptors. Furthermore, GB1 failed to produce a functional $GABA_B$ receptor at the cell surface (Couve et al., 1998). An explanation for these findings was then provided by the discovery that the $GABA_B$ receptor exists as a heterodimer, with a companion protein (GB2) linked in a 1:1 stoichiometry to the GB1 through coiled-coil domains at C-termini (Jones et al., 1998; Kaupmann et al., 1998; Kuner et al., 1999; White et al., 1998). Thus, co-expression of GB1 and GB2 proteins results in the functional expression of $GABA_B$ receptor in plasma membrane, exhibiting a pharmacological profile equivalent to wild-type $GABA_B$ receptors in brain (White et al., 1998). In this regard, while the GB1 subunit has been implicated in ligand binding (Galvez et al., 2001, 2000; Malitschek et al., 1999; Margeta-Mitrovic et al., 2001a), the GB2 subunit has been implicated in G protein coupling of the receptor (Margeta-Mitrovic et al., 2001b; Robbins et al., 2001).

A range of approaches have recently provided evidence that G protein-coupled receptors can exist both as homo- and heterodimer complexes (Angers et al., 2002; Milligan and White, 2001; Rios et al., 2001). The precise implications of G protein-coupled receptor dimerization in receptor

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doi:10.1016/j.ejphar.2003.09.002

signaling have yet to be elucidated, nevertheless, the functional consequences of $GABA_B$ receptor heterodimerization are more profound since the co-expression of both GB1 and GB2 subunits is required for a fully functional $GABA_B$ receptor (Bowery and Enna, 2000).

Cellular responses to agonists are usually rapidly attenuated. This agonist-mediated loss of functional response, termed desensitization, is the consequence of a combination of different mechanisms, such as the uncoupling of the receptor from heterotrimeric G proteins in response to receptor phosphorylation, and the endocytosis of cell surface receptors to intracellular membranous compartments (Bohm et al., 1997; Ferguson, 2001). The $GABA_B$ receptor heterodimer represents a totally new principle of receptor processing and signal transduction in that two non-functional receptor proteins associate to form a functional G protein-coupled receptor. In this context, whether endocytosis and internalization processes are relevant to the heterodimeric $GABA_B$ receptor physiology remains to be established.

The aim of this work was to study the endocytic process of human $GABA_B$ receptors expressed in Chinese hamster ovary (CHO-K1) cells after agonist stimulation, and to elucidate the role of GB1 and GB2 subunits and their cellular trafficking in the internalization of $GABA_B$ receptor heterodimers. We constructed, and semi-stably expressed, GB1 and GB2 subunits that have a modified form of the 27-kDa green fluorescent protein (GFP) derived from *Aequorea victoria* added in-frame to their C-terminal tail. We used these constructs in intact living cells to directly visualize agonist regulation of the levels of the subcellular distribution of the $GABA_B$ receptor subunits.

2. Materials and methods

2.1. Materials

[^{3}H]cAMP was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). GABA and forskolin were from Sigma (St. Louis, MO). [*S*-(*R**,*R**)]-[3-[[1-(3,4-Dichlorophenyl)ethyl]amino]-2-hydroxypropyl](cyclohexylmethyl) phosphinic acid (CGP 54626A) was obtained from Tocris (Ellisville, MO). 3-Isobutyl-1-methylxanthine (IBMX) was from Calbiochem (San Diego, CA).

2.2. Construction of epitope-tagged and cyan fluorescent protein (CFP)/yellow fluorescent protein (YFP)-tagged forms of the $GABA_B$ receptor subunits

For epitope tagging of receptors, cDNAs encoding Myc and haemagglutinin (HA) epitopes were fused in-frame to the 5′ end of cDNAs encoding GB1 and GB2. In each case, the native signal sequence was removed and replaced with that for CD33 (Myc-GB1) or T8 (HA-GB2), (Simmons and Seed, 1988).

GB1 and GB2 were modified at their C-terminal tails by the addition of enhanced CFP and YFP, respectively, using standard polymerase chain reaction (PCR) technology, and incorporating the Clontech™ N-terminal enhanced fluorescent protein (FP) expression vectors.

2.3. Semi-stable transfection of CHO-K1 cells

Cells were seeded in six-well plates at 10^5 cell/plate and grown overnight. Transfections were achieved using 1 μg of the GB1 constructs and/or 2 μg of the GB2 constructs per well using LipofectAMINE reagent (Life Technologies, UK) according to the manufacturer's instructions. To generate the semi-stable cell lines, cells were grown in monolayer culture in Dulbecco's modified Eagle's medium (DMEM)/Hams F-12 (1:1) mix supplemented with 2 mM glutamine, 9% Foetal bovine serum, 400 μg/ml hygromicin, and 500 μg/ml geneticin. Cultured cells were maintained at 37 °C in a 5% CO_2/humidified air atmosphere and detached from the culture flasks for passaging every 3–5 days by brief (<2 min) incubation with trypsin (0.5 mg/ml) and EDTA (0.2 mg/ml) in phosphate-buffered saline (PBS).

2.4. Immunofluorescence studies of epitope-tagged receptors

Cells expressing HA-GB1 and/or Myc-GB2 were grown in a six-well plate on glass coverslips (which had been sterilized in absolute ethanol and air dried). The media was removed and the cells were fixed with 3% formaldehyde in PBS for 15 min. The coverslips were washed with 3 × 2 ml PBS before being treated for 10 min with 0.2% Triton X-100 to permeabilize the cell membrane to antibodies (if required). Coverslips were further washed with PBS and incubated in PBS containing 3% horse serum, 1% bovine serum albumin (blocking buffer) to reduce non-specific binding. Primary antibodies mouse anti-HA and rabbit anti-Myc (Cambridge Biosciences, UK) were added at dilutions 1:1000 and 1:200, respectively, and incubated for 60 min at room temperature. After washing with blocking buffer (3 × 2 ml), the cells were incubated with the secondary antibodies fluorescein isothiocyanate (FITC)-conjugated antimouse 1:200 and Cy3-conjugated antirabbit 1:1000 (Vector Laboratories, Burlingame, CA) for 60 min at room temperature. After further washing (PBS, 6 × 2 ml), the coverslips were dried and treated with anti-fade before being fixed onto glass slides. Slides were examined by laser scanning confocal microscopy (Zeiss LSM510). The images were analysed with Zeiss LSM510.

2.5. Visualization of CFP/YFP receptor chimeras in intact living cells

Cells were observed using laser scanning microscopy as described above. The CFP–GB1 and YFP–GB2 chimeras were excited using a 458- and 488-nm argon/krypton laser,

respectively. The band pass filters were 505–530 and 505–550 nm for CFP–GB1 and YFP–GB2 detection, respectively. Two different protocols for preparation of the cells were used. Before the studies in intact living cells, control experiments for FP-receptor expression and auto-fluorescence were performed. The cells on glass coverslips were fixed, washed and mounted onto glass slides as described above. When examining the effect of the agonist on $GABA_B$ receptor endocytosis, live cells were used. The cells were grown on glass coverslips and mounted on the imaging chamber. The cells were maintained in DMEM/HEPES and the temperature was controlled at 37 °C. Endosomes were detected with transferrin conjugated to Cy3 according to the manufacturer's instructions (Amersham Pharmacia Biotech, Buckinghamshire, UK). Living cells were incubated in the presence of 100 μM GABA for 2 h, and laser scanning was performed every 30 min. The imaging chamber was gently washed with DMEM/HEPES (37 °C) 5 min before scanning and, thereafter, fresh media containing both agonist, and Cy3-transferrin (0.2 μg/ml), were added.

2.6. Adenylate cyclase assay

CHO-K1 cells stably expressing wild-type $GABA_B$ receptors were grown to confluence in 24-well plates. In order to study the agonist-induced receptor desensitization, the cells were rinsed with DMEM/HEPES (2 × 2 ml) and preincubated in the absence, or in the presence of GABA (100 μM) for different times. After washing (DMEM/HEPES, 2 × 2 ml), cells were incubated for 15 min with 0.5 mM IBMX. Forskolin (10 μM, final concentration) was added with GABA at the appropriate concentration and incubated for 10 min at 37 °C in a final volume of 0.3 ml. The incubation was terminated by the addition of 10 μl of HCl (10 M) followed by 10 μl NaOH (10 M) and 200 μl of 1 M Tris–HCl, pH 7.4. The lysated cells were pelleted by centrifugation at 12,000 × *g*. The supernatant containing released cAMP was removed and stored at −20 °C.

cAMP was quantified through inhibition of the binding of 1 nM [^{3}H]cAMP to protein kinase A (PKA; Sigma, 5 μg per tube) and comparison with a standard calibration curve of known cAMP concentrations (10 μM–10 pM, 10 concentrations). The assay buffer contained 50 mM Tris–HCl, pH 7.4, 4 mM EDTA, 100 mM NaCl, and 0.5% bovine serum albumin. The reaction mixture was incubated for 2 h at 4 °C, and the incubation was terminated by filtration through polyethyleneimine-soaked GF/B filters in a Packard 96-tube Filtermate followed by three rinses with 1 ml of ice-cold distilled water. The filters were dried and counted in 50 μl Microscint O with a Canberra-Packard TopCount scintillation counter.

In order to test the effect of CGP 54626A on the ability of GABA to inhibit the forskolin-induced increase of cAMP levels, CHO-K1 cells stably expressing wild-type $GABA_B$ receptors were seeded in 96-well plates at 150×10^3 cells/well and allowed to reattach overnight. Cells were washed in PBS and preincubated in DMEM-F12 medium containing 300 μM IBMX for 45 min at 37 °C. Increasing concentrations of antagonist were added to cells, and plates were returned to 37 °C for 30 min. Forskolin (10 μM) and 1 μM of GABA were added and cells were incubated for a further 15 min. GABA potency was determined by construction of concentration–response curves carried out in the absence of antagonist. cAMP was measured following extraction with 0.1 mM ClH for 1 h at 4 °C. Assays were neutralized with 0.1 mM $KHCO_3$ and cAMP levels were determined using scintillation proximity assays (Biotrak Kit, Amersham Pharmacia), and were counted on Packard Topcount counter. The K_i values for CGP 54626A were estimated using the Cheng and Prusoff equation.

2.7. Data analysis

Statistical comparisons of the effect of the different preincubation times in the presence of GABA on the inhibition of forskolin-stimulated adenylate cyclase activity by $GABA_B$ receptors were performed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Further, to display the pharmacological parameters of agonist-induced inhibition of the forskolin-stimulated adenylate cyclase activity in GABA-preincubated and control cells, concentration–respose curves were performed, and their parameters were calculated by nonlinear regression analysis. The effect of GABA preincubation on the pharmacological parameters was analysed by two-way ANOVA. All mathematical calculations were done by using the computer software GraphPad Prism™. Data are expressed as mean ± S.E.M. of three different experiments performed in duplicate. The level of significance was chosen as $P = 0.05$.

All laser scanning confocal microscopy experiments were performed at least three times using different batches of cells.

3. Results

3.1. Expression of recombinant human $GABA_B$ receptor subunits in CHO-K1 cells

To initiate our studies of agonist-induced desensitization and endocytosis of $GABA_B$ receptors, we first investigated the subcellular localization of the GB1 and GB2 subunits in the absence of agonist stimulation. We introduced the HA- and Myc-epitope tags at the N-terminus of the GB1 and GB2 subunits, respectively. These constructs were semi-stably expressed in CHO-K1 cells and detected with FITC (anti-HA)- and Cy3 (anti-Myc)-conjugated antibodies by confocal microscopy. Visualization of fixed and detergent-permeabilized CHO-K1 cells expressing HA-GB1 receptors showed significant amount of the protein detected in intracellular compartments (Fig. 1A). When recombinant HA-GB1 receptors were immunodetected in non-permeabilized cells, no

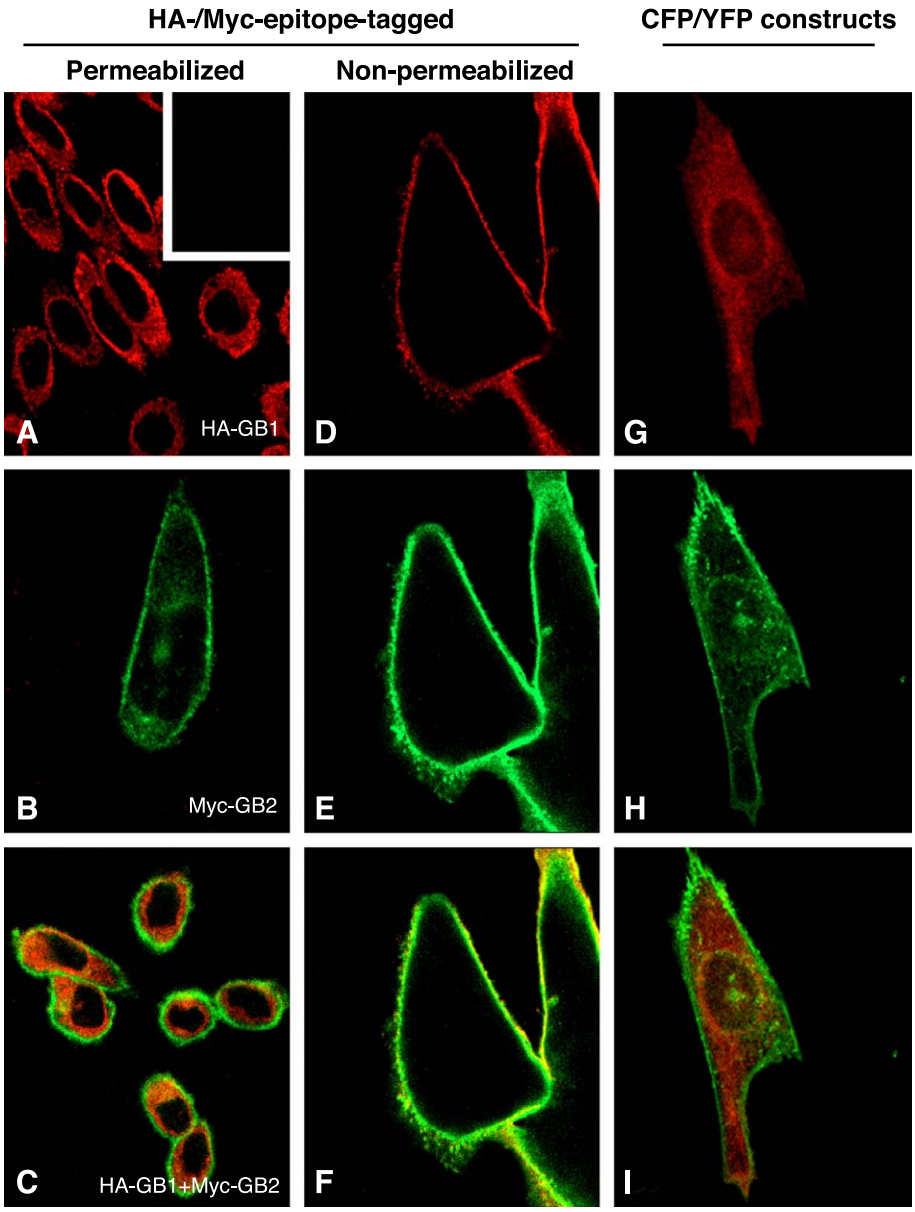

Fig. 1. Localization of GB1 and GB2 receptor subunits in CHO-K1 cells. (A–F) HA-GB1 and/or Myc-GB2 epitope-tagged receptors were semi-stably expressed and immunolocalized by confocal microscopy. To detect total cell epitope-tagged protein, cells were permeabilized prior to incubations with the primary (mouse anti-HA and rabbit anti-Myc) and the secondary (FITC-antimouse and Cy3-antirabbit) antibodies. To detect only cell surface-expressed epitope-tagged protein, cells were not permeabilized to the antibodies. (A) Permeabilized cells transfected only with HA-GB1 (red) (insert: non-permeabilized cells). (B) Permeabilized cells transfected only with Myc-GB2 (green). (C) Permeabilized cell co-transfected with HA-GB1 and Myc-GB2. (D–F) Co-localization of HA-GB1 and Myc-GB2 in the plasma membrane of non-permeabilized cells (D, HA-GB1; E, Myc-GB2; F, overlay). (G–I) CFP–GB1 and YFP–GB2 constructs semi-stably co-expressed and detected by confocal microscopy (G, CFP–GB1; H, YFP–GB2; I, overlay).

surface staining was shown in intact CHO-K1 cells (Fig. 1A, inset). On the contrary, when Myc-GB2 receptor was heterologously expressed, both membrane and cytoplasm labelling was detected (Fig. 1B). The co-expression of HA-GB1 and Myc-GB2 subunits of the $GABA_B$ receptor showed the GB2-mediated trafficking of GB1 to the plasma membrane (Fig. 1C). In order to confirm this plasma membrane localization of HA-GB1 when co-expressed with Myc-GB2, only cell surface-expressed receptors were visualized in non-permeabilized cells (without Triton X-100 incubation, see Section 2). Both HA-GB1 and Myc-GB2 were detected in the plasma membrane of non-permeabilized cells (Fig. 1D–F). HA-GB1 receptors were not detected in the cell surface of non-permeabilized cells in the absence of Myc-GB2 (see above). Similar results were obtained in the neuroblastoma × glioma hybrid cell line NG108 cells (data not shown).

In order to study the protein trafficking of the two $GABA_B$ receptor subunits after agonist activation in intact

living CHO-K1 cells, we linked the cDNAs encoding two different variants of the GFP from *A. victoria*, and either GB1 or GB2 receptor subunits. These fusion proteins were anticipated to encode single open reading frames in which the C-terminus of the $GABA_B$ subunit was directly ligated to the N-terminus of the respective fluorescent protein. Thus, we synthesised CFP–GB1 and YFP–GB2 chimeras that were transiently expressed and detected by confocal microscopy in fixed cells to control the expression pattern and the autofluorescence of the constructs. The subcellular localization of the chimeric proteins did not differ to that described above for epitope-tagged receptors. Thus, only YFP–GB2 was detected in the cell surface when the constructs were expressed individually (data not shown). Moreover, in cells co-expressing the two constructs, both CFP–GB1 and YFP–GB2 were detected in the plasma membrane (Fig. 1G–I). In this regard, confocal microscopy performed in CHO-K1 cells co-expressing CFP–GB1 and YFP–GB2 grown on a glass coverslip demonstrated substantial amounts of the YFP–GB2-derived fluorescence to be plasma membrane delineated (Fig. 1H). Both fluorescent chimeric subunits were detected co-localized at the cell surface (Fig. 1I), with CFP–GB1 showing a significant signal in the intracellular milieu (Fig. 1G). In the absence of agonist, discrete intracellular vesicles were detected in CHO-K1 cells expressing either GB2 (Fig. 1B), or $GABA_B$ receptor heterodimers (Fig. 1C/I), where both subunits appeared as co-localized. These results demonstrate that the addition of FP to the C-terminal tails of GB1 and GB2 does not significantly alter the unique subcellular localization and trafficking of these proteins, allowing their use in functional studies.

3.2. Desensitization of $GABA_B$ receptor

In order to investigate the effect of chronic agonist activation on $GABA_B$ receptor desensitization, we analysed the ability of GABA to inhibit the forskolin-induced increase of cAMP level in intact CHO-K1 cells stably expressing wild-type $GABA_B$ receptors with or without preincubation with 100 μM GABA for 1, 2, and 6 h. As shown in Fig. 2A, 10^{-7} M GABA caused an inhibition of forskolin-stimulated cAMP accumulation, which was subsequently reduced in a time-dependent manner by the preincubation with 100 μM GABA [$F(3,8)=8.11$, $P<0.01$]. This reduction reached the level of statistical significance at 2 h (Dunnett's post hoc test).

GABA reduced forskolin-stimulated cAMP levels in a concentration–response manner (Fig. 2B). Preincubation of the $GABA_B$-CHO-K1 cells with 100 μM GABA for 2 h shifted the concentration–response curve to the right [log EC_{50} values: vehicle-pretreated cells, -7.13 ± 0.11; GABA-pretreated cells, -6.47 ± 0.15; $F(3,56)=8.45$, $P<0.001$] (Fig. 2B). These results demonstrate that the functional coupling of $GABA_B$ receptors to adenylate cyclase was desensitized by preincubation with agonist.

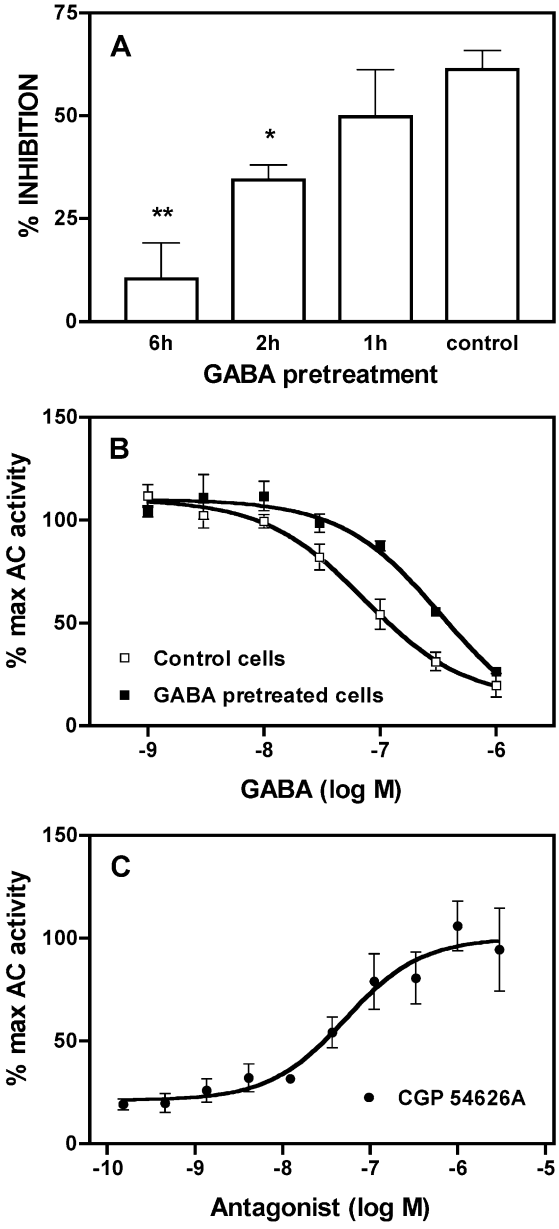

Fig. 2. Desensitization of the wild-type $GABA_B$ receptor-mediated inhibition of the adenylate cyclase (AC) activity after continuous agonist exposure in intact cells. (A) CHO-K1 cells stably expressing $GABA_B$ receptors were preincubated for the indicated times in the presence of GABA (100 μM). The effect of 10^{-7} M GABA on the adenylate cyclase activity was calculated as shown in Section 2. (*$P<0.05$, **$P<0.001$, Dunnett's post hoc test of one-way ANOVA). (B) Concentration–response curves of GABA inhibiting the adenylate cyclase activity after the preincubation of the cells in the absence (□) or in the presence (■) of GABA (100 μM) for 2 h. Data were normalized to the cAMP levels obtained in the absence of GABA. (C) Concentration–response curve of the $GABA_B$ receptor antagonist CGP 54626A blocking the GABA-mediated inhibition of the adenylate cyclase activity. Data were normalized to the estimated maximal cAMP levels. Data are means ± S.E.M. of three experiments performed in duplicate.

The selective $GABA_B$ receptor antagonist CGP 54626A blocked the GABA-mediated inhibition of the forskolin-stimulated cAMP levels in a concentration–response man-

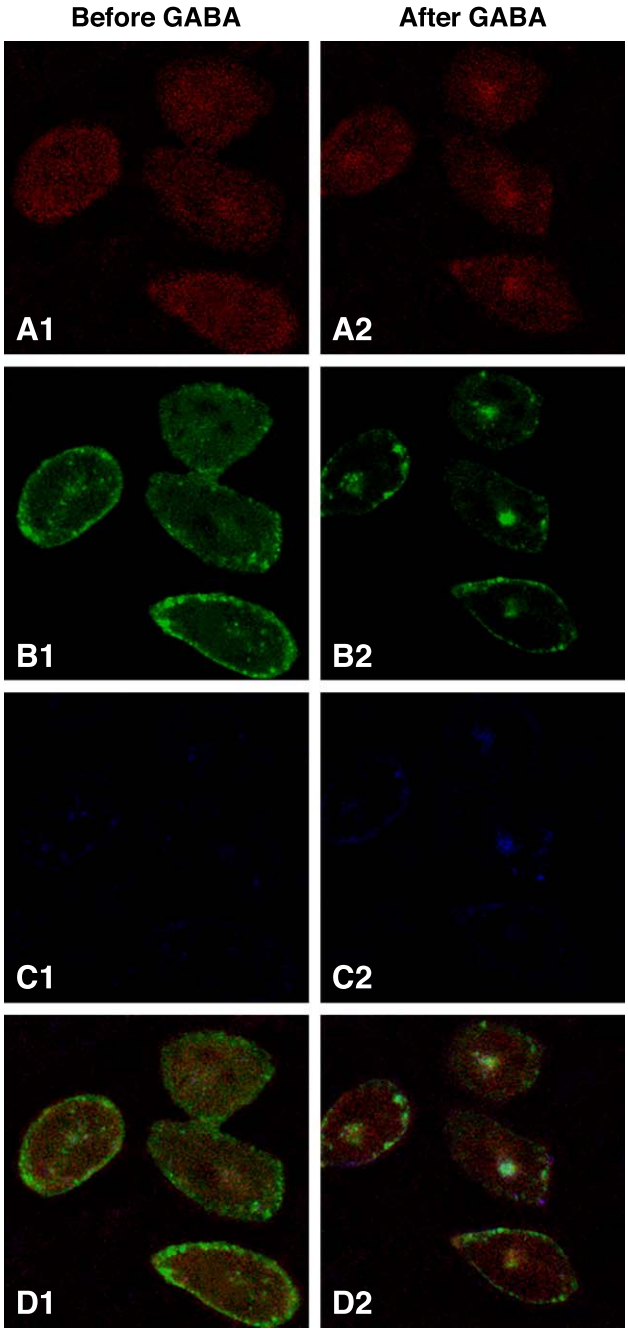

Fig. 3. Agonist-induced endocytosis of $GABA_B$ receptors in CHO-K1 cells. Intact living cells semi-stably co-expressing CFP–GB1 (red) and YFP–GB2 (green) constructs were imaged in the confocal microscope and the endosomes were detected with Cy3-transferrin (blue) as shown in Section 2. (A) CFP–GB1; (B) YFP–GB2; (C) Cy3-transferrin; (D) overlay; (1) before GABA; (2) after GABA (100 μM, 2 h).

ner (Fig. 2C). Parallel control GABA concentration–response curves inhibiting forskolin-stimulated cAMP levels were performed yielding EC_{50} values that were not different to that calculated above in vehicle-pretreated cells (log EC_{50}: -7.34 ± 0.64). Thus, the K_i for CGP 54626A was estimated (log K_i: -8.56 ± 0.68). These results demonstrate that the GABA-induced inhibition of adenylate cyclase activity in $GABA_B$-CHO-K1 cells is mediated by $GABA_B$ receptors.

3.3. Endocytosis of $GABA_B$ receptor

Internalization of G protein-coupled receptors is proposed to be a mechanism for receptor desensitization. Therefore, we studied the endocytic mechanisms in living CHO-K1 cells semi-stably co-expressing the cDNA constructs CFP–GB1 and YFP–GB2 using confocal microscopy as described in Section 2. The cellular endosomes were immunodetected with Cy3-transferrin. In unstimulated cells expressing both $GABA_B$ receptor subunits, a relatively slow endocytosis of receptors from the surface to endosomes was detected (Fig. 3). This basal recycling of the receptor proteins was also shown for the epitope-tagged $GABA_B$ receptors (see above) and in CHO-K1 cells stably expressing wild-type $GABA_B$ receptors (data not shown). In the presence of 100 μM GABA, the rate of endocytosis increased dramatically, and significant amounts of CFP–GB1 (Fig. 3A1 and A2) and YFP–GB2 (Fig. 3B1 and B2) were trafficked intracellularly, co-localizing in a discrete endosome together with Cy3-transferrin (Fig. 3C1 and C2). Thus, both $GABA_B$ receptor subunits were internalized together to the endosome (Fig. 3D1 and D2), and the fluorescence detected at the plasma membrane was decreased after 2 h of agonist stimulation (Fig. 3). Similar receptor redistribution was obtained in experiments performed without Cy3-transferrin labelling of the endosomes (data not shown).

When experiments were carried out in the presence of the selective $GABA_B$ receptor antagonist CGP 54626A (1 μM), GABA (100 μM) did not induce receptor endocytosis much above basal levels (Fig. 4). This result confirmed the agonist-dependent mechanism of $GABA_B$ receptor endocytosis.

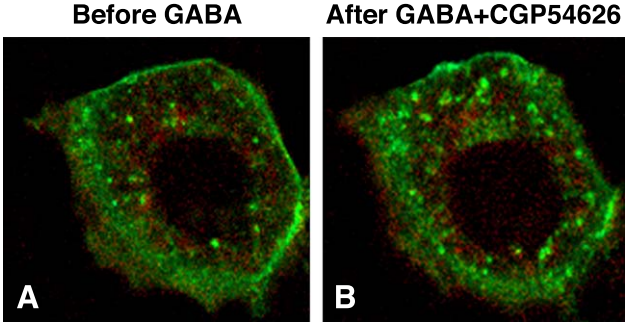

Fig. 4. Antagonist blockade of the agonist-induced endocytosis of $GABA_B$ receptors in CHO-K1 cells. Intact living cells semi-stably co-expressing CFP–GB1 (red) and YFP–GB2 (green) constructs were imaged in the confocal microscope before (A) and after (B) 2 h in the presence GABA (100 μM) and the selective $GABA_B$ receptor antagonist CGP 54626A (1 μM).

4. Discussion

In order to respond to the rapid physiological changes in agonist concentration, activated G protein-coupled receptors must be inactivated and then restored for stimulation. The acute regulation at the level of receptor, termed desensitization, is characterized by the waning of a stimulated response in the presence of continuous agonist exposure. An important aspect of the receptor desensitization process is the endocytosis of agonist-activated receptors into the intracellular membrane compartments of the cell (Tsao et al., 2001).

The generation and expression of fusion proteins containing modified forms of the GFP from *A. victoria* have recently revolutionized protein imaging studies and provided a powerful tool to study the kinetics and regulation of protein distribution and trafficking in intact living cells (Kallal and Benovic, 2000). In this regard, the study of ligand-induced G protein-coupled receptor endocytosis has been actively imaged in real time after expression of forms of receptors with FP attached to their C-terminal tails (Barak et al., 1997; Bremnes et al., 2000; McLean et al., 1999; Tarasova et al., 1997). In the present study, we have constructed and semi-stably expressed C-terminally FP-tagged forms of the $GABA_B$ receptor subunits GB1 and GB2 in CHO-K1 cells to directly study the effect of agonist activation on receptor redistribution. Although it might be anticipated that attachment of a 27-kDa polypeptide could significantly interfere with $GABA_B$ receptor function, it has been reported that the modified β2-adrenoceptor displays essentially unaltered pharmacology, and interacts with G proteins to initiate second messenger regulation (McLean et al., 1999). Furthermore, agonist-induced internalization has been recorded for a range of such constructs. Nevertheless, further work is necessary to completely characterize the effect of the attached FP to the $GABA_B$ receptor physiology. Since the C-terminal tails of the $GABA_B$ receptor subunit have been implicated in important functional characteristics of the receptor such as heterodimerization (Kammerer et al., 1999), intracellular retention of GB1 (Margeta-Mitrovic et al., 2000), and direct interaction with transcription factors (White et al., 2000), the localization of the fluorescent chimeras was compared to that obtained with epitope-tagged receptors. Our results confirmed that, as previously demonstrated in several systems (Jones et al., 1998; Kaupmann et al., 1998; Kuner et al., 1999; White et al., 1998), GB1 is retained in the cytoplasm when expressed alone and trafficked to the plasma membrane by GB2 (Margeta-Mitrovic et al., 2000). Thus, extracellularly exposed epitopes were detected at in the plasma membrane in non-permeabilized cells only when HA-GB1 was co-expressed with Myc-GB2. On the contrary, GB2 trafficked to the cell surface independently of GB1. This pattern of distribution observed for epitope-tagged receptors was not altered by the linkage of CFP/YFP to the C-terminus of the $GABA_B$ subunits.

Whereas the heterodimerization and activation mechanisms of the $GABA_B$ receptor have been extensively studied in recent years (Hammond, 2001), little is known about the receptor dynamics to continuous agonist exposure. We first studied the functional response of wild-type $GABA_B$ receptors after different lengths of agonist exposure. Our results showed that only after 2 h in the presence of the agonist, the $GABA_B$ receptor displayed a significant desensitization of cAMP level regulation in intact cells. In this regard, the concentration–response curves of GABA inhibition forskolin-stimulated adenylate cyclase activity were shifted to the right after 2 h in the presence of the agonist. In this context, Couve et al. (2002) have recently reported that repeated application of short pulses of the $GABA_B$ receptor agonist baclofen for 20 min resulted in a pronounced reduction in the activation of K^+ channels in rat hippocampal neurons, this receptor desensitization being reduced by PKA phosphorylation. Since the reported time frames over which other G protein-coupled receptor phosphorylation and uncoupling from G proteins by arrestins occur usually range within seconds (Ferguson, 2001), and since $GABA_B$ receptor phosphorylation has been shown to reduce the desensitization process (Couve et al., 2002), we studied the endocytosis properties of human $GABA_B$ receptors to determine whether agonist-induced receptor internalization could be involved in the desensitization that we have observed in CHO-K1 cells.

Semi-stable co-expression of CFP–GB1 and YFP–GB2 and monitoring of their intrinsic fluorescence in non-stimulated living cells indicated the presence of both subunits colocalized in discrete intracellular vesicles. Receptors for transferrin capture the nutrient at the cell surface and endocytose to enable the nutrient to dissociate from the receptor in the acidic environment of the endosomes (Koenig et al., 1998). Therefore, we used Cy3-transferrin to detect the endosomes in the living cells. The location of the $GABA_B$ receptor subunits together with transferrin demonstrated the existence of a basal rate of endocytosis. This basal endocytosis, together with the constitutive recycling of the receptors, controls the proportions between the cell surface- and the intracellular-located receptors (Koenig and Edwardson, 1997). Addition of GABA produced a substantial redistribution of CFP–GB1 and YFP–GB2 subunits from the plasma membrane to a discrete cellular endosome, which could be monitored in intact cells and in real time. Thus, after 2 h in the presence of GABA, the fluorescence of the co-internalized subunits appeared as being increased in the endosome, and significantly decreased in the plasma membrane delineated signal. The involvement of G protein activation and second messenger generation in the triggering of endocytosis remains controversial (Szekeres et al., 1998b). Indeed, it has been reported that an antagonist at the cholecystokinin receptor can stimulate internalization (Roettger et al., 1997). The blockade of agonist-induced receptor internalization observed following exposure with the antagonist CGP 54626A demonstrated that receptor activation, and not only ligand binding, was required for the internalization of the $GABA_B$ receptor.

G protein-coupled receptor internalization was originally considered to be a primary mechanism of receptor desensitization. However, it has been reported that pharmacological treatments that block receptor internalization do not alter the desensitization profile of β2-adrenoceptors (Pippig et al., 1995) or muscarinic receptors (Szekeres et al., 1998a). Moreover, it has been observed that internalization plays a role in the resensitization of receptor responsiveness (Szekeres et al., 1998a; Zhang et al., 1997). In this work, we find that continuous agonist exposition induces, in a similar time frame, both receptor desensitization of the adenylate cyclase inhibition and internalization of the $GABA_B$ heterodimer from the plasma membrane. Although further work is necessary to completely characterize the time courses and causal relation between $GABA_B$ receptor endocytosis and desensitization, the observed results suggest that receptor internalization may play an important role in controlling $GABA_B$ receptor desensitization. Similar experiments using FP-receptor chimeras have been performed for several G protein-coupled receptors (Barak et al., 1997; Bremnes et al., 2000; McLean et al., 1999; Tarasova et al., 1997). Nevertheless, the rate at which receptors internalize seems to be receptor specific, showing important differences between closely related receptors (Ferguson et al., 2000; Koenig and Edwardson, 1996; Smalley et al., 2001). Moreover, intrinsic activity of the ligands has been shown to correlate with their ability to increase receptor endocytosis (Szekeres et al., 1998b). Therefore, these kinetic differences may complicate comparison of the results obtained in different systems (Couve et al., 2002).

The present data represent the first clear visualization of the heterodimeric $GABA_B$ receptor becoming internalized in response to agonist activation. We found that both GB1 and GB2 subunits co-localize in endosomes after chronic agonist exposure, extending the knowledge of the functional and operational characteristics of this intriguing receptor. New physiological roles of receptor endocytosis in signal transduction are being discovered (Ferguson, 2001; Hall et al., 1999), therefore, the study of the $GABA_B$ receptor trafficking may help to identify novel signaling pathways and potential therapeutic targets.

Acknowledgements

JAK is grateful to the Isaac Newton Trust for the financial assistance for confocal microscopy facilities. Dr S.C. Sealfon is thanked for useful discussions. JGM was a recipient of a predoctoral fellowship from Spanish Ministry of Education.

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