Dye-Labeled Benzodiazepines: Development of Small Ligands for Receptor Binding Studies Using Fluorescence Correlation Spectroscopy

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To investigate benzodiazepine receptor binding studies by fluorescence correlation spectroscopy (FCS), the four fluorophores fluorescein, tetramethylrhodamine, Oregon Green 488, and Alexa 532 were coupled to the benzodiazepine Ro 07-1986/602 (Ro). Binding assays to polyclonal antibodies to benzodiazepines and at the native benzodiazepine receptor on the membrane of rat hippocampal neurons were established to examine the dye-labeled ligands for their benzodiazepine character and their binding behavior. Both the fluorescein and the Oregon Green488 moiety led to a loss of the benzodiazepine receptor binding of the corresponding Ro derivatives. Antibody recognition and interactions to the receptor were observed for the tetramethylrhodamine derivative ($K_D = 96.0 \pm 9.5$ nM) but with a high amount of nonspecific binding at the cell membrane of about 50%. In saturation experiments a K_D value of 97.2 ± 8.5 nM was found for the Alexa Fluor 532 derivative-antibody interaction. Investigation of the binding of this ligand to the benzodiazepine receptor in FCS cell measurements led to confrimation of high specific binding behavior with a K_D value of 9.9 ± 1.9 nM. A nonspecific binding of <10% was observed after coincubation with 1 μM of midazolam. The different properties of the labeled benzodiazepine derivatives and the requirements of the fluorophore in small dye-labeled ligands in FCS binding studies, at the membrane of living cells, are discussed.

Introduction

The investigation of ligand receptor interactions is one of the main tools in pharmacological research. Conventional methods, using, for example, radioactively labeled ligands, are being increasingly replaced by steadily improved fluorescence techniques. Fluorescence correlation spectroscopy (FCS) allows the investigation of molecular interactions in solution and in biological systems, e.g. in living cells.[1−4] (For a review of FCS applications to biological and chemical systems, see also Hess et al., 2002[5]). In a confocal volume element of about 0.1−1 fl, fluorophores are excited with a focused laser beam and the fluorescence intensity is detected.[6−8] The autocorrelation analysis of the fluorescence fluctuation over the time results in a diffusion time constant and a particle number of the observed fluorescent molecule. If the diffusion coefficients differ sufficiently, the signal allows fast and slow diffusing molecules to be differentiated.[9−11] A major requirement for FCS experiments is a well suited fluorophore, but very few structures under investigation emit sufficient endogenous fluorescence. Thus, there is a need for introducing suitable dye moieties into ligand structures.

Drugs with binding activity at the benzodiazepine receptor are of important clinical use in the therapy of insomnia, anxiety, muscle tension, epileptogenic activity, and fear.[12] This wide therapeutical use is due to the spectrum of actions initiated by drugs, which act at the modulatory benzodiazepine binding site, at the chloride channel in the central nervous system (CNS). Recent results have brought out a deeper insight into the influence of regulative processes after ligand binding to the benzodiazepine receptor.[13,14] To elucidate the subsequent dynamics of receptor regulation in living cells, a highly sensitive and noninvasive method like FCS is capable of providing a better understanding into the involved mechanisms. Ligands used for benzodiazepine receptor binding studies are of small molecular weight far below 1 kDa.[15,16] For the development of small dye-labeled ligands, the selection of an optimal fluorophore is of high relevance. Particularly in small molecules the introduced dye can crucially affect the functionality by intramolecular influences.[17] Measurements of interactions to specific antibodies is a fast and reliable method to detect, for example, an altered binding behavior of the dye-labeled ligand. The recognition of small nonimmunogenic structures by antibodies has been well characterized.[18] Immobilized antibodies are in routine use in diverse immunoassay techniques to quantify low concentrations of small molecules.[19,20]

This work describes the binding characteristics of the four dye-labeled Ro 07-1986/602 (Ro) derivatives, fluorescein-Ro (Flu−Ro), tetramethylrhodamine-Ro (TMR−Ro), Oregon Green 488-Ro (OG−Ro), and Alexa Fluor 532-Ro (Alexa−Ro), both to a polyclonal antibody to benzodiazepines and to the benzodiazepine receptor at

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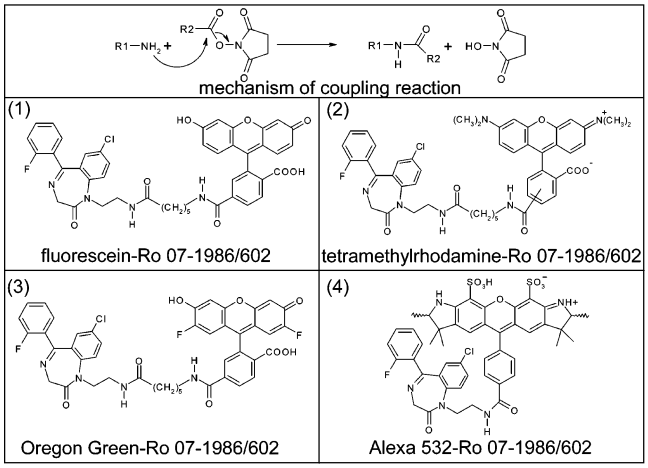

Figure 1. Synthesis and structures of the dye-labeled Ro 07-1986/602 derivatives.

hippocampal neurons to select an optimal fluorescent dye for cell measurements.

Results

Four dye-labeled benzodiazepine derivatives were synthesized by reaction of Ro with each of 6-(fluorescein-5-carboxamido)hexanoic acid, succinimidyl ester (Flu), 6-(tetramethylrhodamine-5-(and-6)-carboxamido)hexanoic acid, succinimidyl ester, mixed isomers (TMR), Oregon Green 488-X, succinimidyl ester 6-isomer (OG), and Alexa Fluor 532 carboxylic acid, succinimidyl ester (Alexa) (Figure 1). The purity of the products was analyzed by HPLC and was between 92 and 95%. Neither the nonlabeled Ro nor the free dyes contributed to the impurities in the four isolated products by HPLC. The identity of each compound was confirmed by high-resolution mass spectrometry. The relative quantum yield of the ligand in comparison to the corresponding dyes was 1.08 for FLU−Ro/FLU, 0.81 for TMR−Ro/TMR, 1.09 for OG−Ro/OG, and 1.04 for Alexa−Ro/Alexa. To test the benzodiazepine character of the dye-labeled compounds, an antigen antibody reaction was performed using a polyclonal antibody against benzodiazepines. The binding behavior to the benzodiazepine receptor was investigated by FCS experiments using prenatal rat hippocampal neurons. Radioligand binding studies with [^{3}H]flunitrazepam ([^{3}H]FNT) were performed to confirm the specific benzodiazepine recognition by the polyclonal antibody (Figure 2C). The antigen binding sites of a 1:500 dilution of the antibody preparation were saturated at a [^{3}H]FNT concentration of approximately 1 μM. A K_D value of 273 nM was calculated. A nonspecific binding of about 23% was found by displacement experiments using a 1000-fold excess of midazolam. The scatchard analysis of the binding data followed a characteristic polynomial shape, indicating the polyclonal character of the used antibody with nonuniform binding affinity to the antigen (insets in Figure 2A−C). In FCS experiments no interactions between the antibody and Flu−Ro were detected within the concentration range of 1 nM and 100 nM. Additionally Flu−Ro showed strong photobleaching during experiments at a laser power of 3.9−12.5 μW/cm^2, which demonstrated a low photostability of the dye. The more photostable TMR coupled to Ro revealed a dye labeled

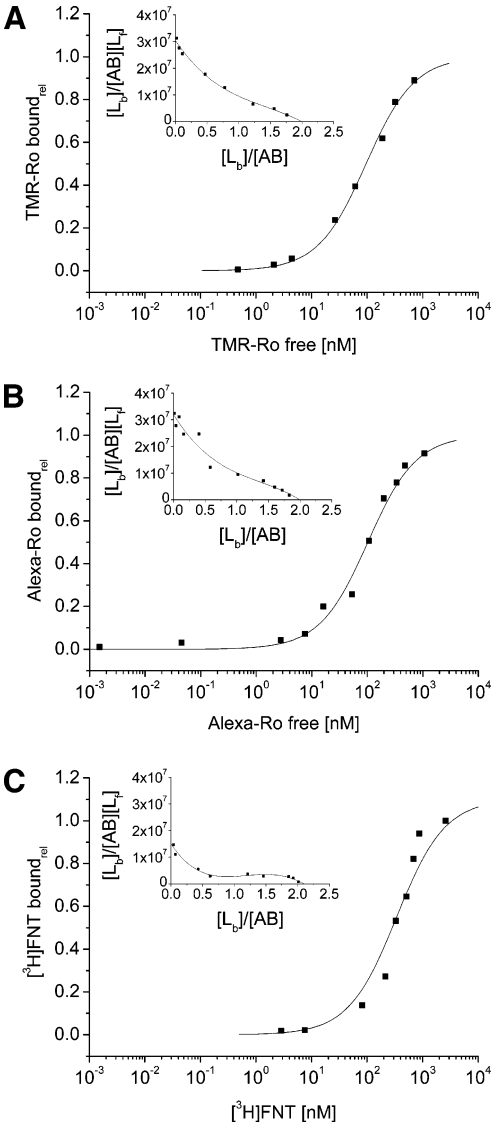

Figure 2. Graphical representation of the binding of TMR−Ro (A), Alexa−Ro (B), and [^{3}H]FNT (C) to the polyclonal antibody to benzodiazepines (dilution: 1:500): abscissa, concentration of the free ligand [L_f] in equilibrium; ordinate, relative binding of the ligand to the antibody to B_{max}. The binding constants K_D were found to be 96.0 ± 9.5 nM for TMR−Ro, 97.2 ± 27.5 nM for Alexa−Ro, and 272.7 ± 53.7 nM for [^{3}H]FNT. The nonlinear scatchard plots (insets) of [L_b]/[AB]-[L_f] vs [L_b]/[AB] calculated by eq 6, demonstrate the nonuniform binding affinity of the polyclonal antibody to the antigen.

ligand which showed a minor photobleaching and which was specifically recognized by the antibody with a K_D = 96.0 ± 9.5 nM (Figure 2A). An almost unchanged relative quantum yield of 0.92 for the TMR−Ro/antibody-complex could be found. Representative FCS binding curve for TMR−Ro bound to the antibody is given in Figure 2A.

In FCS cell measurements the TMR−Ro binding to hippocampal neurons was not saturable within the concentration range of 1 nM to 1 μM (n = 3). Displacement of 20 nM TMR−Ro with a 1000-fold excess of midazolam resulted in a nonspecific binding of about 50% (n = 3), which suggests a strong interaction between the lipophilic dye moiety and the biomembrane of the cells. To avoid high nonspecific binding, the hydrophilic and similar photostable Oregon Green 488

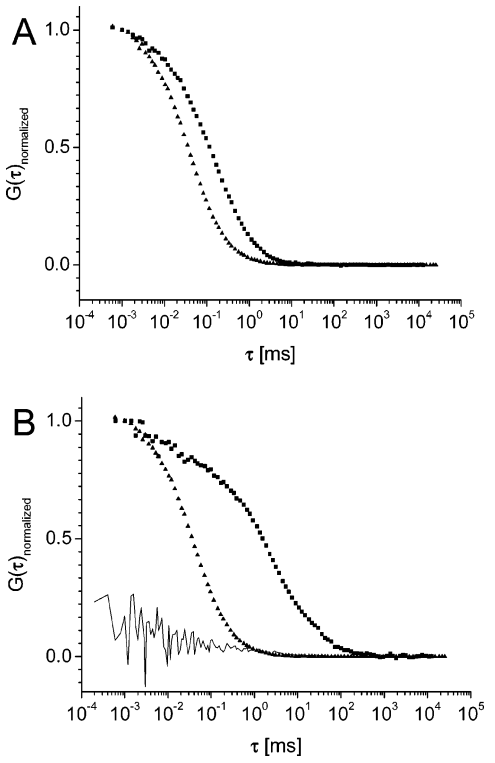

Figure 3. Normalized autocorrelation curves of FCS binding experiments. (A) Free Alexa−Ro (▲) shows a diffusion time constant (τ_D) of 41 μs. Alexa−Ro bound to the polyclonal antibody to benzodiazepines (■) revealed a τ_D of 322 μs. (B) Free Alexa−Ro (▲) again with the τ_D of 41 μs. Alexa−Ro bound to the benzodiazepine receptor at the membrane of the hippocampal neuron (■) revealed two different diffusion time constants of 7.2 ms and 362 ms. The solid line represents the relative autofluorescence background signal of the non incubated cell (—).

was coupled to Ro. In FCS binding studies OG−Ro did not show any interaction to the cell membrane of the neurons ($n = 3$) and neither was it recognized by the antibody ($n = 10$). Both an antibody recognition $K_D = 97.2 \pm 27.5$ nM ($n = 10$) (Figure 2B) and a high specific binding to the benzodiazepine receptor at the neurons were found for Alexa−Ro. (rel Q_i for the Alexa−Ro/antibody-complex: 0.91) An incubation of the hippocampal neurons with 2 nM Alexa−Ro led to a 90% binding of the ligand ($n = 7$) (Figure 3).

Two different mobilities of the receptor ligand complex within the cell membrane were found by a detailed evaluation of the FCS experiments. The diffusion coefficients $D_{bound1} = (1.32 \pm 0.26)\ \mu m^2/s$ ($n = 20$, obtained from three to four cells from seven different cell preparations) and $D_{bound2} = (2.63 \pm 0.63) \times 10^{-2}\ \mu m^2/s$ ($n = 22$, obtained from three to four cells from seven different cell preparations) were calculated from the observed average diffusion time constants of 7.2 ms and 361 ms.

Free diffusing Alexa−Ro showed a diffusion coefficient of $D_{free} = (2.34 \pm 0.63) \times 10^2\ \mu m^2/s$, respectively, a diffusion time constant of 41 μs (Figure 3B). The receptor binding was saturated at a concentration of about 120 nM, and a K_D value of (9.9 ± 1.9) nM was obtained (Figure 4). A nonspecific binding of about 7−10% was found by displacing Alexa−Ro by midazolam ($n = 5$).

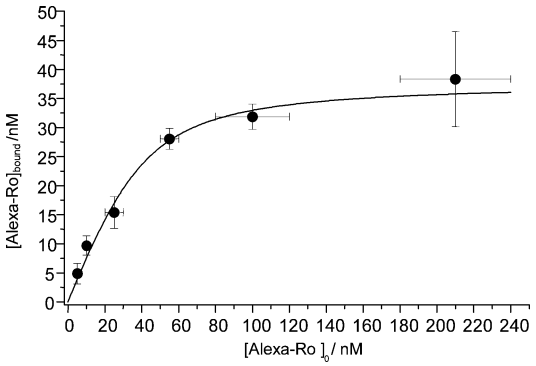

Figure 4. Averaged bound Alexa−Ro concentration (recalculated in particles/volume) versus the total Alexa−Ro concentration and fitted line according to eq 5. The bound Alexa−Ro fraction was determined from the autocorrelation function for different Alexa−Ro concentrations. Each point is the average of seven experiments (mean ± SE).

Discussion

In contrast to conventional radioligand receptorassay techniques, fluorescence methods based on confocal microscopy, like FCS, have shown several advantages.[24] A high sensitivity for the detection of single molecules and no need for separation to distinguish between free and bound ligand are the major ones for the investigation of ligand−receptor interactions. Further, FCS is a noninvasive technique that allows measurements on complex biological systems, particularly on living cells.[11,25−27] A major requirement for FCS investigations is the availability of a suitable dye-labeled ligand, since most structures of interest do not show sufficient native fluorescence. The fluorophores commonly used for the labeling procedure differ widely both in their photochemical properties and in their influence on the binding behavior of the ligand.[17,28] One can expect an increasing influence, the smaller the labeled molecule is.

Thus the functionality of the four synthesized dye-labeled ligands were proven by antibody recognition and receptor binding studies on living cells. By the coupling of Ro to fluorescein (Flu), the recognition by the polyclonal antibody to benzodiazepines and the binding affinity to the benzodiazepine receptor of hippocampal neurons were lost. Additionally a high photobleaching during the FCS measurements was observed, which led alternatively to the use of the more photostable tetramethylrhodamine (TMR). Because of the different substitution pattern of this xanthene analogue, TMR−Ro was actually bound both by the benzodiazepine antibody with a K_D value of 96.0 ± 9.5 nM (Figure 2A) and by the benzodiazepine receptor of the neurons. Because of the strong lipophilic character of TMR, high nonspecific binding of about 50% to the cell membrane was found, which limits the use of this ligand in receptor binding studies. To avoid this effect, a more hydrophilic dye, Oregon Green 488 (OG), was coupled to the benzodiazepine. Remarkably, OG−Ro did not show any affinity to the antibody nor to the benzodiazepine receptor. The strong structural similarity of OG to Flu (Figure 1) suggests that the oxygen functions at the xanthene moiety prevent a binding to the benzodiazepine antibody and receptor. Therefore, Alexa Fluor 532 (Alexa) was chosen, because of its hydrophilic character and a substitution pattern free of the hydroxyl and keto functions. A K_D value of 97.2 ± 27.5 nM was found for

the antibody recognition of Alexa−Ro (Figure 2B), comparable to TMR−Ro (Figure 2A). In FCS binding studies, where only single molecules are excited by the laser beam, the quantum yield of the bound dye-labeled ligand should be high enough to distinguish the emitted fluorescence light from the autofluorescence of an antibody or of the cell membrane. An almost unchanged relative quantum yield of 0.91 was found for the Alexa−Ro/antibody complex by detecting light quanta per complex in comparison to the measured light quanta per free diffusing Alexa−Ro molecule in solution which suggested that the fluorescence intensity of Ro-Alexa was not quenched significantly after antibody binding. The benzodiazepine receptor binding at the neurons was saturated at a concentration of about 120 nM, and a K_D value of 9.9 ± 1.9 nM was obtained (Figure 4). In contrast to TMR−Ro, an acceptable nonspecific binding of about 7−10% was found for Alexa−Ro in cell experiments by displacement with midazolam. A detailed evaluation of the specific Alexa−Ro receptor interaction at the hippocampal neurons revealed two different mobilities of the receptor−ligand complex within the cell membrane. Standard operations such as parametrization and fitting of the autocorrelation function $G(\tau)$, performed with nonlinear least squares minimization according to the Marquardt algorithm,[29] were implemented in the FCS evaluation program. The quality of the fit was judged by the χ^2 criterion which was 0.019493 for the three-component model which suggested free diffusing Alexa−Ro and two Alexa−Ro−benzodiazepine receptor complexes with different lateral mobilities. In contrast, the fit of a two-component model representing free diffusing Alexa−Ro and only one Alexa−Ro−benzodiazepine receptor complex with a distinct lateral mobility revealed a 2-fold higher χ^2 value of 0.039914, which clearly indicated that the three component model was more appropriate to fit the data. Thus, diffusion coefficients of $D_{bound1} = (1.32 \pm 0.26)$ $\mu m^2/s$ and $D_{bound2} = (2.63 \pm 0.63) \times 10^{-2}$ $\mu m^2/s$ were calculated from the two diffusion time constants for different states of receptor ligand complexes. Competition experiments suggested the same binding affinity for both the receptor ligand complex with D_{bound1} and D_{bound2} by a monophasic dissociation behavior (data not shown). Recently, two different lateral mobilities ($D_{bound1} = (2.8 \pm 0.9)$ $\mu m^2/s$ and $D_{bound2} = (0.14 \pm 0.05)$ $\mu m^2/s$) of $GABA_A$ receptors occupied with a fluorescently labeled muscimol derivative was found on hippocampal neurons. An increase in the level of specific ligand binding was demonstrated by the positive cooperative activity of coincubated midazolam, which was selectively found in $GABA_A$ receptor ligand complexes with D_{bound2}.[30] Obviously, distinct states of mobilities of the receptor ligand complex can be differentiated by FCS, which depend on regulatory processes and which may lead to a clearer insight into the subcellular mechanisms of receptor regulation and signaling. A similar observation was made by Pramanik et al., 2001, describing two different diffusion time constants of about 22 ms and 700 ms for the occupied galanin receptor in the membrane of human insulinoma cells.[31]

Ro has been used previously for the synthesis of fluorescent benzodiazepine derivatives for the labeling with different dyes.[17,32] K_i values in the nanomolar range were found, describing a high binding affinity to the benzodiazepine receptor in competition experiments using radioreceptor assays. Janssen et al., 2000, investigated several Ro derivatives and k_i values of 4.9 nM for the nonlabeled Ro, 67 nM for a Bodipy-FL-derivative, 51 nM for a 7-nitrobenz-2-oxa-1,3-diazol-4-yl derivative (NBD-Ro), and in contrast to our results a k_i value of 74 nM for the fluorescein derivative was found. In radioreceptor assays they used calf brain homogenates and a fluorescein-labeled Ro derivative which was characterized by a spacer of three methylene groups between the benzodiazepine moiety and the dye moiety. In our FCS binding studies the spacer of Flu−Ro consisted of five methylene groups. The differences in the method of investigation and in the tissue and ligand that were used may explain the different findings in the receptor binding studies. In fluorescence recovery after photobleaching experiments Velazquez et al., 1989, used the NBD-Ro derivative to determine the diffusion behavior of the benzodiazepine receptor in the membrane of rat spinal cord neurons. They confirmed the specific NBD-Ro binding to the benzodiazepine receptor by a k_i value of 79 nM, obtained in radioreceptor assays. In FCS experiments, where the dye-labeled ligand is used in nanomolar concentrations, NBD, fluorescein, and bodipy FL are not recommended because of the fluorescence quenching in aqueous solutions and the low photostability. Therefore, in addition to a retained and proven functionality, the specificity of the derivative is essential for performing experiments, particularly in complex biological systems. The use of a water-soluble, hydrophilic, and photostable fluorophore is recommended for the synthesis of dye-labeled ligands to perform receptor binding studies on living cells and to observe subsequent dynamics after receptor occupation. We have shown by careful characterization of dye-labeled ligands that an optimization of the ligand properties can be achieved for the use in FCS experiments, even in complex biological systems.

Experimental Section

Synthesis, Purification, and Identification of Ligands. Ro 07-1986/602 (Ro) (Novartis; Basel, Switzerland) ((1) 5.40 mg, (2) 3.14 mg, (3) 0.44 mg, (4) 0.176 mg) and (1) 4.99 mg of 6-(fluorescein-5-carboxamido)hexanoic acid, succinimidyl ester, (2) 5.4 mg of 6-(tetramethylrhodamine-5-(and-6)-carboxamido)-hexanoic acid, succinimidyl ester, mixed isomers, (3) 1.00 mg of Oregon Green 488-X, succinimidyl ester, 6-isomer, and (4) 0.100 mg of Alexa Fluor 532 carboxylic acid, succinimidyl ester (all fluorescent dyes: Molecular Probes; Leiden, Netherlands) were dissolved separately in 0.5 to 1 mL ethanol (99.5%) (Merck; Darmstadt, Germany). While stirring the benzodiazepine solution, the dye was added dropwise. The stirring was continued for at least 12 h. The reaction mixtures 2, 3, and 4 were purified by high performance liquid chromatography (HPLC) (column: LiChrospher RP-select B (5 μm, 4 × 125 mm), (Merck; Darmstadt, Germany)), eluents: (2): mixture of acetonitrile (55%), H_2O (8%), methanol (36%), and H_3PO_4 85% (1%) (pH = 2.00), flow rate: 1 mL/min; (3): mixture of acetonitrile (39%), H_2O (40%), methanol (20%), and H_3PO_4 85% (1%) (pH = 1.97), flow rate: 1 mL/min; (4): mixture of acetonitrile (27.1%), H_2O (64.4%), methanol (8%), and H_3PO_4 85% (0.5%) (pH = 1.93), flow rate: 0.9 mL/min (all solvents: HPLC grade (Merck; Darmstadt, Germany), water was taken from Millipore Milli-Q System (Millipore GmbH; Eschborn, Germany)), detection: 254 nm parallel to 488 nm (OG and Flu) or 514 nm (TMR and Alexa). The calculated yield was found to be between 12 and 30%. Identity was confirmed by UV−vis

spectroscopy and high-resolution mass spectrometry for OG−Ro (*N*-(5-{2-[7-chloro-5-(2-fluorophenyl)-2-oxo-2,3-dihydro-benzo[*e*][1,4]diazepin-1-yl]ethylcarbamoyl}pentyl)-2-(2,7-difluoro-6-hydroxy-3-oxo-3*H*-xanthen-9-yl)terephthalamic acid) (810.2900 measured; 810.3067 calcd), for TMR−Ro ({9-[2-carboxy-5-(5-{2-[7-chloro-5-(2-fluorophenyl)-2-oxo-2,3-dihydro-benzo[*e*][1,4]-diazepin-1-yl]ethylcarbamoyl}pentylcarbamoyl)phenyl]-6-(dimethylamino)xanthen-3-ylidene}dimethylammonium) (measured 858.4050; 858.3978 calcd), and for Alexa−Ro (5-(3-{2-[7-chloro-5-(2-fluorophenyl)-2-oxo-2,3-dihydro-benzo[*e*][1,4]-diazepin-1-yl]ethylcarbamoyl}phenyl)-2,3,3,7,7,8-hexamethyl-10,12-disulfo-3,7,8,9-tetrahydro-2*H*-11-oxa-9-aza-1-azonia-dicyclopenta[*b,i*]anthracene) (939.5041 measured; 939.5093 calcd). Reaction mixture 1 was purified by high performance thin-layer chromatography (HPTLC) (HPTLC plates Si-60 w/o F254 indication, 20 × 20 cm, 2 mm layer (Merck; Darmstadt, Germany), eluent: mixture of methanol (55%) and chloroform (45%) (all solvents: HPLC grade, Merck; Darmstadt, Germany). Detection was performed by parallel HPTLC analysis of pure (6-(fluorescein-5-carboxamido)hexanoic acid, succinimidyl ester, and Ro. Identity of Flu−Ro(*N*-(5-{2-[7-chloro-5-(2-fluorophenyl)-2-oxo-2,3-dihydro-benzo[*e*][1,4]diazepin-1-yl]ethylcarbamoyl}pentyl)-2-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)terephthalamic acid) was confirmed by HRMS (measured 811.2390; 811.2346 calcd).

Fluorescence Correlation Spectroscopy. Experimental Setup. FCS measurements were performed in a ConfoCor instrument (Zeiss; Jena, Germany). The sample was excited with the 488 or 514 nm line of an argon ion laser (LGK 7812 ML 2,Lasos; Jena, Germany) through a water immersion objective (C-Apochromat, 63x, NA 1.2, Zeiss; Jena, Germany). The power density in the focal plane, measured before the objective, was $p_{488\ nm} = 12.5$ kW/cm^2 and $p_{514\ nm} = 14.2-109$ kW/cm^2 (confocal volume element: 0.2 fl). The emitted fluorescence was collected by the same objective. After passing a dichroic mirror, a band-pass filter, and a pinhole, the emitted photons were detected by an avalanche photodiode (SPCM-AQ Series, EG & G Optoelectronics Canada Inc.; Vaudreuil, Quebec, Canada). The SPCM had a decay time of 32 ns (max. 60 ns). With a dark count rate of max. 140 Hz and a max. count rate of 10 MHz, the measurements all were within the sample range of the module. The fluorescence signal was correlated with a digital hardware correlator (ALV-5000, ALV; Langen, Germany).

Data Analysis. In a three-dimensional Gaussian volume the autocorrelation function *G(t)* for *j* different diffusing components is given by the following equation:[21]

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

with

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

and

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

(N_j: average number of molecules of the species *j* in the volume element; τ_{Dj}: diffusion time constant of the species *j*; ω_0: radius of the observation volume in the focal plane; z_0: radius of the observation volume in the *z*-direction; D_j: translational diffusion coefficient of the species *j*; Q_j: quantum yield factor; σ_j: absorption cross section; η_j: fluorescence quantum yield; g_j: fluorescence detection efficiency of the species *j*).

When all fluorescent components *j* have the same count rate per molecule (i.e. all Q_j are the same) and diffuse on a cell surface then eq 1 simplifies:

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

(y_j: fraction of the species *j* to the autocorrelation amplitude; τ_{Dj}: diffusion time constant of the species *j*; *N*: total number of molecules in the detection volume element).

For FCS experiments at the cell membrane, in equilibrium, the concentration of bound ligand ($[L_b]$) is given by the following equation:

$$[L_b] = ([L_0] + B_{max} + K_D \pm \sqrt{([L_0] + B_{max} + K_D)^2 - 4[L_0]B_{max}})/2 \quad (5)$$

($[L_0]$: total amount of ligand in the sample; B_{max}: total number of binding sites in the sample; K_D: dissociation constant of the ligand−receptor complex).

The bound ligand concentration ($[L_b]$) was plotted versus the total amount of ligand in the sample ($[L_0]$) for each single cell. From this the dissociation constant (K_D) and the maximum number of binding sites (B_{max}) were obtained by nonlinear curve fitting to eq 6. To compare different cell experiments each bound ligand concentration ($[L_b]$) was normalized by the number of binding sites of the individual cell (B_{max}). The relative values of $[L_b]/B_{max}$ were averaged, multiplied by the arithmetic average of $B_{max} = (37.8 \pm 7.7)$ nM ($n = 7$), and the resulting values of $[L_b]$ vs $[L_0]$ were fitted to eq 5, keeping B_{max} constant.

Before the experiments, the volume element of observation was calibrated. For this purpose, a defined concentration of Rhodamine 6G was added to the extracellular solution, and the autocorrelation function for the dye diffusion was recorded. From the determined diffusion time constant for Rhodamine 6G and a diffusion coefficient of 280 μm^2/s, the radii $\omega_0 = 0.18$ μm and $z_0 = 1.5$ μm of the confocal volume element were determined (according to eqs 1 and 2).[22]

Antibody Assay. The polyclonal antibody to benzodiazepines (antigen: oxazepam, host: sheep), achieved from Bio Trend Chemikalien GmbH, Cologne, Germany (Cat.Nr.: 1100-5004), was diluted 1:500 in PBS (0.26 g of KH_2PO_4, 2.17 g of NaH_2PO_4, 8.71 g of NaCl; pH 7.4) and stored at −20 °C in 1 mL aliquots. Two hundred microliters of the diluted antibody was incubated with increasing amounts of dye-labeled ligand (TMR−Ro: 1 nM to 800 nM, Alexa−Ro: 1.5 nM to 1.2 μM, OG−Ro: 0.5 nM to 100 nM, Flu−Ro: 0.5 nM to 100 nM). Incubation time was 30 min at room temperature. FCS data acquisition: every data point represents the mean of at least 10 measurements of 30 s runs.

For the validation of the antibody assay, the computer-controlled robot system FILTERPREP (Ismatec, Wertheim-Mondfeld; Switzerland) was used. The radioligand [^{3}H]-flunitrazepam ([^{3}H]FNT) was incubated to the antibody dilution (1:500 in PBS, pH 7.4, 4 °C) in concentrations between 10 and 800 nM for 45 min. The incubation was stopped in equilibrium and separated through a glass fiber filter band (Whatman GF/B; Whatman, Maidstone, UK) followed by two washing steps with PBS at 4 °C under reduced pressure (500 mbar). The radioactivity of the bound antibody retained by the filter was measured with a liquid scintillation counter (TRI-CARB 1900 TR, Canberra Packard, Frankfurt a. M., Germany) in Ultima Gold MV scintillation buffer (Perkin-Elmer, Wellesley, MA). Experiments were performed in triplicate, and results are given as mean values.

All data were analyzed by fitting with a one site binding model. (software: Origin 6.1, Origin Labs; Northampton).

Scatchard analysis was performed using eq 6 for antibody binding with two identical potential binding sites per antibody (insets Figure 2A−C).

$$\frac{[L_b]}{[AB][L_f]} = k_a\left(2 - \frac{[L_b]}{[AB]}\right) \quad (6)$$

($[L_b]$: bound ligand concentration, [AB]: concentration of antibody in the assay, $[L_f]$: free ligand concentration and k_a: association rate constant for the ligand-antibody binding)

Cell Culture. Hippocampal neurons from rat were prepared on embryonic day 18 by microdissection.[23] The cells were triturated and seeded to 18 mm, poly-D-lysine (Sigma; Taufkirchen, Germany)-coated coverslips (approximately 2.5 $\times$ 10^5 cells/cm^2) and cultured in a multiwell-plate (12$\times$) (Nunc; Wiesbaden, Germany) for 14 days in Start-V Medium (Biochrome; Berlin, Germany). Neuronal cultures were used between 8 and 14 days ex vivo.

Cell Experiments. Before FCS cell measurements the neurons were washed three times with Locke's solution (Hepes 5 mM, NaCl 154 mM, KCl 5.6 mM, $MgCl_2$ 1 mM, Na_2CO_3 3.6 mM, glucose 20 mM, $CaCl_2$ 2.3 mM. pH 7.4) at 37 °C. The coverslips with cells were mounted on a coverslip carrier with 300 μL of Locke's solution. The laser beam was placed on the cell soma of a single neuron (motor step size in *x*- and *y*-direction: 1 μm). The focal plane was positioned to the upper membrane by motor aided scanning through the neuron in the *z*-direction (optoelectronical DC-servodrives, motor step size 0.1 μm). Different concentrations of ligand (5 nM, 10 nM, 25 nM, 50 nM, 100 nM, and 210 nM Alexa−Ro) were incubated for 5 min at room temperature. For the evaluation of different lateral mobilities of receptor ligand complexes, all data were analyzed by fitting with two and three-component model (software FCS ACCESS; Zeiss, Jena Germany). The quality of the fit was judged by the χ^2 criterion (observed $G(\tau)$ versus expected values calculated from the pooled data). Nonspecific binding to the cells was determined with 10 nM Alexa−Ro after a preincubation with 10 μM midazolam for 30 min. Displacement experiments were performed by preincubation with 10 nM Alexa−Ro and a following incubation with 10 μM midazolam. The quantum yield of the dyes in solution and of the corresponding dye-labeled ligands in binding studies was determined by detecting light quanta per molecule using FCS.

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