Endocytosis of $GABA_B$ receptors modulates membrane excitability in the single-celled organism *Paramecium*

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Summary

$GABA_B$ receptors modulate swimming behavior in *Paramecium* by inhibiting dihydropyridine-sensitive Ca^{2+} channels via G-proteins. Prolonged occupancy of $GABA_B$ receptors by baclofen results in a decrease in $GABA_B$ receptor functions. Since changes in the number of cell-surface $GABA_A$ receptors have been postulated to be of importance in modulating inhibitory synaptic transmission in neurons, we have studied the cell-surface expression and maintenance of $GABA_B$ receptors in *P. primaurelia*. In this study, we use immunostaining in electron and confocal microscopy to demonstrate that constitutive internalization of $GABA_B$ receptors in *P. primaurelia* is mediated by clathrin-dependent and -independent endocytosis. Indeed, $GABA_B$ receptors colocalize with the adaptin complex AP2, which is implicated in the selective recruitment of integral membrane proteins to clathrin-coated vesicles, and with caveolin 1, which is associated with uncoated membrane invaginations. Furthermore, when endocytosis is blocked with hypertonic medium, cytosol acidification, filipin or with a peptide that disrupts the association between amphiphysin and dynamin, the effect of baclofen on swimming is increased. These results suggest that $GABA_B$ receptor endocytosis into clathrin-coated and -uncoated vesicles represents an important mechanism in the modulation of swimming behavior in *Paramecium*.

Key words: $GABA_B$ receptor, Endocytosis, Clathrin, Adaptin complex, Caveolin, Ciliated protozoa

Introduction

In *Paramecium*, the internal Ca^{2+} concentration increase coupled to membrane depolarization induces a reversal in the direction of ciliary beating and, consequently, a reversal in swimming direction (reviewed by Dryl, 1974; Machemer, 1988; Preston and Saimi, 1990). The duration of ciliary reversal is correlated to the amount of internal Ca^{2+}, and addition of drugs that block the Ca^{2+} current leads to a reduced duration of the backward swimming (Hennessey and Kung, 1984). We have found that baclofen modulates ciliary reversal by a G-protein (G_o or G_i)-mediated inhibition of dihydropyridine-sensitive Ca^{2+} channels (Ramoino et al., 2003). We have also observed that, in *Paramecium*, prolonged treatments with baclofen lead to a decreased function of $GABA_B$ receptors.

It is well known that in cultured neurons, prolonged occupancy of $GABA_A$ receptors by agonists results in a decrease in the density and function of the surface $GABA_A$ receptors, a process defined as downregulation (Tehrani and Barnes, 1991). Among the mechanisms proposed for modification of the $GABA_A$ receptor activity, one of the simplest is a change in the number of $GABA_A$ receptors on the surface membrane. It was found that internalization of muscarinic acetylcholine receptors, β-adrenergic receptors, AMPA receptors and $GABA_A$ receptors from the neuronal surface occurring in response to agonist exposure is mediated by clathrin-dependent endocytosis (Chuang et al., 1986; Silva et al., 1986; Tehrani and Barnes, 1997; Carroll et al., 1999). Clathrin-coated vesicles are the initial vehicles for sequestration of surface receptors, which are ultimately degraded or recycled.

Dynamin-dependent endocytosis was shown to be important in the regulation of cell surface levels of a number of integral membrane proteins, including opioid receptors (Chu et al., 1997), ionotropic glutamate receptors (Carroll et al., 1999) and β-adrenergic receptors in mammalian cells (Pitcher et al., 1998) as well as in the ciliated protozoon *Paramecium* (Wiejak et al., 2004a). Endocytosis of such membrane proteins involves a series of steps beginning with the clustering of receptors at specific sites of the plasma membrane, regions that later turn into clathrin-coated pits. Receptors do this by recruiting cytosolic AP2 adaptor complexes through their cytoplasmic tails. The protein-adaptor complex binds amphiphysin (Marks and McMahon, 1999), which is a key element of the

endocytotic machinery. Dynamin then self assembles into ring-like structures collaring the neck of the invaginating vesicles, an event that leads ultimately to their pinching off (Hinshaw and Schmid, 1995).

It was also evidenced that endocytosis of receptors may occur through other membrane structures, including noncoated membrane invaginations and caveolae (Tsao and von Zastow, 2001). The β_2-adrenergic receptor, which is endocytosed by clathrin-coated pits in several cell types (von Zastrow and Kobilka, 1994; Zhang et al., 1996), is endocytosed by membrane invaginations resembling to caveolae in other cells (Raposo et al., 1989; Dupree et al., 1993). Cholecystokinin receptors have been observed in both clathrin-coated pits and caveolae in the same cells (Roettger et al., 1995). Caveolae are cholesterol- and sphingolipid-rich smooth invaginations of the plasma membrane that partition into raft fractions and the expression of which is associated with caveolin 1.

This study is focused on mechanisms of GABA$_B$ receptor internalization in *Paramecium*. We provide evidence that constitutive internalization of GABA$_B$ receptors in *Paramecium* is mediated by clathrin-dependent and -independent endocytosis. Moreover, inhibition of endocytosis affects the ciliary reversal duration, resulting in an enhancement of GABA$_B$ receptor functions.

Results

Inhibition of KCl-evoked ciliary reversal by GABA$_B$ receptor activation decreases in response to agonist exposure

Membrane depolarization induced in *Paramecium* by external KCl triggers a ciliary reversal that can be divided into two phases: an initial continuous ciliary reversal (CCR), yielding the backward swimming, and a successive partial ciliary reversal (PaCR), giving rise to a spiral-like cell movement (Dryl, 1974). In this study we have only measured CCR. Baclofen, a GABA$_B$ receptor agonist, reduces the duration of CCR in a dose-dependent manner (Ramoino et al., 2003). At 100 μM, baclofen induces a 20% reduction of CCR duration. The effect of baclofen is cancelled after prolonged (60 minutes) exposure (Fig. 1a).

Inhibition of endocytosis increases the function of GABA$_B$ receptors

Baclofen-induced downregulation of the GABA$_B$ receptor in *Paramecium* cells might be due to an agonist-induced reduction in the number of receptors on the cell surface. Downregulation is counteracted by endocytosis inhibitors such as 150 mM sucrose (P<0.01) and 0.1 μg/ml filipin (P<0.01) (Fig. 1b). Furthermore, the block of endocytosis with cytosol acidification causes an increase in the reducing effect of baclofen on the duration of CCR (43%, P<0.01, Fig. 1d). A similar effect also occurs when clathrin-independent endocytosis is blocked by filipin (P<0.01) or when cells are treated with 50 μM P4 (P<0.01), a peptide that disrupts the association between amphiphysin and dynamin (Shupliakov et al., 1997; Wigge et al., 1997) (Fig. 1d). Baclofen alone inhibits 20% of CCR duration (Fig. 1a) and 47% or 45% when cells are preincubated with filipin (P<0.01) or the peptide P4 (P<0.01), respectively (Fig. 1d). Sucrose, that per-se antagonizes KCl-induced CCR duration, greatly improves the effect of baclofen (Fig. 1c). Baclofen addition after the inhibition of endocytosis with sucrose leads to a 65% reduction in the duration of CCR (P<0.01, Fig. 1d). Cytosol acidification and filipin show little effect on CCR duration in the absence of baclofen (Fig. 1c).

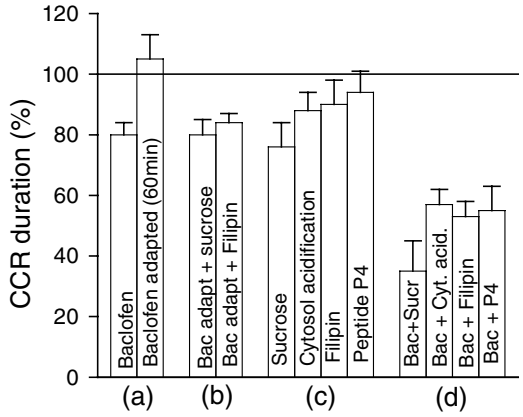

Fig. 1. Interference of the duration of CCR evoked by 40 mM KCl. (a) Baclofen (100 μM) causes a 20% inhibition of CCR (P<0.01) but its effect is abolished after long exposures (60 minutes). (b) Baclofen-induced downregulation of GABA$_B$ receptors is counteracted by sucrose (150 mM) and filipin (0.1 μg/ml); P<0.01 compared with baclofen adapted cells. (c) Effect on CCR by 150 mM sucrose, cytosol acidification (pH 5), 0.1 μg/ml filipin and 50 μM P4 peptide. (d) The inhibitory effect of baclofen is enhanced when cells are treated with 150 mM sucrose, 10 mM acetic acid (pH 5.0), 0.1 μg/ml filipin and 50 μM P4 peptide; P<0.01 compared with baclofen non-adapted cells. Tests were carried out on 15 cells and were repeated on four different occasions over several weeks.

GABA$_B$ receptor internalization is mediated by clathrin-coated pits

Constitutive endocytosis of GABA$_B$ receptors occurs in *Paramecium*, as shown by confocal and electron microscopy. The involvement of clathrin-coated vesicles in this process was visualized using immunofluorescence and confocal microscopy. Cells were double labeled with a guinea pig anti-GABA$_B$ receptor R1-subunit antibody and with a monoclonal anti-clathrin antibody and visualized with Alexa Fluor 594-conjugated anti-guinea pig and Alexa Fluor 488-conjugated anti-mouse secondary antibodies, respectively. Staining with an anti-clathrin antibody led to a punctuate pattern throughout the cytoplasm representing endocytic vesicles. The expression of GABA$_B$ receptors and clathrin-coated vesicles exhibited a clustered distribution on the cell membrane and inside the cytoplasm (Fig. 2a). Importantly, GABA$_B$ receptor and clathrin-coated vesicle clusters were partly colocalized (yellow fluorescence). Colocalized pixels are shown in blue in the 2D cytofluorogram reported in Fig. 2b. The left side of Fig. 3 shows the first (a), middle (b) and last (c) images of a 40-plane 3 μm thick z-stack. Shown on the right side of the same figure is the z-profile of the fluorescence intensity of three different double-stained vesicles of each plane. The similarity of green and red profiles demonstrates colocalization also along the z-axes. The colocalization percentage of GABA$_B$ receptors with proteins involved in the endocytosis is shown in Table 1.

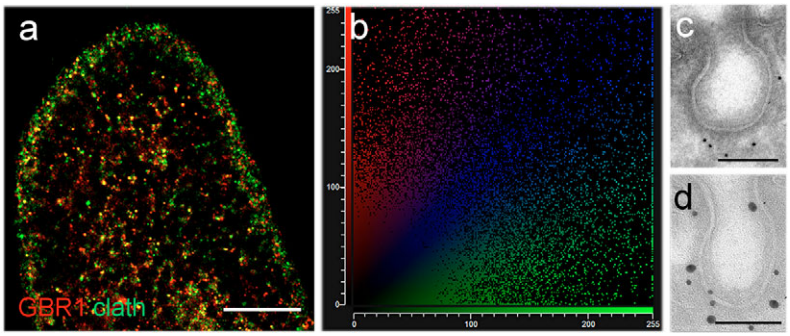

Fig. 2. (a) Colocalization of $GABA_B$ receptors and clathrin. In cells labeled with a polyclonal antibody against $GABA_B$ receptor (red) and a monoclonal antibody against clathrin HC (green), a clustered distribution of fluorescence is detected on the plasma membrane and inside the cytoplasm. $GABA_B$ receptors and clathrin vesicles are partly colocalized (yellow fluorescence). Bar, 10 μm. (b) 2D cytofluorogram. Colocalized pixels are visualized in blue. (c) Ultrastructural localization of $GABA_B$ receptors in *Paramecium* cells using 10 nm gold particles in electron microscopy. $GABA_B$ receptor immuno-analogue is present in coated pits. Bar, 100 nm. (d) Immunogold labeling in the transmission electron microscopy confirms the colocalization of $GABA_B$ receptors (15 nm gold particles) and clathrin (10 nm gold particles) in coated pits. Bar, 100 nm.

Immunogold labeling in the transmission electron microscopy confirms the colocalization of $GABA_B$ receptors and clathrin in coated pits (Fig. 2d) and shows that the receptors are localized on the cytoplasmic side (Fig. 2c). This subcellular localization is consistent with that observed in mammalian neuronal cells (Boyes and Bolam, 2003; Kulik et al., 2003). Treatment of cells with 150 mM sucrose or cytosol acidification significantly inhibited the internalization of receptors, as shown by the considerable reduction in receptors inside the cytoplasm (Fig. 4b,c) when compared with the control (Fig. 4a). This observation strongly suggests that $GABA_B$ receptor internalization in *Paramecium* is mediated by clathrin-dependent endocytosis. In these experiments phagocytosis was blocked by trifluoperazine, a calmodulin antagonist that inhibits the formation of food vacuoles (phagosomes) and stimulates endocytosis (Fok et al., 1985; Allen et al., 1992).

The clathrin adaptor protein AP2 colocalizes with the $GABA_B$ receptor

After observation of a significant clathrin-mediated endocytosis of $GABA_B$ receptors, we analyzed the colocalization between the receptor subunits and the endocytic adaptor complex AP2. Adaptor complexes have been implicated in the selective recruitment of integral membrane proteins into clathrin-coated pits (Schmid, 1997). AP2 is a key component of the endocytotic machinery that links cargo membrane proteins to the clathrin lattice, selects molecules for sorting into clathrin-coated vesicles and recruits clathrin to the plasma membrane (for reviews, see Kirchhausen, 1999; Kirchhausen, 2002; Takei and Hauke, 2001; Traub, 2003). It is composed of subunits α, β_2, μ_2 and σ.

The distribution of $GABA_B$ receptors with the adaptor complex was analyzed by using double immunolabeling in confocal and

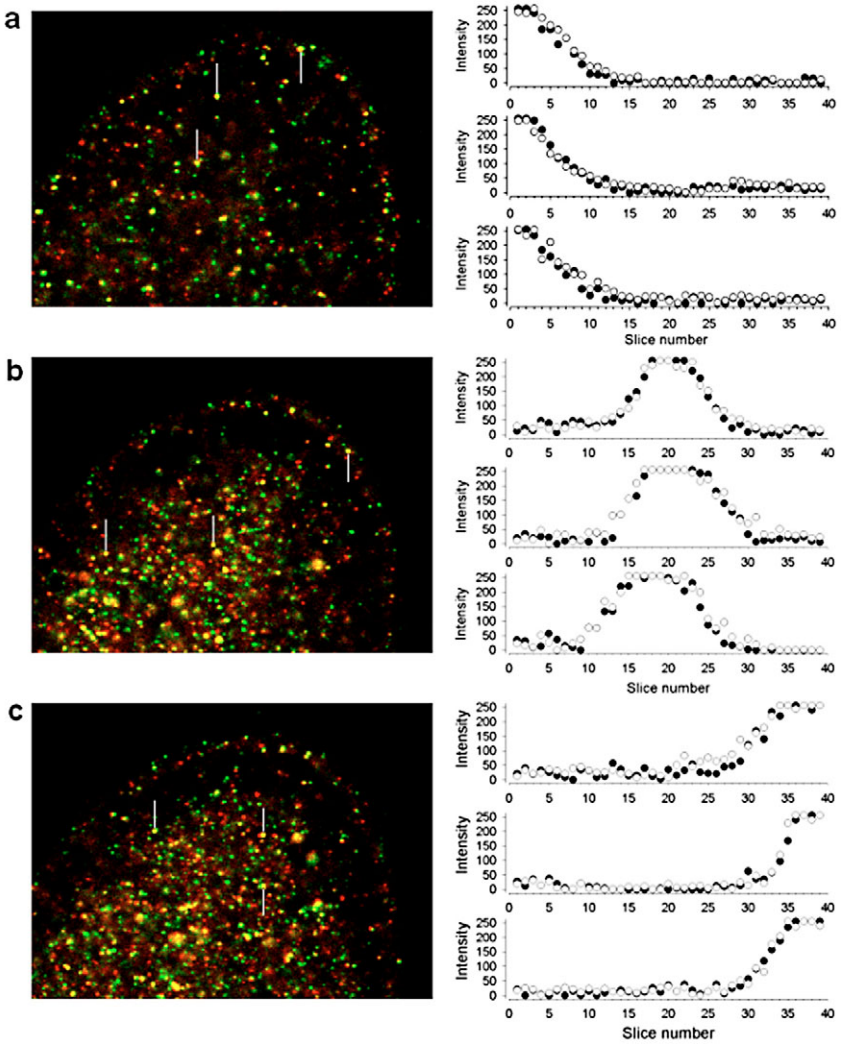

Fig. 3. *z*-Stack profile of fluorescence intensity of double-labeled vesicles. The left side of the figure shows three optical planes (a, first; b, middle; c, last) from a stack of 40 images (total thickness 3 μm). For each focal plane a sample of three vesicles that show colocalization (yellow) of $GABA_B$ receptor (red) and clathrin (green) is selected (white bars). The right side of the figure shows fluorescence-intensity distribution along the *z*-axis for each selected vesicle (green, ○), (red, ●).

Table 1. Colocalization of $GABA_B$ receptors with proteins involved in endocytosis.

Protein	Red vs green (%)	Green vs red (%)	Total
β Adaptin	27±5	25±6	20±5
Caveolin 1	21±6	25±4	17±5
Clathrin	18±3	19±3	12±4

$GABA_B$ receptors were always labeled with red fluorophores and proteins with green dyes. Every colocalization value is the average from four optical sections of ten cells. Data were calculated as the mean ± s.e.m. and are given in percent.

electron microscopy, and the antibody anti-adaptin β, which recognizes the β_2 subunit, i.e. the AP2 region binding to clathrin. In confocal microscopy the antibodies revealed clusters of fluorescence both on the cell surface and inside the cytoplasm (Fig. 5a). These clusters possibly show β_2 adaptin (green stain) in clathrin-coated vesicles on the plasma membrane and during their endocytic pathway, respectively. $GABA_B$ receptor clusters, colocalized with the AP2 complex, were detected on the plasma membrane as a yellow staining (Fig. 5a, Table 1). The colocalization in coated pits was evidenced by electron microscopy (Fig. 5c,d). On western blots of proteins derived from *Paramecium* cells, the adaptin β antiserum detected one band with an estimated molecular mass of ~105 kDa (Fig. 5e, line 1). This value is consistent with that obtained in Jurkat cells under the same experimental conditions (Fig. 5e, line 2).

$GABA_B$ receptor clathrin-independent endocytosis

Constitutive endocytosis of $GABA_B$ receptors in *Paramecium* also involves a clathrin independent mechanism. The presence of the immuno-analogue of caveolin-1, one of the defining components of caveolae in the plasma membrane, was detected using western blotting, electron and confocal laser scanning microscopy. On western blots of proteins derived from *Paramecium* cells (Fig. 6e, line 1) and from human endothelial lysate (Fig. 6e, line 2), the caveolin-1 antiserum recognized two bands with estimated molecular masses of ~24 and 21 kDa (Fig. 6e), corresponding to the α- and β-splicing forms, respectively (Scherer et al., 1995). When observed by confocal microscopy, caveolin-1 was revealed in distinct classes of

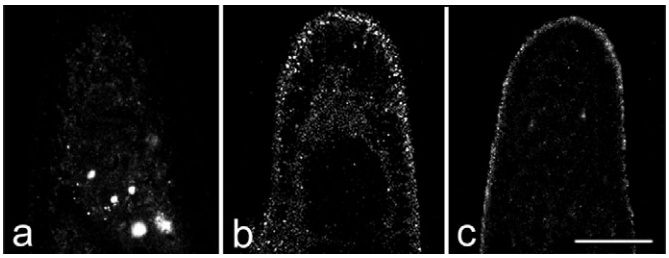

Fig. 4. $GABA_B$-receptor internalization is mediated by clathrin-coated vesicles. In cells whose phagocytic activity is blocked by trifluoperazine, 20-minute treatment with 150 mM sucrose (b) or cytosol acidification (c) inhibits receptor internalization, which can be seen by receptor accumulation on the cell membrane and receptor reduction inside the cytoplasm. Controls (a) are cells incubated with the anti-$GABA_B$ receptor antibody in the absence of inhibitors; the antibody is localized in endosomes and phagosomes. Incubation temperature, 25°C. Bar, 20 μm.

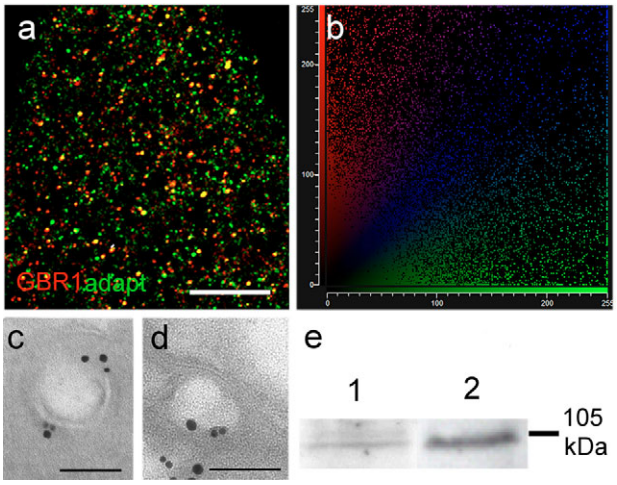

Fig. 5. $GABA_B$ receptors colocalize with the clathrin-adaptor protein AP2. (a) Cells labeled with an anti-$GABA_B$ receptor antibody (red) and an anti-adaptin β antibody (green). Colocalization of $GABA_B$ receptors and AP2 proteins is seen as a yellow fluorescence (image acquired at cell-surface level). Bar, 10 μm. (b) 2D cytofluorogram. Colocalized pixels are visualized in blue. (c,d) Colocalization of $GABA_B$ receptors (15 nm gold particles) and β_2 adaptin (10 nm gold particles) in electron microscopy. (d) Proteins from *Paramecium primaurelia* (line 1) and Jurkat cells (line 2) were subjected to SDS-PAGE and western blotting. A protein band with an estimated molecular mass of 105 kDa was detected with an anti-adaptin β antibody. The position of the molecular mass marker is shown on the right. Bars, 100 nm.

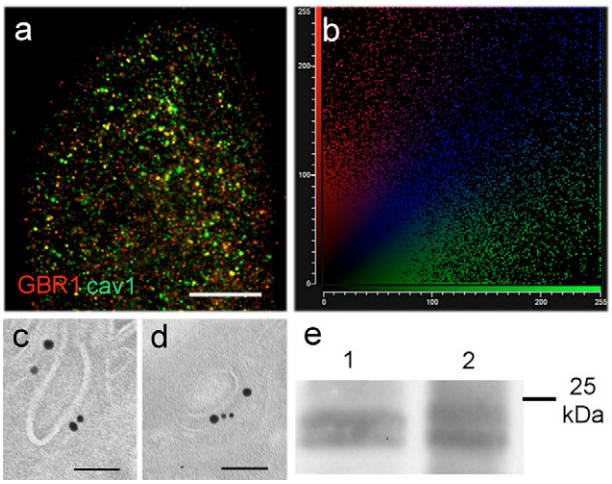

Fig. 6. $GABA_B$ receptors interact with caveolin 1. (a) Cells labeled with an anti-$GABA_B$ receptor antibody (red) and an anti-caveolin 1 antibody (green). Colocalization is seen as a yellow fluorescence. Bar, 10 μm. (b) 2D cytofluorogram. Colocalized pixels are visualized in blue. (c,d) Immunogold labeling in the transmission electron microscopy confirms the colocalization of $GABA_B$ receptors (15 nm gold partcles) and caveolin 1 (10 nm gold particles). Bars, 50 nm. (e) Proteins from *Paramecium primaurelia* cells (line 1) and human endothelial lysate (line 2) were subjected to SDS-PAGE and western blotting. Two protein bands with estimated molecular masses of 24 and 21 kDa were detected with an anti-caveolin 1 antibody, corresponding to the α and β splicing forms, respectively. The position of the molecular mass marker is shown on the right.

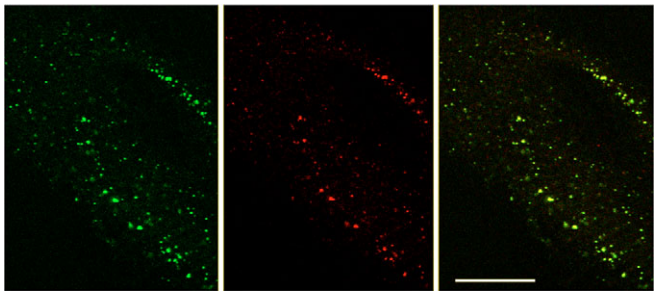

Fig. 7. $GABA_B$ receptors are internalized by clathrin-independent endocytosis. Cells blocked in their phagocytic activity by trifluoperazine (2.5 μg/ml for 15 minutes) were incubated for 5 minutes at 25°C with anti-$GABA_B$ receptor antibody (dilution 1:100) and dextran-coupled Texas-Red (0.02% w/v). Colocalization of $GABA_B$ receptors and dextran is seen inside the vesicles located on the cell membrane (yellow). Bar, 20 μm.

intracellular endosomes. Colocalization of $GABA_B$ receptors and caveolin-1 was observed by indirect immunofluorescence of endogenous caveolin-1 and $GABA_B$ receptors both on cell surface and throughout the cytoplasm (Fig. 6a, Table 1). A simultaneous localization of $GABA_B$ receptors and caveolin was also observed in the same pits and in vesicles, by using gold particles and electron microscopy (Fig. 6c,d).

To further assess whether $GABA_B$ receptors are internalized by clathrin-independent mechanisms, cells were incubated for various times both with the antibody anti-$GABA_B$ receptors and with dextran, a fluid-phase endocytosis marker. This revealed a colocalization of $GABA_B$ receptors and dextran in *Paramecium* cells (Fig. 7).

Moreover, when endocytosis was blocked by filipin (0.1 μg/ml) or by nystatin (2 μg/ml), sterol-binding agents that disrupt caveolar structure and function (Schnitzer et al., 1994), the receptor internalization decreased (Fig. 8a-e). In these experiments cells were incubated in the anti-$GABA_B$ receptor antibody for 30 minutes at 4°C (a temperature inhibiting phagosome and endosome formation) (Fok et al., 1984), so that receptors were accumulated on the cell membrane (Fig. 8a). After removal of the excess of antibody, cells were incubated at 25°C. 84% receptors were internalized in untreated cells after 20 minutes incubation at 25°C (Fig. 8e), as shown both by the reduction of cell membrane fluorescence intensity and by the fluorescence localization into endosomes and phagosomes (Fig. 8b). Only 37% and 46% fluorescence was internalized in filipin ($P<0.01$) and nystatin-treated cells ($P<0.01$), respectively (Fig. 8e). These results suggest that both endocytic processes (clathrin-dependent and/or -independent) occur in *Paramecium* and that the amount of receptors internalized through the two pathways is practically equivalent.

$GABA_B$ receptor internalization is enhanced by baclofen

When paramecia are incubated with the anti-$GABA_B$ receptor antibody, the initially formed endosomes are only a few, then they partly fuse with phagosomes (see Fig. 4a). To evidence the effect of agonists on receptor internalization, cells were incubated at 25°C for 5-10 minutes in the anti-$GABA_B$ receptor antibody with and without baclofen. Then, the total fluorescence inside the cell was measured. The intensity values in controls [281±16 arbitrary units (AU)] and in baclofen-

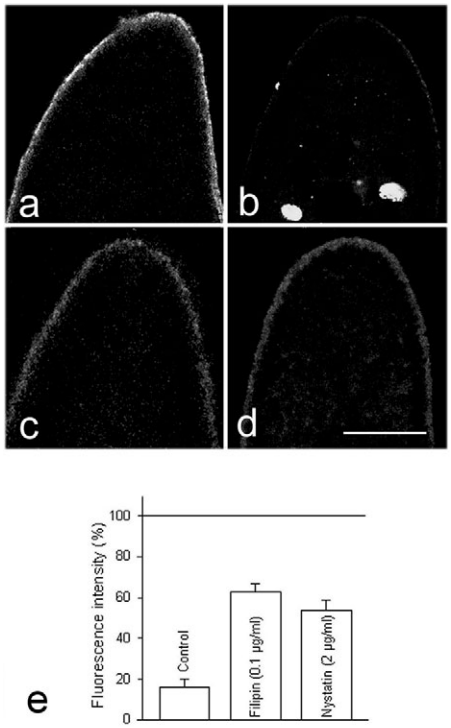

Fig. 8. $GABA_B$ receptors are internalized by non-coated endocytosis. Cells preincubated at 4°C for 30 minutes and labeled with anti-$GABA_B$ receptor antibody (dilution 1:100) for 30 minutes (a) are fixed after a 20-minute chase at 25°C in the absence (b) or in the presence of non-coated-pit endocytosis inhibitors filipin (c) and nystatin (d). (e) Measurement of the internalization representatively shown in b-d. Constitutive receptor internalization is partially inhibited by filipin (0.1 μg/ml) and nystatin (2 μg/ml) (47% and 38%, respectively); $P<0.01$, Student's *t*-test). Data were normalized to cells before internalization at 25°C (shown in a). Bar, 20 μm.

treated cells (868±33 AU) already differ after 5 minutes incubation with the antibody: baclofen addition leads to a 300% increase in the fluorescence intensity. In cells incubated for 15 minutes, fluorescence intensity in the baclofen-treated cells (1873±46 AU) was about 4.5 times of that of controls (396±25 AU).

Discussion

In this study, we are interested in understanding the endocytic properties of $GABA_B$ receptors in *Paramecium*. Although most G-protein-coupled receptors undergo endocytosis, the conditions and mechanisms of this process vary from receptor to receptor. Many of them are endocytosed by clathrin-coated pits, but some are not (for reviews, see Nichols and Lippincott-Schwartz, 2001; Johannes and Lamaze, 2002). Some have an agonist-induced endocytosis, some are continuously endocytosed even in the absence of stimulation, whereas others exhibit both a constitutive and a stimulated endocytosis (Dale et al., 2001; Nichols and Lippincott-Schwartz, 2001; Tsao and von Zastrow, 2001). Currently, very little is known about the targeting and trafficking mechanisms of $GABA_B$ receptor in cells. In the past few years, attention has mainly been focused on endocytosis of ionotropic GABA ($GABA_A$) receptors. It has been shown that $GABA_A$ receptors are internalized by a

clathrin-coated-pit-mediated process in hippocampal neurons and in A293 cells (Kittler et al., 2000) and in a clathrin-independent manner in HEK-293 cells (Cinar and Barnes, 2001). Using a dominant-negative dynamin construct K44A Herring et al. showed that constitutive endocytosis of $GABA_A$ receptors in HEK-293 cells is dynamin-mediated (Herring et al., 2003), whereas Cinar and Barnes found that it is dynamin-independent (Cinar and Barnes, 2001). It was also shown that both recombinant and neuronal $GABA_A$ receptors can constitutively recycle between the cell surface and an intracellular endosomal compartment (Connolly et al., 1999).

Here, we have demonstrated by immunogold and electron microscopy that $GABA_B$ receptor internalization in *Paramecium* occurs both at clathrin-coated pit and at caveolin-labeled invagination level, and that the localization of $GABA_B$ receptors is similar to that observed in mammalian cells. Furthermore, we have shown that $GABA_B$ receptors are removed from the plasma membrane by clathrin-dependent and -independent endocytosis. Indeed, internalization of receptors is blocked by hypertonic sucrose, a classic inhibitor of clathrin-mediated endocytosis (Heuser and Andersen, 1989). However, it has recently been found that sucrose inhibits $GABA_A$ receptor endocytosis that is not mediated by clathrin-coated pits (Cinar and Barnes, 2001). Therefore, we have also used cytosol acidification with acetic acid to inhibit clathrin-mediated endocytosis (Sandvig et al., 1987). Cytosol acidification inhibits clathrin-mediated endocytosis by interfering with clathrin-adaptor interactions (Hansen et al., 1993b), or by altering the structure of clathrin itself (Heuser, 1989; Hansen et al., 1993a). Furthermore, $GABA_B$ receptor internalization in *Paramecium* is blocked by the cholesterol-binding drugs filipin and nystatin. The sensitivity of endocytosis to nonacute cholesterol depletion with agents such as filipin and nystatin, distinguishes caveolae and raft pathways from clathrin-dependent and constitutive pinocytosis pathways (for a review, see Nabi and Le, 2003). In addition, the receptor endocytosis through a clathrin-independent pathway was also demonstrated by $GABA_B$ receptor internalization together with dextran-coupled Texas-Red in living cells. In *Paramecium* both clathrin-dependent and -independent internalization mechanisms are present (Ramoino et al., 2001) and a dynamin- and clathrin-dependent pathway has been observed (Wiejak et al., 2004b). Colocalization values reported in Table 1 and experiments carried out in living cells (Fig. 8e) suggest that $GABA_B$ receptors are internalized through the two pathways in a similar quantity.

To further elucidate the mechanism by which $GABA_B$ receptors are selectively recruited to clathrin-coated vesicles in *Paramecium*, we have investigated whether these receptors could associate with proteins implicated in the recruitment of integral membrane proteins to clathrin-coated vesicles. $GABA_B$ receptors were found to associate with the adaptor complex AP2. Indeed, $GABA_B$ receptors colocalize with β_2 adaptin in a number of sites on the plasma membrane.

A homologue of dynamin has recently been identified in *Paramecium* (Wiejak and Wyroba, 2002). A gene fragment of this dynamin reveals 74% similarity to human dynamin 2 mRNA and the deduced amino acid sequence shows 61.1% identity in a 175 amino acid overlap to the N-terminal region of human, mouse and rat dynamin. The cloned gene fragment encodes the conserved region of the GTP-ase domain including all three GTP-binding motifs: QSAGKSS, DLPG and TKLD (Surmacz et al., 2003). For the clathrin heavy chain and for the AP2 complex, sequences are cloned from *Paramecium* (International *Paramecium* genomic project) (Dessen et al., 2001).

Our results also suggest the presence of an agonist-induced internalization of $GABA_B$ receptors. To investigate the role of endocytosis in regulating the number of $GABA_B$ receptors, we analyzed the effects of loading cells with reagents that block endocytosis. Baclofen-induced downregulation of the $GABA_B$ receptor in *Paramecium* cells was blocked by sucrose and filipin, suggesting that the agonist-induced desensitization is due to an internalization-dependent reduction in the number of receptors. Furthermore, we loaded paramecia with a peptide that blocks endocytosis by disrupting the interaction between dynamin and amphiphysin, and with agents blocking clathrin- and caveolin-mediated endocytosis. This resulted in a significant 'run-up' in the effect of baclofen on the duration of CCR, consistent with an accumulation of surface $GABA_B$ receptors.

The agonist-induced $GABA_B$ receptor internalization is a still debatable. A basal rate of $GABA_B$ receptor internalization by clathrin-dependent endocytosis was demonstrated in Chinese hamster ovary (CHO) cells, in which the agonist-induced receptor internalization produced a substantial redistribution of receptors from plasma membrane to cellular endosomes (Gonzàlez-Maeso et al., 2003). A robust desensitization, induced by GABA and baclofen, in cerebellar granule cells endogenously expressing $GABA_B$ receptors was also reported (Perroy et al., 2003). Conversely, Fairfax et al. demonstrated that $GABA_B$ receptors are remarkably stable at the plasma membrane because they show little basal endocytosis and do not undergo agonist-induced phosphorylation or internalization in cultured cortical and hippocampal neurons (Fairfax et al., 2004). Furthermore, no agonist-promoted endocytosis or desensitization was observed in HEK293 cells heterologously expressing $GABA_B$ receptors (Perroy et al., 2003), whereas co-expression of $GABA_B$ heterodimer with the $GABA_A$ receptor γ2S subunit can confer to $GABA_B$ receptors the ability to internalize in response to agonist stimulation (Balasubramanian et al., 2004).

In conclusion, we have shown by western blotting, and immunodetection in confocal and electron microscopy the presence in *Paramecium* of caveolin 1 and β_2 adaptin, proteins involved in the first steps of endocytosis in mammalian cells. According to our results, $GABA_B$ receptors undergo constitutive endocytosis by clathrin- and caveolin-dependent mechanisms. This means that a system typical of mammalian neuronal cells is already present in the single-celled organism *Paramecium*, suggesting that a general mechanism has been maintained through evolution.

Materials and Methods

Antibodies

Monoclonal antibodies anti-clathrin (1:400), anti-adaptin β (1:1000), and anti-caveolin 1 (1:500) were purchased from Transduction Laboratory (BD Biosciences, San Jose, CA), the polyclonal guinea pig anti-$GABA_B$-receptor R1 antibody from Sigma (Steinheim, Germany) and the polyclonal goat anti-$GABA_B$-receptor R1 antibody from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); the secondary antibodies anti-guinea-pig Alexa Fluor 594, anti-goat Alexa Fluor 488 and anti-mouse Alexa Fluor 488 were obtained from Molecular Probes, Invitrogen (Carlsbad, CA).

The polyclonal guinea pig anti-$GABA_B$-receptor R1 antibody was developed

using an immunogenic synthetic peptide (PSEPPDRLSCDGSRVHLLYK) corresponding to the C-terminal amino acid sequence of the rat $GABA_B$ receptor common to both the $GABA_B$ 1a and $GABA_B$ 1b types. It was used in confocal and electron microscopy on fixed cells.

The polyclonal goat anti-$GABA_B$-receptor R1 antibody was raised against a peptide mapping at the N-terminus of $GABA_B$ R1α of rat origin; it was used for in vivo experiments.

Chemicals

Dextran-coupled Texas-Red was obtained from Molecular Probes, prestained molecular mass markers were obtained from Amersham (Buckinghamshire, England). All other chemicals were from Sigma when not otherwise specified in the text.

Cell culture

Paramecium primaurelia stock 90 was grown at 25°C in lettuce medium (pH 6.9) bacterized with *Enterobacter aerogenes*. Cells were harvested in mid-log phase of growth.

Behavioral studies

Cells were adapted for 30 minutes in a solution containing 1 mM CaCl2 and 1 mM HEPES (pH 7.2). They were then transferred into a test solution (adaptation solution plus 40 mM KCl) containing baclofen (100 μM). KCl in the test solution depolarizes the cell membrane, triggering ciliary reversal and backward swimming. Response to drugs was determined by forcing individual cells from a micropipette into a test solution. Cell responses were noted under low power magnification (12×), and then the duration of backward swimming was recorded with a stopwatch.

Inhibitors

Paramecia were treated with the following inhibitors of endocytosis: sucrose (Heuser and Andersen, 1989) 150 mM, cytosol acidification (10 mM acetic acid, pH 5.0) (Sandvig et al., 1987), nystatin (Rothberg et al., 1992) 2 μg/ml, filipin (Schnitzer et al., 1994) 0.1 μg/ml, the peptide P4 (QVPSRPNRAP; a kind gift of Harvey McMahon, Laboratory of Molecular Biology; Cambridge, UK) 50 μM.

Test of inhibitor effect

On backward swimming

Cells adapted for 15-30 minutes in an inhibitor-containing solution were tested in the depolarizing solution in the presence of both baclofen (100 μM) and inhibitors. Controls were non-adapted cells tested in absence of inhibitors.

On $GABA_B$ receptors

Cells blocked in their phagocytic activity by trifluoperazine (2.5-5 μM) for 15 minutes and pre-adapted in the inhibitors for 15-30 minutes were incubated at 25°C for 15-30 minutes in a culture medium containing the polyclonal antibody anti-$GABA_B$-receptor R1 and the inhibitor, then fixed and processed for immunolabeling. Controls were cells without inhibitor. In additional experiments, cells, pre-adapted at 4°C for 30 minutes, were incubated with the anti-$GABA_B$ receptor antibody for 30 minutes at 4°C, washed in a cold lettuce medium (Sonneborn, 1970), fixed after a chase of 20 minutes at 25°C with or without the inhibitors, and processed for immunolabeling.

Statistical analysis

Data are expressed as mean ± s.e.m. The significance of differences between means was valued by Student's *t*-test (GraphPad Prism, GraphPad, San Diego, CA). Statistical tests were performed on raw data, but to emphasize any changes in cell response to test solutions, the duration of backward-swimming was normalized to control values. Tests were carried out on 15 cells and were repeated on four different occasions over several weeks.

Immunolabeling for confocal microscopy

Cells were fixed in 4% paraformaldehyde in PBS buffer (0.01 M, pH 7.4) for 30 minutes, washed three times with PBS and incubated for 60 minutes with 3% bovine serum albumin (BSA) and 1% Triton X-100 in PBS. The blocking permeabilizing buffer was removed and the cells were incubated overnight at 4°C with both the polyclonal antibody against the $GABA_B$ receptor (1:2000 dilution) and one of the monoclonal antibodies. After three washes in 1% BSA in PBS plus 0.1% Triton X-100 for 10 minutes each, a cocktail of the secondary antibodies anti-guinea-pig IgG conjugated to Alexa Fluor 594 (dilution 1:300) and anti-mouse IgG conjugated to Alexa Fluor 488 (dilution 1:300) were applied for 2 hours at 37°C. After extensive washing with PBS, cells were mounted in glycerol-buffer.

In control experiments, the absence of cross-reactivity between the secondary antibodies was verified by omitting one of the primary antibodies during incubation. Moreover, for every combination of double labeling, single labeled vesicles were always observed in the cells.

Immunofluorescence staining with signal amplification

Caveolin 1 immunofluorescence was amplified by using the Alexa Fluor 488 signal amplification kit for mouse antibody (Molecular Probes). Cells removed from blocking buffer were incubated overnight at 4°C in primary antibodies (anti-caveolin 1 and anti-$GABA_B$ receptor). After three washes in 1% BSA in PBS plus 0.1% Triton X-100 for 10 minutes each, 10 μg/ml Alexa Fluor 488 rabbit anti-mouse (component A) and Alexa Fluor 594 goat anti-guinea-pig (dilution 1:300) were applied for 30 minutes at 37°C. Cells were then washed for three times with 1% BSA in PBS plus 0.1% Triton X-100 and incubated for 30 minutes at 37°C with 10 μg/ml Alexa Fluor 488 goat anti-rabbit (component B) and Alexa Fluor 594 goat anti-guinea-pig. After extensive washing with PBS cells were mounted in glycerol-buffer.

Confocal image acquisition

Images (512×512×8 bit) were acquired by a confocal laser scanning microscope Nikon C1 (Nikon Instr., Florence, Italy), mounted on an inverted optical microscope Nikon Eclipse TE 300. An argon-ion laser (488 nm, 514 nm) and a He-Ne laser (543.5 nm) provided the excitation beams. Emission was observed through the standard filter sets for fluorescein fluorescence (excitation, 488 nm; emission, 515-530 nm) and Texas-Red fluorescence (excitation, 543.5 nm; emission, 620 nm). Serial optical sections were taken through the cell at a *z*-step of 75 nm with a laser power of 1.5 mW and illumination attenuated by a 50% transmission neutral-density filter to reduce photobleaching, a 1 Airy unit pinhole diameter, and an oil immersion objective 100× /NA=1.3. The image acquisition through red and green channels was performed according to a time-sequential protocol to reject possible cross-talk artefacts. The software program EZC1 (Coord, Amsterdam, NL) was used for image acquisition, storage and analysis.

Labeling experiments were repeated three to four times and images are representative of observations of an average of 30 cells in each sample. Illustrations were prepared with PhotoShopPro 7.

Image analysis

Cells tested for the analysis of the inhibitory effects on receptor internalization were processed for the quantification of cell membrane fluorescence. Image processing was performed by home-made analysis routines (macro), integrated into the MATLAB (The MathWorks Inc, Natick, MA) platform. The total fluorescence inside the cell (using an average of 10 cells) was measured by using a threshold algorithm to assess the amount of green fluorescence per unit area. The amount of fluorescence was calculated as the mean ± s.d. and fluorescence intensity was expressed in arbitrary units (AU). The analysis of cell membrane fluorescence was carried out in 20 different areas (4 μm^2) from 10 cells. Data were normalized to cells before receptor internalization at 25°C.

The quantitative estimation of colocalized proteins was performed calculating the 'colocalization coefficients' (Manders et al., 1993). They express the fraction of colocalizing molecular species in each component of dual-color image and are based on the Pearson's correlation coefficient, a standard procedure for matching one image with another in pattern recognition (Gonzales and Wintz, 1987). If two molecular species are colocalized, the overlay of their spatial distribution has a correlation value higher then that expected by chance alone. Costes et al. developed an automated procedure to evaluate the correlation between the green and red channels with a significance level >95% (Costes et al., 2004). The same procedure automatically determines an intensity threshold for each color channel based on a linear least-square fit of the green and red intensities in the image's 2D correlation cytofluorogram. Costes' approach was accomplished by macro routines integrated as plugins (WCIF Colocalization Plugins, Wright Cell Imaging Facility, Toronto Western Research Institute, Canada) in the ImageJ 1.34f software (Wayne Rasband, Nat. Inst. of Health, USA). Sequential acquisition was performed to avoid cross-talk effects between the color channels.

Western blot analyses

Cells were centrifuged at 600 ***g*** to a density of 200×10^3 cells/ml and resuspended in water containing a protease inhibitor cocktail (Sigma). Samples were homogenized, sonicated and subjected to SDS-polyacrylamide gel electrophoresis followed by western blotting. 100 μg of *Paramecium* protein and 10 μg of Jurkat cell or human endothelial lysate per lane, as controls, were used. Prestained appropriate molecular mass markers were run concomitantly. Transfer to nitrocellulose (NC) membranes was performed electrophoretically at 100 V in 75 minutes. After saturation with 5% non-fat dry milk in TTBS buffer (0.9% Nacl, 0.1% Tween 20, 10 mM Tris pH 7.4), the NC membranes were incubated 1 hour with the primary antibodies mouse anti-adaptin β (1:5000) and mouse anti-caveolin 1 (1:5000). The membrane was incubated with horseradish-peroxidase-conjugated (sheep) anti-mouse 1:2000 in TTBS supplemented with 5% non-fat dry milk and coated using the ECL western blotting detection system for 1 minute. The membrane was immediately exposed to autoradiography film (Amersham) at room temperature for various periods (5 seconds to 60 minutes) in a film cassette.

Electron microscopy

For immunoelectron microscopy, 10% gelatine-embedded, 2.3 M sucrose-infused blocks of aldehyde-fixed *P. primaurelia* cells were frozen in liquid nitrogen. Ultrathin cryosections were obtained with a Reichert-Jung Ultracut E with FC4E

cryo-attachment and collected on copper-formvarcarbon-coated grids. Single and double immunogold localization on ultrathin cryosections was performed as described previously (Schiaffino et al., 1999). In particular, for double labeling, the sections immunostained with the anti-$GABA_B$ receptor antibody, followed by staining with protein A-gold (10 nm), were incubated with 1% glutaraldehyde in 0.1 PBS, to quench free protein A. Sections were then incubated alternatively with antibodies against clathrin, adaptin β or caveolin 1. After washing, an appropriate rabbit anti-mouse bridging-antibody was used (Dako), followed by staining with protein A-gold (15 nm). Control sections were incubated with an unrelated antibody or without first antibodies. To determine quenching efficiency, sections incubated with anti-$GABA_B$ receptor antibody were quenched with 1% glutaraldehyde in 0.1 M PBS, and challenged with protein A. No labeling was detected in any of the control sections. Sections were examined with a ZEISS EM 902 electron microscope.

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