Lateral Mobility and Specific Binding to $GABA_A$ Receptors on Hippocampal Neurons Monitored by Fluorescence Correlation Spectroscopy†

Oliver Meissner‡ and Hanns Häberlein*,§

Department of Pharmaceutical Biology, Philipps-University, Marburg, Germany, and Department of Physiological Chemistry, Rheinische Friedrich-Wilhelms University, Bonn, Germany

Received June 20, 2002; Revised Manuscript Received November 20, 2002

ABSTRACT: The binding behavior of a fluorescently labeled muscimol derivative to the $GABA_A$ receptor was analyzed at rat hippocampal neurons by fluorescence correlation spectroscopy. After muscimol had been labeled with the fluorophore Alexa Fluor 532, specific binding constants for binding of the dye-labeled ligand (Mu-Alexa) to the $GABA_A$ receptor were determined. We found a high specific binding affinity of Mu-Alexa with a K_D value of 3.4 ± 0.5 nM and a rate constant of ligand−receptor dissociation (k_{diss}) of (5.37 ± 0.95) × 10^{-2} s^{-1}. A rate constant of ligand−receptor association (k_{ass}) of (1.57 ± 0.28) × 10^7 L mol^{-1} s^{-1} was calculated. The following diffusion coefficients were observed: $D_{free} = 233 \pm 20$ $\mu m^2/s$ ($n = 66$) for free diffusing Mu-Alexa, $D_{bound1} = 2.8 \pm 0.9$ $\mu m^2/s$ ($n = 64$) for the lateral mobility, and $D_{bound2} = 0.14 \pm 0.05$ $\mu m^2/s$ ($n = 56$) for the hindered mobility of the $GABA_A$ receptor−ligand complex in the cell membrane. Saturation of Mu-Alexa binding was observed at a concentration of 50 nM. A maximum number of binding sites [$B_{max} = 18.4 \pm -0.4$ nM ($n = 5$)] was found. Similar K_i values of 4.5 ± 1.0 nM for nonlabeled muscimol and 8.8 ± 1.8 nM for Mu-Alexa were found by RRAs using [^{3}H]muscimol as a radioligand. A concentration-dependent increase in the level of specific Mu-Alexa binding was demonstrated by the positive cooperative activity of co-incubated midazolam, which was selectively found in $GABA_A$ receptor−ligand complexes with hindered mobility.

$GABA_A$ receptor binding studies have been performed mainly by radioreceptor assays (RRAs)[1] which need radioactively labeled ligands (*1*), such as [^{3}H]GABA (*2*) and [^{3}H]-muscimol (*3*). To study the binding affinity of pharmacologically active compounds by RRAs, a separation of free from bound radioligand is required. Interactions of ligands with rapid binding kinetics are often not detectable, because the half-life of the receptor−ligand complex is shorter or analogous to the time required for the separation process. Thus, separation by, for example, filtration works only for ligand−receptor binding processes with K_D values of approximately $\leq 10^{-8}$ nM (*1*). Furthermore, cell membrane preparations are mainly used in the RRA technique and not receptors which are embedded in their native environment of living cells. This may lead to altered receptor−ligand interactions. Using fluorescence correlation spectroscopy (FCS), one can study receptor−ligand interactions at the molecular level on living cells (*4*). The FCS technique, which allows the illumination of a very small open volume element (0.1−2 fL), measures statistical fluctuations in the fluorescence intensity of singly dye-labeled molecules, which are excited by a sharply focused laser beam. Several receptor binding studies have been carried out directly on the membrane surface of cells without any separation procedures (*5*−*8*). Besides binding and kinetic properties of a particular molecular interaction, the mobility of receptor−ligand complexes in the cell membrane can be studied simultaneously in real time. Like other transmembrane proteins, receptors can move freely through the fluid bilayer (*9*, *10*), are associated via protein−protein interactions with the cytoskeleton (*11*, *12*), or are clustered in the cell membrane for a following internalization process (*13*−*15*). Additionally, receptor proteins can be associated with functional entities of a higher order, so-called "lipid rafts", which float within the cell membrane (*16*).

Neurotransmitters (e.g., GABA) or drugs which bind to CNS receptors are often nonfluorescent structures with low molecular masses. Due to the prerequisite of fluorescently labeled ligands for FCS, the influence of the coupled dye on the binding behavior of small ligands has to be weak. In this study, we describe a $GABA_A$ receptor binding assay at the surface of living hippocampal neurons, which provides additional information about the lateral mobility of the $GABA_A$ receptor−ligand complex using a fluorescently labeled muscimol derivative.

MATERIALS AND METHODS

Materials. Alexa Fluor 532 carboxylic acid, succinimidyl ester was purchased from Molecular Probes (Leiden, The Netherlands). Midazolam, bicuculline methiodide, and muscimol hydrobromide were obtained by Sigma (Taufkirchen, Germany).

† This work was financially supported by Bionorica AG (Neumarkt, Germany).

* To whom correspondence should be addressed: Department of Physiological Chemistry, Nussallee 11, D-53115 Bonn, Germany. E-mail: haeberlein@institut.physiochem.uni-bonn.de.

‡ Philipps-University of Marburg.

§ Rheinische Friedrich-Wilhelms University of Bonn.

[1] Abbreviations: FCS, fluorescence correlation spectroscopy; RRA, radioreceptor assay; Mu-Alexa, muscimol-bound Alexa Fluor 532; HPLC, high-performance liquid chromatography; HRMS, high-resolution mass spectrometry; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; Mu-Bodipy, muscimol-bound bodipy TMR-X.

10.1021/bi0263356 CCC: $25.00
Published on Web 01/24/2003

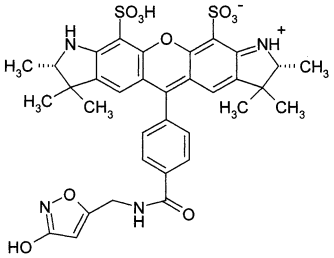

FIGURE 1: Molecular structure of the Alexa 532-labeled muscimol derivative (Mu-Alexa).

Synthesis of Mu-Alexa. The Alexa 532-labeled muscimol derivative (Mu-Alexa) (Figure 1) was obtained from the reaction of muscimol with Alexa Fluor 532 carboxylic acid, succinimidyl ester in DMSO at room temperature. The fluorescently labeled ligand was purified by HPLC on a LiChrospher 60 RP-select B column (5 μM, 4 mm × 125 mm, Merck) using the H_2O/AcCN/CH_3OH/H_3PO_4 (64/27/8/1) mixture as the eluent, at a flow rate of 1 mL/min, and with detection wavelengths of 240 and 525 nm.

Preparation of Brain Membranes. Three-month-old adult Sprague-Dawley rats were decapitated, and the cerebral cortexes were dissected and frozen. The cortical tissues of two rats were pooled and homogenized with a Polytron homogenizer in 50 mL of ice-cold 50 mM Tris-citrate buffer (pH 7.4) supplemented with 1 mM EDTA. The homogenate was centrifuged at 20000*g* for 20 min, and the pellets were resuspended in the same buffer and then recentrifuged five times. The final suspension was prepared in 50 mM Tris-citrate buffer (pH 7.4), divided into aliquots, and stored at −80 °C.

Radioreceptor Assay. Frozen membranes were thawed, resuspended, and centrifuged once before final resuspension in 50 mM Tris-citrate buffer. The final membrane suspension (300 μL) was used with 100 μL of [^{3}H]muscimol (DuPont de Nemours) at a concentration of 6 nM and 100 μL of Mu-Alexa or muscimol hydrobromide at varying concentrations in the range of 0.01 nM to 10 μM. After a 60 min incubation period at 0 °C, bound and free ligands were separated by rapid filtration of the membranes onto Schleicher & Schuell no. 52 glass fiber filters. The samples were rinsed twice with 5 mL of 10 mM ice-cold Tris-HCl (pH 7.4). The filters were immersed in 4 mL of scintillation fluid (Packard Ultima Gold), and the amount of radioactivity was determined in a Beckmann scintillation counter. All experiments were performed in triplicate. Origin 6.1G software (OriginLab Corp., Northampton, MA) was used for the data evaluation.

FCS Instrumentation. FCS measurements were performed via confocal illumination of a volume element of 0.20 fL in a ConfoCor instrument (Zeiss/Evotec). A water immersion objective (C-Apochromat, 63×, NA 1.2, Zeiss) was used for the optics. A dichroic filter and a band-pass filter separated the excitation light from the emitted fluorescence. Sample excitation was performed with the 514 nm line of an argon laser (550W50 interference filter). The power of the laser beam entering the sample was between 32 and 100 μW. The intensity fluctuations were detected by an avalanche photodiode (SPCM-AQ Series, EG&G Optoelectronics) and were correlated with a digital hardware correlator (ALV-5000, ALV). To calibrate the volume element of observation for the experiments, a defined concentration of rhodamine 6G was used in the extracellular solution above the cells. From the determined diffusion time constant of rhodamine 6G and the diffusion coefficient D of 280 μm^2/s, the radii ω_0 (0.20 μm) and z_0 (0.82 μm) of the volume element were determined.

Volume element positioning to the upper membrane of the cell soma was performed both by viewing in the x and y directions (resolution of 1 μm) and by motor-aided scanning through the neuron in the z direction (optoelectronical DC-servodrives, resolution of 0.1 μm).

Data Evaluation. The autocorrelation function $G(\tau)$ for j different diffusing components in a three-dimensional Gaussian volume element is given by the following equation (*17*):

$$G(\tau) = 1 + \frac{\sum_{j=1}^{n} Q_j^2 N_j}{\left(\sum_{j=1}^{n} Q_j N_j\right)^2} \frac{1}{1 + \tau/\tau_{D_j}} \sqrt{\frac{1}{1 + (\omega_0/z_0)^2 \tau/\tau_{D_j}}} \quad (1)$$

with

$$\tau_{D_j} = \frac{{\omega_0}^2}{4D_j} \quad (2)$$

and

$$Q_j = \sigma_j \eta_j g_j \quad (3)$$

where N_j is the average number of molecules of species j in the volume element, τ_{D_j} is the diffusion time constant of species j, ω_0 is the radius of the observation volume in the focal plane, z_0 is the radius of the observation volume in the z direction, D is the translational diffusion coefficient, Q_j is the quantum yield correction factor, σ_j is the absorption cross section, η_j is the fluorescence quantum yield, and g_j is the fluorescence detection efficiency of species j.

If it is assumed that the receptor−ligand complex diffuses in a two-dimensional manner ($z \rightarrow \infty$) and all fluorescent components j show the same count rate per molecule, i.e., all Q_j values are the same, eq 1 can be simplified to

$$G(\tau) = 1 + \frac{1}{n} \sum_{j=1}^{n} \frac{y_j}{1 + \tau/\tau_{D_j}} \quad (4)$$

where y_j is the fraction of species j of the autocorrelation amplitude and N is the average number of molecules in the detection volume element. Equation 4 was used for the data evaluation of all FCS experiments, because the difference between the three- and two-dimensional autocorrelation functions was <6% for the calculated diffusion time constants and <1% for the calculation of the corresponding particle numbers.

For parametrization and fitting of the autocorrelation function $G(\tau)$, a nonlinear least-squares minimization was performed according to the Marquardt algorithm (*18*).

Cell Culture and Binding Studies. Hippocampal rat neurons were prepared on embryonic day 18 by microdissection (*19*). The cells were trituated and seeded at a density of approximately 2.5 × 10^5 on 18 mm poly-D-lysine-coated

coverslips. The coverslips were placed in a Nunclon multiwell (12 wells) plate, and the cells were cultured for 8 days in Start V Medium (Biochrom, Berlin, Germany). Cells in culture were used for experiments from day 9 until day 14.

Prior to the FCS measurements, cells were washed three times with Locke's solution [5 mM HEPES, 154 mM NaCl, 5.6 mM KCl, 1 mM $MgCl_2$, 3.6 mM Na_2CO_3, 20 mM glucose, and 2.3 mM $CaCl_2$ (pH 7.4)] at 37 °C. For the binding studies, the coverslips were mounted on a coverslip carrier and the cells were incubated for 15 min with 300 μL of Locke's solution containing different concentrations of ligand (1, 2.5, 5, 10, 20, 50, and 100 nM Mu-Alexa**)**. The concentration of bound ligand ($[L^*]_{bound}$) can be calculated with the following equation:

$$[L^*]_{bound} = \frac{[L^*]_0 + B_{max} + K_D \pm \sqrt{([L^*]_0 + B_{max} + K_D)^2 - 4[L^*]B_{max}}}{2} \quad (5)$$

where $[L^*]_0$ is the total amount of ligand in the sample, B_{max} is the total number of binding sites in the sample, and K_D is the dissociation constant of the dye-labeled ligand.

By plotting $[L^*]_{bound}$ versus $[L^*]_0$, we could obtain the parameters K_D and B_{max} for each cell by nonlinear curve fitting to eq 5.

To determine the level of nonspecific binding, 7.5 nM Mu-Alexa was added to cells which had been preincubated with 10 μM muscimol hydrobromide or 100 μM bicuculline methiodide for 30 min. A time-dependent displacement experiment was performed using 10 μM muscimol hydrobromide and cells preincubated with 7.5 nM Mu-Alexa for 10 min. The decrease in the fraction of bound Mu-Alexa was plotted against time, as shown in the following model:

$$[L]_{bound} = y_0 + ae^{-tk_{diss}} \quad (6)$$

where $[L]_{bound}$ is the amount of the receptor−ligand complex in percent, a is the amplitude, y_0 denotes the level of nonspecific binding of Mu-Alexa, and k_{diss} is the rate constant of dissociation of the receptor−ligand complex.

To measure the allosteric modulatory influence of the benzodiazepine agonist, midazolam, on the binding properties of Mu-Alexa, the hippocampal neurons were preincubated with 7.5 nM Mu-Alexa for 15 min followed by a 15 min incubation with increasing amounts of midazolam (0.75 nM, 7.5 nM, 75 nM, 750 nM, 7.5 μM, and 75 μM).

RESULTS

The ligand Mu-Alexa was obtained by labeling muscimol with Alexa 532 carboxylic acid, succinimidyl ester (Figure 1). The structure of Mu-Alexa was confirmed by HRMS: $[M^+]$ = 722.6162 (observed), 722.6150 (calculated). The purity of Mu-Alexa was 96%, as demonstrated by HPLC analysis.

The K_i values of nonlabeled muscimol and Mu-Alexa were 4.5 ± 1.0 and 8.8 ± 1.8 nM (n = 6), respectively, as analyzed by RRAs using [^{3}H]muscimol as the radioligand and a rat cortex preparation containing the $GABA_A$ receptor (Figure 2). Thus, the bulky dye did not significantly influence the specific binding behavior for binding of Mu-Alexa to the $GABA_A$ receptor.

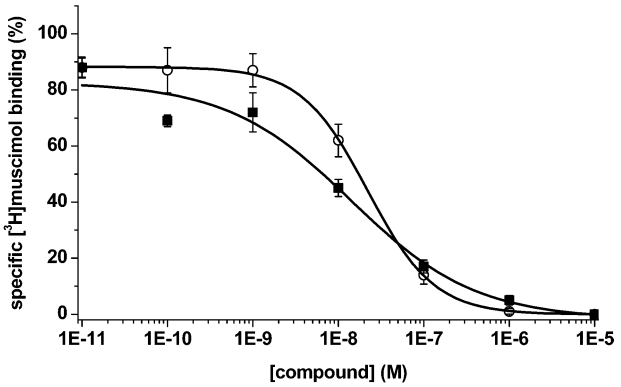

FIGURE 2: Displacement of [^{3}H]muscimol from rat cortex preparations by muscimol hydrobromide (■) and Mu-Alexa (○).

The diffusion time constant and particle number of endogenous cellular components varied between 0.37 and 40.1 ms and between 9.12 and 99.6 particles, respectively, for different hippocampal neurons. In the example depicted in Figure 3A, a diffusion time constant of the fluorescent cell components (τ_D) of 35.6 ms and a particle number (N) of 41 were obtained by fitting the fluorescence fluctuation to eq 4. The average count rate was 0.97 kHz, which results in a count rate of 0.024 kHz/molecule. In the presence of 2.5 nM Mu-Alexa (N = 0.49), a count rate of 6.585 kHz/molecule was obtained. Amplitude autocorrelation signal-to-background ratios of 7, 10, 16, 25, 51, 113, and 185 were observed in the presence of 1, 2.5, 5, 10, 20, 50, and 100 nM Mu-Alexa, respectively.

The illuminated volume element was positioned on the upper membrane of the cell soma of hippocampal neurons. Fifteen minutes after addition of 7.5 nM Mu-Alexa, two diffusion time constants of 3.98 ms (τ_{bound1}) and 74.0 ms (τ_{bound2}) were measured for different states of lateral mobility of the receptor−ligand complex in the cell membrane (Figure 3B). The diffusion time constant τ_{free} of 42 μs for free diffusing Mu-Alexa in solution was measured in independent experiments and was kept constant in all fitting procedures. Incubation of the cells with pure Alexa 532 carboxylic acid, succinimidyl ester did not show any binding of the dye to the cell membrane. Diffusion coefficients were calculated from the diffusion time constants using eq 2 (Table 1): D_{free} = 233 ± 20 $\mu m^2/s$ (n = 66) for free diffusing Mu-Alexa, D_{bound1} = 2.8 ± 0.9 $\mu m^2/s$ (n = 64), and D_{bound2} = 0.14 ± 0.05 $\mu m^2/s$ (n = 56) for different states of mobility of the receptor−ligand complex. Saturation of Mu-Alexa binding was observed at a concentration of approximately 50 nM. The dissociation constant K_D [3.4 ± 0.5 nM (n = 5)] and the maximum number of binding sites B_{max} [18.4 ± 0.4 nM (n = 5)] were obtained from a plot of the bound ligand versus the total amount of the ligand by nonlinear curve fitting (Figure 4). The maximum concentration of bound ligand (B_{max}) is the same as the maximum number of binding sites in the sample and corresponds to receptor density in the cell membrane. For an average observed membrane area of 0.122μm^2, a receptor density in hippocampal neurons (r) of 16.3 ± 2.7 receptors/μm^2 (n = 7) was found.

Following incubation of 7.5 nM Mu-Alexa, 5.78 nM (77%) of the ligand was bound to hippocampal neurons. While 79%

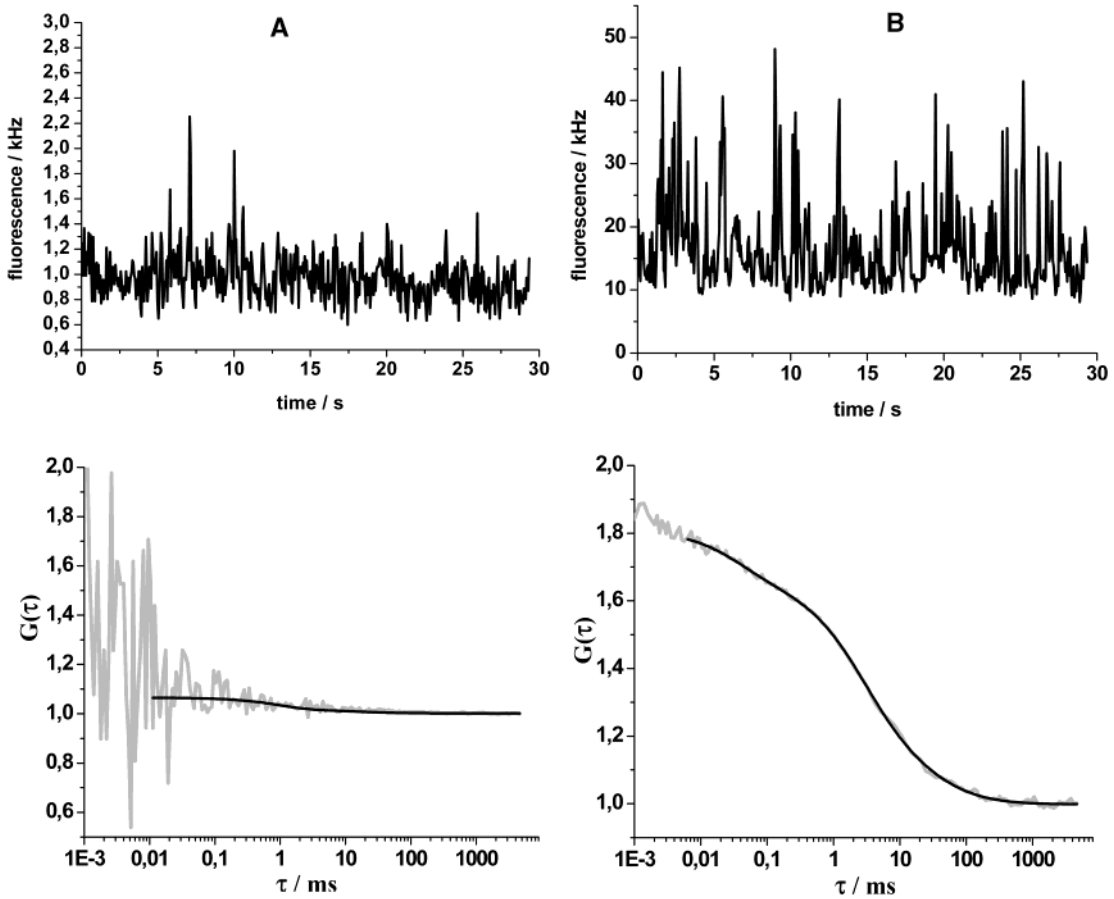

FIGURE 3: Fluorescence time course and corresponding autocorrelation functions for autofluorescence of the cell membrane (A) and in the presence of 7.5 nM Mu-Alexa (B). The autocorrelation functions were fitted by a diffusional model (eq 4). (A) Autofluorescence: $\tau_1 =$ 35.6 ms and $N = 40.58$. (B) Mu-Alexa (7.5 nM): $\tau_1 = 42$ μs (19%), $\tau_2 = 2.9$ ms (66%), $\tau_3 = 39$ ms (15%), and $N = 1.27$.

Table 1: Binding Characteristics of Mu-Alexa

Mu-Alexa or $GABA_A$ receptor−Mu-Alexa complex	diffusion time constant (ms)	diffusion coefficient ($\mu m^2/s$)	binding to the $GABA_A$ receptor (%)	occurrence of lateral mobility of $GABA_A$ receptor−Mu-Alexa complexes (%)
free	0.042	233 ± 20	23	−
free lateral mobility	3.98	2.8 ± 0.9	77	79
hindered lateral mobility	74.0	0.14 ± 0.05		21

of the bound ligand showed a rapid mobility, represented by D_{bound1}, 21% were slow diffusing receptor−ligand complexes, represented by D_{bound2}.

Levels of nonspecific binding of approximately 11 and 13%, respectively, were determined by displacement experiments using a 1000-fold excess of muscimol hydrobromide and a 10000-fold excess of bicuculline methiodide. The diffusion time constants of nonspecifically bound Mu-Alexas were 4.25 and 3.40 ms, respectively.

A time-dependent displacement experiment using muscimol hydrobromide led to a receptor−ligand dissociation rate constant k_{diss} of $(5.37 \pm 0.95) \times 10^{-2}$ s^{-1} ($n = 4$) (Figure 5). A delay time of 40 min was observed. By dividing the rate constant of receptor−ligand complex dissociation (k_{diss}) by the dissociation constant (K_D), we calculated a rate constant of receptor−ligand complex association (k_{ass}) of $(1.57 \pm 0.28) \times 10^7$ L mol^{-1} s^{-1} ($n = 4$). The count rate did not decrease during the measurements, thus verifying that photobleaching did not influence the displacement experiment.

To confirm the allosteric increase in the level of $GABA_A$ receptor binding mediated by benzodiazepines, the behavior for the binding of Mu-Alexa to hippocampal neurons was studied by co-incubating the cells with varying concentrations

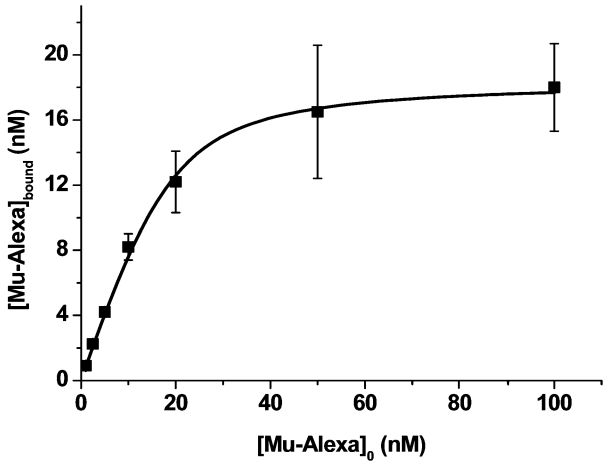

FIGURE 4: Mu-Alexa binding to hippocampal neurons. Averaged bound Mu-Alexa concentration vs the total Mu-Alexa concentration and fitted line according to eq 5. The bound Mu-Alexa fraction was determined from the autocorrelation function for different Mu-Alexa concentrations ($n = 5$).

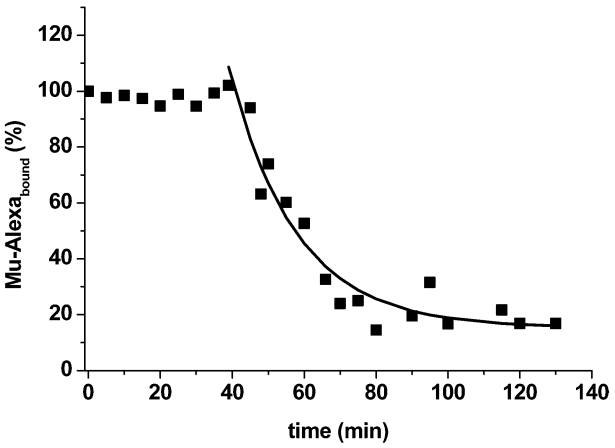

FIGURE 5: Time-dependent displacement of 7.5 nM Mu-Alexa from hippocampal neurons by 10 μM muscimol hydrobromide. The line reflects a monoexponential fit through the experimental data (n = 4).

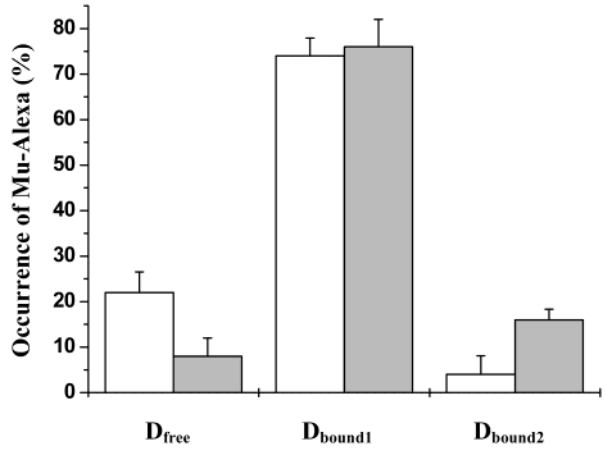

FIGURE 6: GABA$_A$ receptor binding studies on hippocampal neurons. Occurrence of free diffusing Mu-Alexa (D_{free}), lateral mobility (D_{bound1}), and hindered mobility (D_{bound2}) of the receptor−ligand complex (white bars) in the presence of 7.5 nM Mu-Alexa and (gray bars) in the presence of 7.5 nM Mu-Alexa and 7.5 nM midazolam.

of midazolam. The maximal positive cooperative activity of midazolam was observed at a concentration of 7.5 nM and a corresponding Mu-Alexa binding capacity of ~118% ($p < 0.01$, two-tailed t test). A detailed data evaluation of the modulated Mu-Alexa binding properties revealed a selective increase in the number of receptor−ligand complexes with hindered mobility (from 4 to 16%), whereas the free lateral mobility of the receptor−ligand complex (from 74 to 76%) was negligibly influenced by midazolam (Figure 6).

DISCUSSION

We have established a homogeneous GABA$_A$ receptor assay for hippocampal neurons based on FCS using the fluorescently labeled ligand Mu-Alexa. To measure fluorescence intensities at the molecular level, an important parameter is the signal-to-background ratio, especially for experiments with living cells. Background noise may result from scattered laser light, Raman scattering, and spurius fluorescence from the solvent, the optics, and cellular components. FCS binding studies with living cells require a water soluble, fluorescently labeled ligand with a high quantum yield and a high photostability. Because Alexa Fluor 532 fulfills the physical and chemical criteria, we used this dye to lable muscimol, a specific GABA$_A$ agonist (*20*). When using 7.5 nM Mu-Alexa, the signal-to-background ratio was 25 and, therefore, the background fluorescence did not significantly influence the FCS measurements.

A bodipy TMR-X-labeled muscimol derivative (Mu-Bodipy) has previously been used to localize GABA$_A$ receptors in the salamander retina (*21*). The detection of Mu-Bodipy binding on eyecup preparations was evaluated with single-frame images. In our receptor binding studies, we did not use Mu-Bodipy because of the high rate of nonspecific binding of the lipophilic dye moiety to cell membranes. Our results demonstrate specific binding of Mu-Alexa to the GABA$_A$ receptors of hippocampal neurons. The evaluation of a saturation experiment by nonlinear curve fitting yielded a dissociation constant K_D of 3.4 ± 0.5 nM (n = 5) and a maximum number of binding sites B_{max} of 18.4 ± 0.4 nM. Similar results were obtained in binding experiments with [^{3}H]muscimol and crude synaptic membranes of rat brain by RRAs. Dissociation constants (K_D) in the range of 1.75−5.1 nM were found for [^{3}H]muscimol (*2*, *3*, *22*). Bernasconi et al. (*23*) investigated the binding of [^{3}H]muscimol to crude synaptic membranes of mouse brain after treatment with Triton X-100, and they found a dissociation constant K_D of 1.88 nM (*23*).

In FCS experiments using a 1000-fold excess of muscimol hydrobromide, a amount of nonspecific binding of 11% was found for Mu-Alexa, which is similar to the amount of nonspecific binding observed for [^{3}H]muscimol when measured with an RRA (17%) (*3*). It is well-known that muscimol binds to both GABA$_A$ and GABA$_C$ receptors, which are mainly found in the retina (*24*, *25*), but also in hippocampal regions (*26*). For this reason, we performed a displacement experiment using the specific GABA$_A$ antagonist bicuculline methiodide to calculate the level of Mu-Alexa binding to GABA$_C$ receptors (*20*). After treatment with bicuculline methiodide, the amount of nonspecific Mu-Alexa binding was in the same order of magnitude as found by the displacement with muscimol hydrobromide. Although we cannot exclude the possibility of minor binding activity of Mu-Alexa to GABA$_C$ receptors, one can assume that GABA$_A$ receptor binding studies on hippocampal neurons were not significantly influenced by interactions between Mu-Alexa and the other binding site. A time-dependent displacement of Mu-Alexa, achieved by using an excessive amount of muscimol hydrobromide, revealed a receptor−ligand dissociation rate constant k_{diss} of (5.37 ± 0.95) × 10^{-2} s^{-1}. Remarkably, the displacement was observed with a delay time period of 40 min, which can be explained by the hindered dissociation of the bound ligand from the receptor caused, for example, by clustering and internalization of the receptor−ligand complex (*14*, *27*). Delay time periods of approximately 25 and 10 min were also found in displacement experiments with the EGF receptor and benzodiazepine receptor (*28*, *29*). The rate constant of complex association (k_{ass}) was calculated with the k_{diss}/K_D ratio to be (1.57 ± 0.28) × 10^7 L mol^{-1} s^{-1}. Binding studies on crude synaptic membranes of the rat brain revealed both the rate constants (k_{ass} = 1.10 × 10^6 L mol^{-1} s^{-1} and k_{diss} = 1.78 × 10^{-3} s^{-1}) for [^{3}H]muscimol (*22*).

The interactions of Mu-Alexa with hippocampal neurons can be characterized by three different diffusion coefficients.

Because of the monoexponential curve fitting of the time-dependent displacement experiment, only one dissociation constant was observed for one distinct $GABA_A$ binding site. Besides the free diffusing ligand ($D_{free} = 233 \pm 20$ $\mu m^2/s$) in the extracellular solution, two diffusion coefficients ($D_{bound1} = 2.8 \pm 0.91$ $\mu m^2/s$ and $D_{bound2} = 0.14 \pm 0.05$ $\mu m^2/s$) were found for different states of receptor−ligand complexes localized in the cell membrane. D_{bound1} can be assigned to the free lateral mobility of the receptor−ligand complex, because similar diffusion coefficients for the movement of transmembrane proteins within the cell membrane have been previously reported by others (*9*, *10*, *30*). D_{bound2} can be assigned to several receptor−ligand complexes, which may exist in different states of mobility, e.g., in a hindered state of mobility. This may be caused by an association with cytoskeleton proteins possessing diffusion coefficients between 0.19 and 4.4×10^{-2} $\mu m^2/s$ (*11*, *12*), a clustered localization in clathrin-coated pits (*13*, *14*, *27*), internalization via clathrin-dependent endocytosis (*31*), or an association with lipid rafts with a D of 1 $\mu m^2/s$ (*9*, *10*), as well as with a reduced mobility inside the lipid rafts (maximum $D \leq 5 \times 10^{-2}$ $\mu m^2/s$) (*9*). "Immobile fractions" for proteins in cell membrans, often detected during FRAP experiments, cannot be detected by FCS, and therefore, we cannot exclude the presence of an immobile receptor moiety.

The allosteric modulation of the Mu-Alexa binding properties by the benzodiazepine agonist, midazolam, is shown to verify the binding behavior of Mu-Alexa, which was obviously not influenced significantly by the bulky dye. Midazolam was used in the FCS experiments because of its high water solubility and a low intrinsic fluorescence in comparison to those of other benzodiazepines. In the presence of 7.5 nM midazolam, the amount of Mu-Alexa binding increased by ~118.4%, which is in accordance with the results provided by RRA studies investigating benzodiazepine-modulated binding of $GABA_A$ receptor radioligands. Using brain homogenates, 44 and 30% enhancements of the [^{3}H]GABA binding mediated by diazepam and quazepam were observed (*32*, *33*). Bristow and Martin (*34*) showed an increase of ~20% in the amount of [^{3}H]GABA binding to rat brain by co-incubation with 1 μM flunitrazepam (*34*). Remarkably, midazolam selectively increases the number of receptor−ligand complexes with hindered mobility. It may be assumed that one of these complexes is part of the chloride channel, which shows an increased influx of chloride ions mediated by midazolam.

In this study, we demonstrate the potential of the FCS technique as a highly sensitive and noninvasive method for investigating receptor−ligand binding properties on living cells. In addition, this technique is suitable for measuring the lateral mobility of cellular components, e.g., receptors. The simultaneous discrimination of different states of mobility of the receptor−ligand complex in the cell membrane, as provided by FCS, creates a new approach to comparing the mobility of such receptor−ligand complexes with subcellular mechanisms of receptor regulation and signaling.

ACKNOWLEDGMENT

We thank Prof. Dr. H. Lüddens and K. Dämgen for giving us the opportunity to perform the RRA experiments in their laboratory and Dr. C. Culmsee and S. Engel for placing the hippocampal neurons at our disposal.

REFERENCES

1. Bennett, J. P., Jr., and Yamamura, H. I. (1985) in *Neurotransmitter Receptor Binding* (Yamamura, H. I., Enna, S. J., and Kular, M. J., Eds.) pp 61−89, Raven Press, New York.
2. Jordan, C. C., Matus, A. I., Piotrowski, W., and Wilkinson, D. (1982) *J. Neurochem. 39*, 52−58.
3. Beaumont, K., Chilton, W. S., Yamamura, H. I., and Enna, S. J. (1978) *Brain Res. 148*, 153−162.
4. Widengren, J., and Rigler, R. (1998) *Cell. Mol. Biol. 44*, 857−879.
5. Klingler, J., and Friedrich, T. (1997) *Biophys. J. 73*, 2195−2200.
6. Van Craenenbroeck, E., and Engelborghs, Y. (1999) *Biochemistry 38*, 5082−5088.
7. Boonen, G., Pramanik, A., Rigler, R., and Haberlein, H. (2000) *Planta Med. 66*, 7−10.
8. Pramanik, A., Olsson, M., Langel, U., Bartfai, T., and Rigler, R. (2001) *Biochemistry 40*, 10839−10845.
9. Pralle, A., Keller, P., Florin, E. L., Simons, K., and Horber, J. K. (2000) *J. Cell Biol. 148*, 997−1008.
10. Schutz, G. J., Kada, G., Pastushenko, V. P., and Schindler, H. (2000) *EMBO J. 19*, 892−901.
11. Velazquez, J. L., Thompson, C. L., Barnes, E. M., Jr., and Angelides, K. J. (1989) *J. Neurosci. 9*, 2163−2169.
12. Peran, M., Hicks, B. W., Peterson, N. L., Hooper, H., and Salas, R. (2001) *Cell Motil. Cytoskeleton 50*, 89−100.
13. Essrich, C., Lorez, M., Benson, J. A., Fritschy, J. M., and Luscher, B. (1998) *Nat. Neurosci. 1*, 563−571.
14. Baer, K., Essrich, C., Benson, J. A., Benke, D., Bluethmann, H., Fritschy, J. M., and Luscher, B. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 12860−12865.
15. Kittler, J. T., Wang, J., Connolly, C. N., Vicini, S., Smart, T. G., and Moss, S. J. (2000) *Mol. Cell. Neurosci. 16*, 440−452.
16. Simons, K., and Toomre, D. (2000) *Nat. Rev. Mol. Cell Biol. 1*, 31−39.
17. Thompson, N. L. (1991) in *Topics in Fluorescence Spectroscopy* (Lakowicz, J. R., Ed.) pp 337−378, Plenum Press, New York.
18. Marquardt, D. W. (1963) *J. Soc. Ind. Appl. Math. 11*, 431−441.
19. Mattson, M. P., Cheng, B., Culwell, A. R., Esch, F. S., Lieberburg, I., and Rydel, R. E. (1993) *Neuron 10*, 243−254.
20. Johnston, G. A. (1996) *Trends Pharmacol. Sci. 17*, 319−323.
21. Wang, H., Standifer, K. M., and Sherry, D. M. (2000) *Visual Neurosci. 17*, 11−21.
22. Williams, M., and Risley, E. A. (1979) *J. Neurochem. 32*, 713−718.
23. Bernasconi, R., Bittiger, H., Heid, J., and Martin, P. (1980) *J. Neurochem. 34*, 614−618.
24. Cutting, G. R., Lu, L., O'Hara, B. F., Kasch, L. M., Montrose-Rafizadeh, C., Donovan, D. M., Shimada, S., Antonarakis, S. E., Guggino, W. B., Uhl, G. R., et al. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2673−2677.
25. Lukasiewicz, P. D., and Shields, C. R. (1998) *Semin. Cell Dev. Biol. 9*, 293−299.
26. Enz, R., Brandstatter, J. H., Hartveit, E., Wassle, H., and Bormann, J. (1995) *Eur. J. Neurosci. 7*, 1495−1501.
27. Craig, A. M., Blackstone, C. D., Huganir, R. L., and Banker, G. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 12373−12377.
28. Pramanik, A., and Rigler, R. (2001) *Biol. Chem. 382*, 371−378.
29. Hegener, O., Jordan, R., and Häberlein, H. (2002) *Biol. Chem.* (in press).
30. Peters, R., and Cherry, R. J. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4317−4321.
31. Kittler, J. T., Delmas, P., Jovanovic, J. N., Brown, D. A., Smart, T. G., and Moss, S. J. (2000) *J. Neurosci. 20*, 7972−7977.
32. Skerritt, J. H., Willow, M., and Johnston, G. A. (1982) *Neurosci. Lett. 29*, 63−66.
33. Corda, M. G., Sanna, E., Concas, A., Giorgi, O., Ongini, E., Nurchi, V., Pintori, T., Crisponi, G., and Biggio, G. (1986) *J. Neurochem. 47*, 370−374.
34. Bristow, D. R., and Martin, I. L. (1989) *Eur. J. Pharmacol. 173*, 65−73.

BI0263356