The M3/M4 Cytoplasmic Loop of the \( \alpha_1 \) Subunit Restricts GABA\(_A\)Rs Lateral Mobility: A Study Using Fluorescence Recovery After Photobleaching

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A crucial problem in neurobiology is how neurons are able to maintain neurotransmitter receptors at specific membrane domains. The large structural heterogeneity of gamma aminobutyric acid receptors (GABA\(_A\)Rs) led to the hypothesis that there could be a link between GABA\(_A\)R gene diversity and the targeting properties of the receptor complex. Previous studies using Fluorescence Recovery After Photobleaching (FRAP) have shown a restricted mobility in GABA\(_A\)Rs containing the \( \alpha_1 \) subunit. The M3/M4 cytoplasmic loop is the region of the \( \alpha_1 \) subunit with the lowest sequence homology to other subunits. Therefore, we asked whether the M3/M4 loop is involved in cytoskeletal anchoring and GABA\(_A\)R clustering. A series of \( \alpha_1 \) chimeric subunits was constructed: \( \alpha_1 \)CH (control subunit), \( \alpha_1 \)CD (Cytoplasmic loop deleted), \( \alpha_1 \)CD2, and \( \alpha_1 \)CD3 (\( \alpha_1 \) with the M3/M4 loop from the \( \alpha_2 \) and \( \alpha_3 \) subunits, respectively). Our results using FRAP indicate an involvement of the M3/M4 cytoplasmic loop of the \( \alpha_1 \) subunit in controlling receptor lateral mobility. On the other hand, immunocytochemical approaches showed that this domain is not involved in subunit targeting to the cell surface, subunit-subunit assembly, or receptor aggregation. Cell Motil. Cytoskeleton 63:747–757, 2006.

Key words: GABA\(_A\)Rs lateral mobility; FRAP; receptor clustering; anchoring

INTRODUCTION

It is broadly believed that GABA\(_A\)R molecular heterogeneity determines biophysical and pharmacological properties of the receptor [for review see Sieghart, 2000]. In addition, there are strong evidences of the localization of GABA\(_A\)Rs not only at synaptic sites but also at presynaptic and axonal sites [Sakatani et al., 1991; Nusser et al., 1998; Kullmann et al., 2005]. However, it is not clear whether these neurotransmitter receptors are edited and routed to their final postsynaptic domains or if a freely mobile pool of receptors is maintained on the cell surface. Neuronal demands could recruit these free receptors to

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specific regions of the neuronal membrane. Lateral movement in the plasma membrane represents a mechanism by which the local GABA\(_A\)R concentration might be regulated to adjust the efficacy of synaptic inhibitory transmission. In fact, we have previously shown that the diversity of subunit isoforms determines distribution and mobility of GABA\(_A\)R on the cell surface [Perán et al., 2001, 2004]. The use of quantum dot labelling of single glycine receptor molecules [Dahan et al., 2003] or single-molecule fluorescence microscopy of AMPA receptors [Tardin et al., 2003] have confirmed as well, that individual neurotransmitter receptor molecules can be observed moving in and out of synapses independent of endocytosis. Several groups are involved in the study of how GABA\(_A\)R are anchored and maintained at specific membrane domains [review in Lüscher and Keller, 2004]. Their approaches, however, depend on biochemical reconstitution of the system. The study presented here has approached the problem of receptor anchoring and identification of the molecular interactions in a different manner. Thus, attention was focused on the membrane dynamics of the receptor, measuring its lateral mobility by using the Fluorescence Recovery After Photobleaching (FRAP) method. FRAP is a powerful tool that permits the lateral motions of molecules in the membranes of single, living cells to be examined under physiological conditions [Reits and Neefjes, 2001]. In FRAP experiments fluorescently labelled molecules are irreversibly photo bleached by a high-intensity laser beam that briefly illuminates a small area of the cell. Diffusion of surrounding non-bleached molecules leads to recovery of the fluorescence. A higher mobility of the molecules results in a shorter time of recovery. From the recovery curve, it is possible to obtain estimates of the diffusion coefficient and immobile fraction [Axerold et al., 1976].

We have already provided evidence, using FRAP, that GABA\(_A\)Rs containing the \(\alpha_1\) subunit have a restricted lateral mobility [Perán et al., 2001, 2004]. Because the major amino acid sequence divergence between \(\alpha\) subunit isoforms is found in the M3/M4 cytoplasmic domain, we hypothesised that this region might potentially mediate \(\alpha_1\) subunit-specific association with the cytoskeleton and/or confine receptors to specific neuronal domains. The intracellular loop M3/M4 contributes most of the cytoplasmic domain of these receptors and includes multiple interaction sites for putative trafficking and postsynaptic scaffold proteins as well as phosphorylation sites for diverse serine/threonine and tyrosine kinases [Lüscher and Keller, 2004]. In addition, this region of the \(\alpha_1\) subunit might contain a retention motif responsible for the retention of recombinant \(\alpha_1\) subunit homo-oligomers in an intracellular compartment [Perán et al., 2001].

The objective of the present work was to study the possible implication of the \(\alpha_1\) cytoplasmic loop in GABA\(_A\)Rs trafficking and anchoring. To do so several chimeric \(\alpha_1\) subunits were constructed: an \(\alpha_1\) subunit in which the M3/M4 domain had been excised (\(\alpha_1\)CD) and \(\alpha_1\) subunits where the M3/M4 loop was replaced by the loops of the \(\alpha_2\) subunit (\(\alpha_1\)CD2) and the \(\alpha_3\) subunit (\(\alpha_1\)CD3). The \(\alpha_2\) and \(\alpha_3\) were chosen for construction of the chimeric \(\alpha\) subunits because there is evidence that these subunits form co-localised clusters with gephyrin [Koksma et al., 2005].

These chimeric subunits were transiently expressed with wild-type \(\beta_3\) and \(\gamma_2s\) subunits to form recombinant GABA\(_A\) receptors. FRAP approaches and immunocytochemical studies were performed on these transiently expressed receptors. We discovered that the M3/M4 cytoplasmic loop of the \(\alpha_1\) subunit regulates receptor mobility but not receptor trafficking.

The advantages of using FRAP in the study of GABA\(_A\)Rs allowed us to investigate in a non-invasive way the behaviour of these receptors in living cells.

These observations shed more light on the role of the M3/M4 cytoplasmic loop of the \(\alpha_1\) subunit in receptor sorting, and we believe will be very useful for other studies aimed at explaining the role of the different GABA\(_A\)R subunits in physiology.

**MATERIALS AND METHODS**

**Subcloning Strategy in the Production of GABA\(_A\)R \(\alpha_{1CH}, \alpha_{1CD}\) and GABA\(_A\)R \(\alpha_{1CD\alpha}\)**

The following chimeric GABA\(_A\)R \(\alpha_1\) subunits were constructed (see Fig. 1).

1. Chimeric GABA\(_A\)R \(\alpha_1\), \(\alpha_{1CH}\) (control subunit). To create the \(\alpha_{1CH}\) a 0.9 kb Bsp120I - EcoNI S’ restriction fragment (encoding the N’ terminal bd24 epitope) of the bovine GABA\(_A\)R \(\alpha_1\) was ligated to a 0.6 kb 3’ EcoNI- EcoRI restriction fragment of the rat into the mammalian expression vector pCDNAIamp.

2. Cytoplasmic domain deleted GABA\(_A\)R \(\alpha_1\), \(\alpha_{1CD}\). To do so the 3’ end of rat GABA\(_A\)R \(\alpha_1\) was amplified from codon 414 (in effect deleting codons 362–413) using an universal reverse primer (sp6) and a forward primer (5’ aggatctcttctcagcggaggaagtgaact 3’) which encodes two unique restriction sites for BamHI (in bold) and XhoI (italic) at its 5’ end. The 3’ end of GABA\(_A\)R \(\alpha_{1CH}\) was removed by digestion with BamHI and EcoRI and replaced with the BamHI - EcoRI double digested rat PCR product.

3. GABA\(_A\)R \(\alpha_1\) in which the cytoplasmic domain of the \(\alpha_1\) was swapped with the ones of the \(\alpha_2\)
and α3 to give GABA<sub>A</sub>R α<sub>1CD2</sub> and α<sub>1CD3</sub>. To create GABA<sub>A</sub>R α<sub>CD2-3</sub>, GABA<sub>A</sub>R α<sub>CD</sub> was double digested with BamHI and XhoI. The CDs from GABA<sub>A</sub>R α<sub>2-3</sub> were individually amplified using forward primers encoding a BamHI restriction site and reverse primers encoding a XhoI restriction site (Primers sequences used for α<sub>CD2</sub>: Forward: 5′ggatccaggctccgtcatgata 3′; Reverse: 5′gctcgagttgaaagttttcttgg 3′. Primers sequences used for α<sub>CD3</sub>: Forward: 5′ggatcaggacgcccaaccaa 3′; Reverse: 5′gctcgagttgtagttcttgct 3′). Amplified GABA<sub>A</sub>R α<sub>2-3</sub> CDs were double digested with BamHI and XhoI and individually ligated into BamHI and XhoI digested GABA<sub>A</sub>R α<sub>CD</sub>.

Cell Culture. HEK293 cells were cultured in Eagle’s MEM supplemented with 10% fetal bovine serum (FBS). COS7 cells were grown in Dulbecco’s MEM with 10% (FBS).

Transfection. Cells were plated on 35 mm poly-D-lysine coated dishes and transfected with Lipofectamine (GIBCO-BRL) following manufacturers’ instructions. Cells were analysed 48 h post-transfection. Mock transfections were performed with vector only.

Immunocytochemistry

Antibodies. Monoclonal anti-α1 (bd24, Boehringer, Germany) was used at a 1:50 dilution and polyclonal antibody anti-β102/103 for the β3 subunit was used at a 1:200 dilution. Secondary antibodies used were TRITC-conjugated goat anti-mouse and TRITC-conjugated goat antirabbit (Calbiochem, Nottingham, UK) or Cascade blue-conjugated goat anti-mouse antibody (Molecular Probes, Leiden, The Netherlands) at 1:200 dilution.

Labelling of Fixed and Permeabilized Cells. Cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 15 min, washed twice with PBS, blocked in 10% heat inactivated serum (HIS) for 15 min and then incubated for 1 h in primary antibody diluted in buffer A (1 mg/ml BSA, 10% HIS, 0.5% Triton X-100, PBS) at room temperature. Cells were washed three times in PBS and then incubated for 30 min with secondary antibodies in buffer A. Controls were performed with mock transfected cells and using secondary antibody only.

Labelling of Live Cells. The bottom of culture dishes were replaced by coverslips which permitted direct viewing of live cells using a high numerical aperture objective in an inverted microscope. Transfected cells plated in these dishes were washed twice in PBS then incubated at 4°C in primary antibody diluted in growth media for 45 min and secondary antibody for 20 min at room temperature.

Cells visualized with Bodipy-Ro-1986 (a fluorescent benzodiazepine that binds γ subunit-containing GABA<sub>A</sub>Rs, Molecular Probes, Leiden, The Netherlands) and with the primary antibody bd24 were labelled for 30 min with the bd24 diluted in PBS and with 100 nM of...
the fluorescent benzodiazepine together with the secondary antibody diluted in PBS for 30 min.

**Image Capture and Analysis.** Images were obtained using a Nikon FX-35A camera adapted to a Nikon fluorescence microscope. Photographs were recorded through a Zeiss 63× water immersion 1.2 NA objective on Ektachrome film pushed to ASA 3200. Images were transferred to Adobe Photoshop 7.0.

**Production of Fluorescently Labelled Anti-α1 Fab’ Antibody Fragment FRAP Probes**

The monoclonal antibody bd24 (Boehringer) recognizes both the bovine and human α1 subunit of GABA<sub>A</sub>R. The bd24 antibody (10 µg) was digested with 1 µg of papain in 0.2 mM Na-acetate, pH 5, 1 M cysteine and 20 mM EDTA for 15 h at 37°C. Iodoacetamide was then added to a final concentration of 75 mM and incubated for 30 min at room temperature. Fab’ fragments were purified by Protein A column chromatography (Pierce, Northumberland, UK). For direct labelling, Fab’ fragments were conjugated with the fluorophore BODIPY 493/503, SE (Molecular Probes, Leiden, The Netherlands). Fab’ fragments were diluted to 900 µl in 0.1 M sodium carbonate, pH 9.2 and 50 µl of BODIPY 493/503, SE (1 mg/ml) were added in 5 µl aliquots, with gentle, but continuous stirring. The reaction was incubated in the dark for 8 h at 4°C after which unbound dye was removed by Sephadex G-10 column chromatography (Sigma, St. Louis, MO).

**Fluorescence Photobleach Recovery**

Cells for FRAP were washed in phenol red-free MEM prior to experimentation. GABA<sub>A</sub>Rs on living cells were labelled with 100–200 µl of Fab’α<sub>1</sub>-BODIPY (0.01 µg/µl) diluted in 500 µl red-free MEM per 35 mm plate of cells at room temperature for 20 min in dark. The cells were gently washed three times with PBS and transferred to a microscope stage maintained at 22–24°C.

For FRAP experiments performed with Bodipy-Ro-1986 as a probe cells were labelled with Bodipy-Ro-1986 at 40 or 100 nM in PBS-sucrose. Although at these concentrations not all receptors are labelled, these concentrations were chosen based upon the KD of the fluorescent benzodiazepine to help minimise any non-specific binding or lipid partitioning of the fluorescent probe. The signal from the unbound fluorophore is negligible because the fluorescence of the Bodipy conjugates is enhanced upon binding to the receptor. Non-specific binding of Bodipy-Ro-1986 was determined by including chlorazepate (1 mM) in the FRAP assay. The non-specific labelling, based upon photon counts obtained under identical experimental conditions, was found to be less than 10% of total.

For FRAP experiments, a 63× and 1.2 NA water immersion objective was used directly in the culture dish. A Zeiss Universal fluorescence microscope was used with an argon-ion monitoring laser (λ = 498 nm, 5 mW). The beam was focused to a Gaussian radius of 1.2 µm and a ~2 µm<sup>2</sup> region of membrane was illuminated by a brief (10–200 ms) laser pulse (5 mW) photobleaching ~ 75% of the initial fluorescence. Bleaching during the initial monitoring phase is negligible within the time frame of the experiment. The time course of fluorescence recovery was followed with an attenuated monitoring beam. D<sub>C</sub> and F were determined by curve-fitting procedures based on theoretical models described previously [Axelrod et al., 1976]. Single FRAP measurements were taken from each cell. To avoid long exposures of the cells preparations and possible lost of cell vitality, only 10 measurements were carried out for each experiment. Standard mean errors were calculated from the repeated measurements on cultures for all experiments. FRAP experiments performed with the same recombinant receptor combinations were repeated on different days and little variation in the results was found.

Statistical analyses were routinely performed using the statistical package Statistica version 6. A Kolmogorov-Smirnov test of normality was performed and the significance of the difference between data was tested using a Student’s t-test. Data are presented throughout the study as mean ± standard errors of the mean. In all cases, a probability of less than 0.05 was regarded as statistically significant. Pearson-moment correlation was carried out among measured variables.

**RESULTS**

**Localization of the Recombinant Receptor Including the Chimeric Subunit on the Cell Membrane**

The cellular distribution of recombinant α<sub>1</sub>CHβ3γ2s and α<sub>1</sub>CDβ3γ2s receptors was studied by immunocytochemistry in HEK293 cells (data not shown) and COS7 cells (Fig. 2). Both α<sub>1</sub>CHβ3γ2s and α<sub>1</sub>CDβ3γ2s were expressed in clusters on the surface of live HEK293 and COS7 cells.

These results imply that the transfection of the genetic material containing the truncated subunit were able to form stable protein complexes. These chimeric α1 subunits were able to assemble and be expressed on the cell surface in conjunction with β3 and γ2 “wild type” subunits.

**Lateral Mobility of Truncated Receptors**

COS7 and HEK293 cells were transfected with β3 and γ2s subunit cDNAs together with each of the α1 constructs: α<sub>1</sub>CH, α<sub>1</sub>CD, α<sub>1</sub>CD<sub>2</sub> and α<sub>1</sub>CD<sub>3</sub> and the mobility of the expressed receptors were measured by FRAP.
Recombinant $\alpha_1\text{Chimeric}(\beta_3\gamma_2\text{s})$ complexes were labelled with fluorescently labelled Fab' antibody fragment of the $\alpha_1$ subunit specific bd24 antibody ($\alpha_1\text{-Fab'}$ BODIPY).

For $\alpha_1\text{CH}(\beta_3\gamma_2\text{s})$ and $\alpha_1\text{CD}(\beta_3\gamma_2\text{s})$-expressed receptors parallel experiments were performed using fluorescently BODIPY FL Ro-1986, a fluorescent benzodiazepine that labels GABA$_A$Rs containing the $\gamma_2$S subunit.

The non-specific labelling, based upon photon counts obtained in experiments using non-transfected cells, under identical experimental conditions, was found to be less than 10% of total.

No statistically significant difference in the mobile fraction (% Recovery) of recombinant GABA$_A$Rs expressed in HEK293 was found. The recombinant receptors containing the different $\alpha$ constructs were able to freely move on the cell membrane. The mobile fraction of receptors comprising the $\alpha_1$ subunit chimeras ranged from 65 to 71%. These results are in concordance with those obtained before with the wild types of $\alpha$ subunits expressed on HEK293 cells [Perán et al., 2004]. These cells seem to lack those elements necessary to anchor GABA$_A$Rs since the receptors were mobile whichever subunit combination was expressed.

In contrast, when the same receptors where expressed in COS7 cells, a statistically significant difference in the relative mobile fractions of the expressed receptors was found. Using Fab' $\alpha_1$, BODIPY, the mobile fraction of $\alpha_1\text{CH}_3\gamma_2\text{s}$ expressed receptors was 27.7% ± 6% ($n=10$) whilst 76.7% ± 7% of $\alpha_1\text{CD}_3\gamma_2\text{s}$ expressed receptors were freely mobile ($n=10$) (Fig. 3a). Receptors containing the chimeric $\alpha_1$ with the M3/M4 loop were almost immobile, after 60 s only 27% of the fluorescence was recovery. The deletion of the cytoplasmic loop changed the behaviour of GABA$_A$R containing the $\alpha_1$ subunit. The mobile fraction of $\alpha_1\text{CD}_3\gamma_2\text{s}$ expressed receptors went up to 70% in the same time.

When FRAP experiments were performed using BODIPY FL Ro-1986 as a probe the results were very similar: 29% ± 8% for $\alpha_1\text{CH}_3\gamma_2\text{s}$ expressed receptors ($n=12$) and 75% ± 10% for $\alpha_1\text{CD}_3\gamma_2\text{s}$ expressed receptors ($n=10$).

Despite the clear difference in the percentage of relative mobile fractions of $\alpha_1\text{CH}_3\gamma_2\text{s}$ and $\alpha_1\text{CD}_3\gamma_2\text{s}$ receptors expressed in COS7 cells, both receptor types were clustered at the cell surface (Fig. 2). This implies that receptor aggregation does not govern receptor mobility.

For COS7 cells (Fig. 3a) the recovery of the fluorescence signal was 73.6% ± 9% for $\alpha_1\text{CD}_3\gamma_2\text{s}$ and 71.6% ± 8% for $\alpha_1\text{CD}_3\gamma_2\text{s}$ receptors, compared to 27.7% ± 6% for $\alpha_1\text{CH}_3\gamma_2\text{s}$. These results suggest that exchanging...
the cytoplasmic loop of the α1 subunit with the corresponding domain of the α2 or α3 subunits released the receptor complex from the constraints that tethered the receptor on the cell surface. Thus, although α1CD2 and α1CD3 chimeras have an α1 subunit amino-acid backbone the replacement of the cytoplasmic loop transformed the mobility pattern of wild type α1 subunits.

Figure 3b shows the rate of movement of α1CD, α1CH, α1CD2 and α1CD3-containing receptors was typical of most membrane glycoproteins with diffusion coefficients of the range of 0.97–1.44 × 10^-10 cm^2 s^-1 [Jacobson et al., 1983]. It should be noted, however, that although these values are expected using FRAP, when single particle tracking is used, the absolute value of diffusion coefficients can be quite different [Simson et al., 1998]. It is therefore important to qualify these results in the light of the technique used.

To determine whether receptor cluster density has any effect on diffusion parameters, the diffusion coefficient and mobile fractions were plotted against the pre-bleach fluorescence intensity. As can be seen in Fig. 4 there is no evidence for a correlation between pre-bleach intensity and the mobile fraction.

In addition, statistical analysis of the data showed that there were no significant correlations between pre-bleach intensity or bleach intensity versus recovery fraction or half-time of recovery (data not shown). This means that the results of lateral mobility were not influenced by the experimental conditions.

Localisation of the α1 Subunit Chimeras in Transfected COS7 and HEK293 Cells

We have provided evidence that α1 subunit homo-oligomers expressed in HEK293 or COS7 cells are not directed to the cell surface but remain intracellular, unable to exit the ER [Perán et al., 2001].

On that report, we showed that when COS7 cells transfected with α1 are live-stained, no signal is apparent. In contrast, after fixation and permeabilization, α1 signal is clearly intracellularly located. To test whether an ER-retention signal is contained within the M3/M4 cytoplasmic loop the truncated α1 subunit construct lacking this domain, α1CD, was transiently transfected into COS7 and HEK293 cells. Immunocytochemistry was performed to determine where in these cells this subunit was expressed and compared with the distribution of bovine/rat chimera, α1CH. When COS7 and HEK293 cells transiently transfected with the α1CD subunit were stained live with the monoclonal antibody bd24, no surface fluorescence was observed. The same results were obtained with the chimeric subunit, α1CH (data not shown). Immunoreactive product was only visible if the cells were fixed and permeabilised. Hence, α1CH and α1CD chimeric subunits were retained in an intracellular compartment. Figure 5a, shows that the intracellular distribution of α1CH-containing receptors in HEK293 cells, is similar to the pattern shown by α1CD-containing receptors (Fig. 5b) and to the one obtained for α1 subunit homo-oligomers [Perán et al., 2001]. Matching results were obtained in transfected COS7 cells (Fig. 5), the intracellular retention of expressed α1CH and α1CD-containing receptors are shown in Figs. 5c and 5d, respectively.

To test whether the M3/M4 cytoplasmic loops of α2 or α3 subunits could direct α1 subunit homo-oligomers to the cell surface, the distribution of transiently expressed α1CD2 (Fig. 5e) and α1CD3 (Fig. 5f) subunit constructs
were analysed by immunocytochemistry in COS7 cells. All the α1 subunit constructs were found retained intracellularly.

β3 Subunits Re-route α1 Subunits Chimeras to the Cell Surface

We have shown before [Perán et al., 2001] in agreement with [Connor et al., 1998] that the β3 subunit rescues the α1 subunit from its intracellular retention site and re-route it to the cell surface. To determine if the M3/M4 cytoplasmic loop is necessary for α1-β3 subunit assembly, the α1 subunit constructs used in the previous section were transfected into COS7 cells together with the β3 subunit. The lack of a cytoplasmic loop (α1CD) did not prevent α1 subunits from being rescued by β3 subunits. When α1CD subunit cDNA and β3 subunit cDNA were cotransfected into COS7 cells (Figs. 6c and 6d), these subunits were found clustered at the cell surface in a pattern that paralleled that found for α1β3 [Perán et al., 2001] and α1CHβ3 (Figs. 6a and 6b) complexes expressed in these cells. Identical results were obtained with the α1 subunit constructs α1CD2, and α1CD3 when they were cotransfected into COS7 cells with the β3 subunit (Figs. 6e and 6f and Figs. 6g and 6h respectively).

Together these results demonstrate that the α1 subunit constructs (α1CD, α1CD2 and α1CD3) are restricted to the ER when expressed alone as for the wild-type α1 subunit. Only when these subunits were co-expressed with the β3 subunit, they did acquire the signal to leave the ER and form co-localised clusters on the cell membrane.

DISCUSSION

Deletion of the M3/M4 Cytoplasmic Domain of α1 Subunit Does Not Prevent Receptor Assembly or the Rescue of the α1 Subunit From the ER

Functional receptor complexes are formed by diverse subunits. Proper folding and assembly of the subunits that constitute a receptor is required for the delivery of multimeric proteins to the membrane. Therefore, heterodimerization is a mechanism that enables the cells to restrict traffic to the cell membrane only to those receptor properly assembled. In some receptors, a specific ER
There are many studies reporting the localization of ER retention signal in other neurotransmitter receptor channels. For instance, deletion of a retention signal within the I-II loop of the Ca\(^{2+}\) channel \(\alpha_1\) subunit facilitates the expression of this subunit in absence of beta [Bichet et al., 2000]. In NMDA receptors, an RXR-type ER retention/retrieval motif in the C-terminal tail of the NR1 subunit has been described [Scott et al., 2001]. In kainate receptors intracellular retention of the KA2 subunit is mediated through discrete protein trafficking signals, including an arginine-rich ER retention/retrieval motif and a di-leucine endocytic sequence in the C terminus of the KA2 subunit A [Ren et al., 2003].

In addition, studies have been carried out in the type B GABA receptors. Thus, a RXR motif present in GABAB receptor GB1 subunits is masked by assembly
with GB2, ensuring heterodimerization [Marchetto-Mitrovic et al., 2000; Calver et al., 2001; Pagano et al., 2001; Villemure et al., 2005].

In contrast with these findings, much less is known about the mechanisms that restrict the exit of homo-oligomeric α1 subunit GABA_A receptor complex from the ER. In this study we have showed new evidence that the ER retention signal of α1 subunits is not contained within the M3/M4 cytoplasmic domain of the subunit. It appears that this region of the protein does not contain the information that dictates whether the subunit is retained or programmed to leave the ER. Deletion of this cytoplasmic domain did not allow the truncated α1 subunit to be expressed at the cell surface, and when by coexpressed with the β3 subunit the α1 subunit chimeras were able to leave the ER.

Thus, the M3/M4 cytoplasmic loop of the α1 subunit appears not to be involved in subunit targeting to the cell surface, subunit-subunit assembly nor receptor aggregation.

We decided to perform our studies in COS7 cells for a number of reasons. First, COS7 cells have successfully been used to study traffic of neuronal membrane proteins by several groups. For example, the membrane sorting properties of neuregulin receptors [Perron et al., 2006], ionotropic glutamate receptors [Jaskolski et al., 2005], glycine receptors [Hanus et al., 2004], ATP-sensitive potassium channels [Hu et al., 2003], G protein activated inwardly rectified potassium channels [Ma et al., 2002], and metabotropic glutamate receptors [Chan et al., 2001], among others, were studied in COS7 cells. In several of these reports, it was demonstrated that the cellular signals important for traffic in neurons were also functional in COS7 cells. Second, the flat nature of COS7 cells makes them specially suited for our experiments. Finally, we have previously shown that the α1 subunit restricts the lateral mobility of the receptor complex in both COS7 and cerebellar granule cells, which suggests that the anchoring mechanisms are shared between these two types of cells [Perán et al., 2004].

Removal of the M3/M4 Cytoplasmic Domain of the α1 Subunit Releases the Lateral Constraints Imposed on GABA_AR Mobility

Accumulation or clustering of neurotransmitter receptors at postsynaptic sites is believed to be crucial for efficient synaptic transmission. However, the mechanism by which GABA_ARs are delivered to and maintained at synapses is still poorly understood. In a review presented by Lüscher and Keller [2004], GABA_AR synaptic localization seems to be in part encoded by structural determinants present in α1, α2, α3 and γ2 subunits. Nevertheless, the same receptor subunits are also abundant at extrasynaptic sites, suggesting that receptors move in and out of the postsynaptic membrane. Data of lateral diffusion of GABA_AR is still scarce; in this work we presented novel results from FRAP experiments supporting the role of a specific domain within the α1 subunits in controlling lateral mobility of the receptors. In COS7 cells transfected with the α1CD subunit lacking the cytoplasmic loop M3/M4, receptor complexes were almost freely mobile on the cell surface. In contrast, control chimeric α1CH subunit-containing receptors showed restricted mobility. This data suggest that deletion of this sequence prevents the formation of links with the cytoskeletal elements that tether the receptors at the cell surface. This finding agree with Fritschy et al. [2003] who hypothesized that the sorting and synaptic targeting of GABA_AR is determined by interactions between specific associated proteins and appropriate sequence motifs present in particular subunits. The limitation of the lateral diffusion of GABA_AR could be due to interactions with the underlying cytoskeletal network, interactions with others membrane proteins, or interactions with the extracellular matrix.

The fact that not 100% of fluorescence is recovered after photobleach even when the M3/M4 region is deleted might be explained by the mosaicism of the membrane. Single particle tracking data suggested that there are membrane microdomains that are not readily accessible for proteins to migrate into, therefore the effective area or recovery after photobleach might be smaller that the original bleached area [Simson et al., 1998].

The lateral diffusion of glutamate receptors in and out of synaptic sites has been reported [Borgdorff and Choquet, 2002; Sergé et al., 2002; Tardin et al., 2003]. However, the mechanisms that control GABA_ARs mobility have not yet been described. Many groups are involved in the study of proteins that may interact with GABA_ARs and cluster them to the membrane at precise postsynaptic regions. Several candidates such us GABARAP [Chen et al., 2000], Raft1 and Collibistin, Plic1 or dynein light chain 1 and 2 have been identified [revised in Triller and Choquet, 2003]. Yeast two hybrid and immunoprecipitation experiments showed that those proteins directly interact with gephyrin or inhibitory receptors.

Gephyrin is the core protein of the scaffold at inhibitory synapses and responsible for anchoring of the other main inhibitory neurotransmitter receptors, the glycine receptors, [Kirsch et al., 1991; Kirsch and Betz, 1993]. This led to the hypothesis that this protein may be involved in the anchoring of GABA_ARs. Although studies support the hypothesis that gephyrin might be involved in postsynaptic positioning of GABA_A [Craig et al., 1994; Essrich et al., 1998], there is as yet no clear “in vivo” evidence. In fact, the postsynaptic localization of α1 subunit-containing GABA_ARs in cultured hippocampal neurons and the clustering of these receptors in spinal cord sections
from gephyrin knockout mice appear unaffected by loss of gephyrin [Kneussel et al., 2001; Levi et al., 2004]. In addition, GABA\(_A\) receptors can form clusters in immature neurons before being detectably colocalized with gephyrin [Dumoulin et al., 2000; Danglot et al., 2003]. Thus, in contrast to glycine receptors, gephyrin does not appear to interact directly with GABA\(_A\) receptors. Gephyrin may function by trapping receptors, anchoring receptors or otherwise maintaining synaptic receptor clusters, rather than by inserting receptors in the plasma membrane. Experiments showing that disruption of microtubules with colchicine do not affect gephyrin clustering in mature neurons in vitro [Allison et al., 1988; Van Zundert et al., 2002] led to the hypothesis that aggregation of GABA\(_A\)R and gephyrin at synaptic sites reflects a dynamic equilibrium and is not dependent on stable interaction with the tubulin cytoskeleton.

Receptor clustering by scaffold proteins could explain receptor immobility. However, movement of individual clusters of receptors with or without interaction with scaffolding proteins has been shown in real time using single particle tracking in cultured neurons [for review see Triller and Choquet, 2003]. In addition, we have found that the lateral mobility of the receptor was not dependent on receptor clustering. Although this data agree with Sérégé et al. [2002], we did not find the lower diffusion coefficient for clustered receptors that they described. It seems then, that the binding of clustered receptors to rigid elements such as the cytoskeleton is necessary for immobilization. Taking the data presented in this work into account, we conclude that this interaction is mediated by a specific region within the \(\alpha 1\) subunit of the GABA\(_A\)Rs, the cytoplasmic loop M3/M4.

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**REFERENCES**


