Ectopically expressed γ-aminobutyric acid receptor B is functionally down-regulated in isolated lipid raft-enriched membranes

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Abstract

Lipid raft domains have attracted much recent attention as platforms for plasma membrane signalling complexes. In particular, evidence is emerging that shows them to be key regulators of G protein coupled receptor function. The G protein coupled γ-aminobutyric acid receptor B (GABA B receptor) co-isolates with lipid raft domains from rat brain cerebellum. In the present study, we show that the GABA B1a,2 receptor was also present in lipid raft domains when expressed ectopically in a Chinese hamster ovary cell line. Lipid raft-associated receptor was functionally active, displaying a concentration-dependent increase in GTPγS binding in response to the receptor agonist GABA. Compared with whole cell membranes, lipid raft-associated receptor displayed an increased EC50 and a reduced magnitude of response to GABA. We conclude that lipid raft association is an intrinsic property of the GABA B1a,2 receptor and is not cell-type specific. In addition, localisation to lipid raft domains may provide a mechanism to inhibit receptor function.

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Lipid rafts are specialised microenvironments within biological membranes that are enriched in cholesterol and sphingolipids. Interest in rafts has increased greatly since it was recognised that they are centres for receptor-mediated signalling. Lipid rafts concentrate subsets of membrane proteins and exclude others, and thereby act as platforms for the regulation of signalling systems during transport, functional signalling, and/or internalisation of associated components [1–5].

Evidence is accumulating that lipid raft domains are enriched for some G protein coupled receptors [6]. These receptors represent the third largest gene family in the human genome, with an estimated 1% of the genome coding for this receptor superfamily. They play key roles in a range of physiological processes including vision, taste, neurotransmission, and hormonal signalling. It is not surprising, therefore, that they are targets for the majority of pharmaceutical drugs, including therapeutic drugs and drugs of abuse [7].

The association of some G protein coupled receptors with lipid rafts is cell-type specific. The β2-adrenoreceptor, for example, associates with lipid raft-enriched domains in cardiac myocytes [8,9], but is largely excluded from raft membranes in vascular smooth muscle cells [10]. Further, when the human receptor is overexpressed in rat myocytes, it fails to localise with lipid raft domains [11]. Similarly, while the large majority of
adenosine A1 receptors reside in lipid raft fractions isolated from unstimulated cardiac ventricular myocytes [12], only a very small proportion of the receptor is present in lipid rafts isolated from epithelial cells [13].

The modulation of G protein coupled receptor signalling by lipid rafts does not follow a homogeneous pattern. Instead, the lipid raft environment seems to enhance signalling of some G protein coupled receptors, while it inhibits that of others. The G protein coupled gonadotropin-releasing hormone receptor, for example, displays an agonist-induced inositol trisphosphate response when it resides in rafts. However, this response is lost when rafts are disrupted using the cholesterol-binding agent cyclohexextrin [14]. Similarly, the chemokine receptor 5 loses its ligand-binding ability in response to lipid raft disruption [15]. On the other hand, signalling of the G protein coupled β2-adrenoceptor seems to be depressed by localisation to lipid rafts [8,9]. Although this receptor displays functional signalling properties whilst residing in cardiac myocyte lipid raft domains, perturbation of these domains by the raft disrupting drugs cyclohexextrin or filipin leads to enhanced receptor signalling as measured by prolonged and increased contraction rates. Thus, the effect of lipid raft association on G protein coupled receptor function is specific to each receptor, and may enhance the activities of some receptors while inhibiting those of others.

The metabotropic γ-aminobutyric acid receptor type B (GABA_B receptor), which is implicated in pathological conditions ranging from hypertension to epilepsy, is an example of a G protein coupled receptor [16,17]. The amino acid GABA is the major inhibitory neurotransmitter in the mammalian brain and is the ligand for both the ionotropic GABA_A and GABA_C receptors, and for the metabotropic GABA_B receptor. GABA_B receptors couple via G proteins to adenyl cyclase activity, leading to the opening of inwardly rectifying K⁺ channels and the closure of voltage-dependent Ca²⁺ channels. GABA_B receptors are unusual in that the homologous GABA_A1 and GABA_A2 subunits need to heterodimerise in order to reach the cell surface and form fully functional receptors. Different receptor splice variants have been identified and may distribute differentially in neurones, with immunohistochemical studies suggesting that GABA_A1a and GABA_A1b localise pre- and post-synaptically, respectively.

We have shown recently that the GABA_B receptor co-localises with lipid raft-enriched membranes isolated from rat brain cerebellum [18]. Since this was the first study published on the raft association of this receptor, it was not known whether raft localisation was intrinsic. Neither was it known whether the raft-localised receptor pool displayed functional signalling properties. In this current report, we set out to analyse whether the raft distribution of the GABA_B receptor was specific to rat cerebellum or could also be observed in Chinese hamster ovary (CHO) cells stably expressing the receptor. We also tested whether lipid raft association affects receptor signalling by measuring ligand-induced GTPγS binding both in whole membranes and in lipid raft-enriched fractions. We found that the GABA_B receptor associated with lipid rafts in CHO cells, suggesting that segregation into rafts is not cell-type specific but rather intrinsic to the GABA_B receptor itself. Furthermore, we found that, compared with whole membranes, the receptor displayed a reduced functional response to agonist binding in raft-enriched fractions.

**Materials and methods**

*Materials.* Polyclonal antibodies against GABA_A1a and GABA_A2 were kindly donated by Prof. G. Milligan (Division of Biochemistry and Molecular Biology, University of Glasgow, UK). Rabbit anti-Ga2 antibody was obtained from Calbiochem. Polyclonal anti-caveolin antibody and monoclonal anti-transferrin receptor antibody were purchased from BD Transduction Laboratories and Zymed Laboratories, respectively. [35S]GTPγS was purchased from Amersham.

*Cell line.* GABA_A1a and GABA_A2 receptor subunits were cloned into pcDNA3.1(-) (Invitrogen) as previously described [19]. The generation of the stable CHO-K1 cells expressing GABA_B heterodimeric receptors has been described previously [22,23].

*Solubility studies.* Cells were homogenised in ice-cold Mes-buffered saline (MBS; 25mM Mes, pH 6.5, and 0.15M NaCl) and a mixture of protease inhibitors by passing 12 times through a 27 gauge needle, and incubated at 4°C for 60min either with or without 1% (w/v) Triton X-100. Samples were subsequently centrifuged at 4°C and 14,000g for 15min, and pellets were resuspended to the initial sample volume. Equal volumes of resuspended pellets and supernatants were analysed by SDS-PAGE and immunoblotting.

*Isolation of lipid raft-enriched fractions.* Lipid raft-enriched fractions were prepared by adapting a method described previously for brain tissue [18]. All steps were carried out on ice or at 4°C. Cell pellets were resuspended in Mes-buffered saline and a mixture of protease inhibitors, and homogenised using an Ultra-Turrax tissue homogeniser, followed by sonication. Cell lysates were centrifuged at 1000g for 5min to remove nuclei and unbroken cells, and membranes were recovered by ultracentrifugation at 150,000g for 90min. Samples were resuspended by sonication in MBS containing a mixture of protease inhibitors and Triton X-100 at a final protein:detergent ratio of 0.61 (meaning that samples were solubilised in 1% (w/v) Triton X-100 at a protein concentration of 6mg/ml). Subsequent to sonication, samples were adjusted to 40% sucrose, and 1ml of sample was injected under a 10.3ml of 5–30% (w/v) sucrose gradient in MBS. Gradients were centrifuged at 140,000g for 18h at 4°C in a SW 40.1 rotor (Beckman Instruments), and 1ml fractions were subsequently collected from the top of the gradient to the bottom, with the pellet resuspended in 1ml MBS being fraction 13.

*Protein concentration assay.* Total protein was quantified using the bicinchoninic acid assay (Pierce), which colorimetrically measures the production of bicinchoninic acid–copper complexes and is compatible with the presence of up to 5% Triton X-100.

*GTPγS-binding assay.* Assays were performed in duplicate in 96-well clear-bottom plates. CHO-GABA_A membranes (15μg/well) were incubated for 30min at room temperature with [35S]GTPγS (0.3nM final, 1170Ci/mmol) in the presence of a range of 11 GABA concentrations (10⁻²–10⁻⁶ M) in 10mM Tris-Cl, 0.1mM EDTA, 1mM CaCl₂, pH 7.4, and 0.5mM GTP was used to define non-specific
binding. When testing for antagonist effects, membranes were first incubated with the GABA B antagonist CGP54626 (1.6μM) for 30 min at room temperature prior to addition of \[^{[^{35}}S\text{GTPpS}\] and GABA. Wheat germ Agglutinin (WGA) SPA beads (Amersham) (0.5mg/well) were added, and binding was allowed to proceed at room temperature for 30 min on an orbital shaker. Plates were then spun at 1500 rpm for 5 min and membrane-bound radioactivity (corrected counts per min, CPM) was determined on a Wallac Trilux Microbeta Counter.

Cholesterol assay. Cholesterol was quantified colorimetrically using an assay kit purchased from Boehringer–Mannheim/R-Biopharm and following the manufacturers’ instructions.

Results

We have shown previously that the G protein coupled GABA B receptor associates with lipid rafts in the rat cerebellum. Raft association has been shown to be cell type-specific for the G protein coupled β2-adrenoceptor and A1 adenosine receptor. For example, while the large majority of the A1 adenosine receptor associates with lipid rafts in cardiac ventricular myocytes, it does not associate with lipid rafts at all when the human form is ectopically overexpressed in rat myocytes. In order to test whether raft-association is cell-specific for the GABA B receptor, we used a stable ovary CHO K1 host cell line that ectopically expressed the GABA B1a,2 receptor. GABA B1 exists as two main splice variants [20], with GABA B1a and GABA B1b differing in their extracellular N-terminus. No differences in the resulting functional receptor have been reported (for review, see [16,17]), and for the current study we used a GABA B1a,2 receptor-expressing cell line only. This transfected cell line has been characterised previously [21,22], but to date has not been tested for lipid raft isolation or GABA B receptor association with lipid raft-enriched fractions.

In the present study, immunocytochemical analysis revealed co-localisation of GABA B1a and GABA B2 when ectopically expressed in CHO cells (Fig. 1). Some internal staining for GABA B1a was also observed, which is explained by the fact that GABA B1a cannot reach the cell surface without GABA B2, as it has a retention motif that is masked by the formation of a coiled-coil between the C termini of GABA B1 and GABA B2, hence allowing surface expression of the heterodimer.

Ectopically expressed GABA B receptor associates with lipid rafts in CHO cells

In order to test for lipid-raft association of the GABA B receptor in the CHO cells, we first analysed the solubility of the receptor in Triton X-100, as lipid raft components are insoluble in Triton X-100 at 4°C. The stably transfected receptor was found mostly in the Triton X-100-insoluble pellet subsequent to detergent treatment followed by low-speed centrifugation (Fig. 2). The transferrin receptor, which serves as a commonly used control membrane protein that is not associated with rafts [23], was present almost exclusively in the soluble fractions.

To isolate lipid raft-enriched fractions, we adapted conditions used previously for rat cerebellar tissue [18]. CHO cell membranes were prepared as described in Materials and methods, followed by detergent-treatment and sucrose density gradient centrifugation. Subsequent to centrifugation, 12 equal-volume fractions were isolated from the top to the bottom of the gradient (with fraction 13 being the reconstituted pellet). A light-scattering band representing the lipid raft-enriched fractions was observed in fractions 4–6, representing the 15–20% sucrose region of the gradient (not shown). Comparing all isolated fractions for protein content, less than 5% of total protein was found to be present in lipid raft-enriched fractions, with 30% of proteins present in the insoluble pellet and the remainder present in the soluble fractions (fractions 11 and 12).

Immunoblot analysis revealed the large majority of the GABA B receptor to be in the lipid raft-enriched fractions, together with the commonly used raft marker caveolin [24] (Fig. 3). In separate experiments, we routinely found at least 70% of the GABA B receptor associated with these raft-enriched fractions. Furthermore, the Gαi and Gβ subunits of the commonly GABA B receptor-associated heterotrimeric G proteins were also enriched in the lipid raft fractions. This contrasted with the membrane protein transferrin receptor, which was present almost exclusively in the soluble fractions at the bottom of the gradient.

Fig. 1. Distribution of GABA B1 and GABA B2 in GABA B1a,2-transfected CHO cells. GABA B1a,2-transfected CHO cells were stained with primary antibodies for GABA B1 (right panel) and GABA B2 (left panel).

Fig. 2. Solubility of GABA B1a,2 in Triton X-100. GABA B1a,2-transfected CHO cells were incubated with 1% Triton X-100 for 60 min at 4°C and subsequently centrifuged. Pellets were reconstituted to the original sample volume, and equal volumes of detergent-insoluble pellet (P) and supernatant (S) were analysed by SDS-PAGE and immunoblotting for GABA B1, GABA B2, and transferrin receptor.
From these results we conclude first that lipid raft-enriched domains could be isolated successfully from transfected CHO K1 cells, and second that the GABAB receptor associated with lipid raft domains in these cells. The GABAB receptor displays reduced agonist-induced GTP\(\gamma\)S binding in lipid raft-enriched fractions. Having shown that GABA\(B\) receptors constitutively associated with lipid raft domains in transfected CHO cells, this cell line provided us with a good, homogeneous system in which to study receptor function. A common approach to study the effects of lipid rafts on receptor signalling is to manipulate the cholesterol content of membranes using drugs such as cyclodextrin or filipin. However, cholesterol is present in non-raft areas of the cell membrane as well as in rafts, and decreasing the cholesterol content has pleiotropic effects on all membrane areas, including increased membrane permeability and Golgi complex vesiculation [25]. We therefore studied the effects of lipid raft localisation on GABAB receptor function without using cholesterol-altering drugs by comparing receptor signalling in isolated whole membranes with that in lipid raft domains.

Whole membranes and lipid rafts were isolated using essentially the same buffers. Whole membranes were isolated as described in Materials and methods in Mes-buffered saline. Lipid raft membranes were enriched away from whole membranes and Triton X-100 in a Mes-buffered saline sucrose gradient. Both membrane pellets were resolubilised in Tris/EDTA buffer containing 1mM CaCl\(_2\) for functional assays. Because lipid raft isolation necessitates solubilisation of non-raft membranes which are thus unsuitable for functional studies, GABA\(B\) receptor function was compared in raft-enriched versus whole membranes fractions.

We performed the functional studies on whole membranes and on lipid raft-enriched membrane fractions by testing equal amounts of total protein for GTP\(\gamma\)S binding in response to increasing concentrations of the GABAB receptor agonist GABA (Figs. 4A and B). Eleven concentrations of GABA were used ranging from \(10^{-8}\) to \(10^{-3}\)M. The experiments were performed twice in duplicate, and we obtained a dose-dependent increase in GTP\(\gamma\)S binding in response to agonist stimulation both in whole membranes and in raft-enriched membranes. In the presence of the GABAB receptor antagonist CGP54626 (described in [26]), the dose responses to GABA were decreased (Figs. 4A and B), thereby corroborating the specificity of the observed GABA response. Interestingly, the magnitude of the response in lipid raft-enriched fractions was decreased compared with...
that observed for whole membranes, with average decreases of 2.3- and 1.5-fold being observed in the two duplicate experiments, respectively (Figs. 4A and B). Because the magnitude of the response depends on the amount of receptor present, we compared equal amounts of total protein from whole membranes and raft-enriched fractions for the receptor and controls (Fig. 5). In raft-enriched fractions, the receptor and caveolin made up a higher proportion of the total protein content than in the non-raft domains. The same held true for the $G_\alpha$ and $G_\beta$ subunits of the GABA$_B$ receptor-associated heterotrimeric $G$ proteins. The transferrin receptor, on the other hand, was not enriched in lipid raft-enriched fractions. When testing the fractions for cholesterol content in relation to protein content, we observed an approximately 15-fold enrichment in cholesterol in the raft-enriched fractions compared with whole membranes, when testing equal amounts of total protein (results not shown). These results show that the decreased magnitude of response observed in lipid raft-enriched fractions was not due to a reduced amount of receptor or $G$ proteins.

Upon normalisation, we obtained an average EC$_{50}$ value of $9.0 \times 10^{-6}$ ($9.7$ and $8.3 \times 10^{-6}$, respectively) for lipid raft-enriched fractions and of $2.8 \times 10^{-6}$ (for each of the duplicates) for whole membranes (Fig. 4C). The EC$_{50}$ for whole membranes was therefore approximately threefold lower than the EC$_{50}$ that was obtained when using raft-enriched fractions.

Thus, GABA$_B$ receptor signalling was reduced in lipid raft-enriched fractions when compared with whole membrane fractions, showing a reduced magnitude of response and a higher EC$_{50}$ Value. Because we compared lipid raft-enriched fractions with whole membranes rather than non-raft fractions, the observed difference in receptor signalling is probably an underestimation of the actual in vivo difference between raft and non-raft domains.

**Discussion**

*Ectopically expressed GABA$_B$ receptor associates with lipid rafts in CHO cells*

Over the past few years, a range of $G$ protein coupled receptors has been shown to reside in lipid raft microdomains [6]. This localisation is not only receptor- but also cell-type specific, with, for example, the $\beta_2$-adreno-receptor associating with lipid rafts in cardiac myocytes [8,9] but not in vascular smooth muscle cells [10] or when the human form is overexpressed in rat myocytes [11]. Also, while the adenosine A1 receptor resides in raft domains in cardiac ventricular myocytes [12], it is all but absent from epithelial cells [13]. We have shown previously that the GABA$_B$ receptor associates with lipid rafts in rat brain cerebellum [18]. As this was the first report of GABA$_B$ receptor localisation to lipid rafts, it was not known whether this association was specific to neuronal cells. In this current study, we report that association of the GABA$_B$ receptor with lipid raft domains is not specific to neurones, but also occurs when the GABA$_B1a,2$ receptor is ectopically expressed in a non-neuronal cell line. CHO K1 cells were stably transfected with GABA$_B1a,2$ [21], and lipid raft-enriched fractions were isolated from these cells by detergent-treatment and sucrose density gradient floatation. The GABA$_B1a,2$ receptor was found associated with the lipid raft-enriched fractions, which also contained the raft-marker caveolin, but not the non-raft transferrin receptor, confirming the stringency of the isolation procedure. In addition, the majority of $G_\alpha$ and $G_\beta$ which are subunits of the commonly GABA$_B$ receptor-associated heterotrimeric $G$ proteins, was present in lipid raft-enriched domains.

The fact that raft localisation is not restricted to neurones but is equally evident in CHO cells suggests that this constitutive segregation into lipid rafts is not cell-type specific but rather is intrinsic to the GABA$_B$ receptor itself. Interestingly, however, while approximately 30-40% of the GABA$_B$ receptor is present in rafts isolated from rat cerebellum [18], in transfected CHO cells the large majority of the receptor was observed to be raft-associated. This difference could indicate that access to rafts is more stringently regulated in neurones.

*The GABA$_B$ receptor displays reduced agonist-induced GTP$\gamma$S binding in lipid raft-enriched fractions*

As our previous publication was the first account of GABA$_B$ receptor association with lipid rafts, the effects of this localisation on receptor signalling had not yet been elucidated. While the functional signalling of some $G$ protein coupled receptors has been shown to be influenced by their raft localisation, no overall pattern of effects exists. For example, while lipid rafts enhance
signalling of the gonadotropin-releasing hormone receptor [14] and the chemokine receptor type 5 [15], signalling of the β2-adrenoreceptor is inhibited by raft localisation [8,9].

In brain, the different GABAB receptor subtypes are differentially distributed with, for example, the GABA-B1a,2 Receptor reported to locate to presynaptic sites of cerebellar granule cells, and the GABA-B1b,2 Receptor being expressed post-synaptically in cerebellar Purkinje cells [16,17]. Isolation for functional studies of sufficient quantities of a single type of membrane expressing only one type of receptor would thus have been close to impossible from brain. Having established that CHO cell-expressed GABA-B1a,2 receptor localises to lipid rafts, this cell line provided us with an simple and homogeneous system in which to study the effects of raft localisation on receptor function.

The effects of lipid raft domains on receptor signalling are commonly studied by manipulating the cholesterol content of membranes using drugs such as cyclodextrin or filipin. However, cholesterol occurs both in lipid raft and non-raft domains, and sequestration of this molecule changes the physiology of the plasma membrane as a whole rather than being specific for lipid rafts [27]. Thus, removing cell membrane cholesterol will potentially influence the signalling behaviour of both raft-localised and non raft-localised receptors. In addition, cholesterol sequestration has an array of pleiotropic effects ranging from increased membrane permeability to Golgi complex vesiculation [25]. For example, effects such as HIV entry into cells via the CD4 receptor and its G protein coupled co-receptor CCR5 have been shown to be affected by cholesterol depletion without depending on lipid raft domains [28]. We therefore wanted to study the effects of lipid raft association on GABA-B1a,2 receptor function without having to deplete membranes of cholesterol. We did this by comparing signalling in isolated raft-enriched membranes to that in whole membranes. Because non-raft membranes were solubilised subsequent to lipid raft isolation and therefore unsuitable for functional studies, the whole membrane fractions were chosen as a suitable comparison for raft-enriched membrane fractions. In order to ensure that the membranes were treated similarly, we used standardised isolation and reconstitution buffers throughout. Detergent-free methods of raft-enrichment have been described. However, the isolated membrane domains are enriched on the basis of density alone and differ in composition from detergent-isolated raft-enriched fractions [29,30]. Such an isolation procedure was therefore deemed less suitable for our purposes.

We show in the current report that the GABA-B1a,2 receptor is functionally active in lipid rafts isolated from transfected CHO cell, as measured by GTP binding in response to agonist stimulation. However, although the GABA-B receptor displayed functional activity in isolated raft-enriched fractions, the observed magnitude of response was reduced when compared with whole cell fractions. Equal amounts of total protein from raft-enriched and whole membranes were used for the functional studies. In raft-enriched fractions, the GABA-B1a,2 receptor and the G protein Gαi and Gβi subunits represented a larger proportion of total protein than in whole membranes. As a consequence, more receptor and G proteins were available in the raft-enriched fractions. The observed decrease in magnitude of the response was thus not due to decreased amounts of receptor or G proteins being present.

The calculated EC50 value was approximately three-fold higher for the raft-enriched GABA-B receptor pool than for whole membranes, indicating that a higher agonist concentration was required to achieve a response in raft-associated receptors than in non-raft domains. The EC50 value is independent of the amount of protein present and this result strengthens our finding that function is down-regulated when the receptor resides in lipid raft domains. Because we compared raft membranes to whole membranes rather than non-raft membranes, this observed down-regulation is probably an underestimation of that occurring in vivo.

A similar down-regulation is seen for lipid raft-associated β2-adrenoreceptor [8,9]. Also, as is the case for the β2-adrenoreceptor, we do not yet know what causes this receptor down-regulation. Possible mechanisms may include limited access to or uncoupling from components that are important to receptor function. The biophysical properties of the lipid raft microdomains may also feasibly place constraints on receptor function.

In conclusion, the GABA-B1a,2 receptor associated with lipid rafts not only in brain, but also when expressed ectopically in a non-neuronal cell line. The raft-association of the GABA-B receptor is, therefore, not cell-type specific, but seems rather to be a property intrinsic to the receptor. Furthermore, the receptor displayed functional signalling behaviour while in lipid raft-enriched domains. However, although raft-associated GABA-B receptor was functionally active, signalling was down-regulated with respect to the total receptor population. This indicates that localisation to lipid raft domains may inhibit receptor signalling and suggests that changes in membrane environment may regulate receptor function in vivo.

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