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GABA_C RECEPTOR BINDING OF QUANTUM-DOT CONJUGATES OF VARIABLE LIGAND VALENCY

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Abstract

Highly fluorescent CdSe quantum dots (qdots) can serve as a platform for tethering multiple copies of a receptor-targeted ligand, affording study of how the level of multivalency affects receptor binding. We previously showed that qdots conjugated with long PEG chains terminated by muscimol, a known GABA_C agonist, exhibit specific binding to the surface membrane of GABA_C receptor-expressing *Xenopus* oocytes. The present report addresses the effect of varying the number, i.e., valency, of muscimol- (M-) terminated PEG chains attached to the qdot on binding of the resulting conjugate GABA_C receptors. M-PEG-qdots of differing muscimol valency were prepared by conjugating AMP®-CdSe/ZnS qdots with muscimol-terminated and methylamine-terminated PEG chains in proportions designed to yield varying percentages of muscimol-terminated chains among the total ~150–200 chains bound to the qdot. The investigated valencies represented 0%, ~25%, ~50% and 100% loading with muscimol (preparations termed M-PEG-qdot0, M-PEG-qdot25, M-PEG-qdot50 and M-PEG-qdot100, respectively). Binding of a given conjugate to surface membranes of GABA_C receptor-expressing oocytes was analyzed by quantitative fluorescence microscopy following defined incubation with ~30 nM of the conjugate. With 5–20 min incubation, the fluorescence signal resulting from incubation with M-PEG-qdot25 exceeded, by ~6-fold, the fluorescence level obtained with M-PEG-qdot preparations that lacked muscimol-terminated chains (M-PEG-qdot0). M-PEG-qdot50 yielded a net signal roughly similar to that of M-PEG-qdot25, and that produced by M-PEG-qdot100 exceeded, by ~30–50%, those for M-PEG-qdot25 and M-PEG-qdot50. The time course of changes in oocyte surface membrane fluorescence resulting from the introduction of and removal of M-PEG-qdots-in the medium bathing the oocyte indicated only a modest dependence of both binding and wash-out kinetics on muscimol valency. The results demonstrate a dependence of the binding activity of the M-PEG-qdot conjugates on muscimol valency, presumably reflecting higher GABA_C avidity and/or affinity of the muscimol at high valency, and provide insight on the interactions of membrane receptor proteins with qdot conjugates containing multiple copies of a receptor-targeting ligand.

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INTRODUCTION

CdSe/ZnS core-shell nanocrystals (quantum dots; “qdots”) are highly fluorescent, photobleach-resistant particles that can be conjugated with biomolecules and used in a wide range of biochemical and biological imaging applications [for recent reviews, see references (1–4)]. A central feature in the design of qdot conjugates for biological imaging is the attachment to the qdot surface of a ligand that is distally terminated by a small molecular weight compound, or by a macromolecule such as an antibody, that promotes interaction of the conjugate with a specific intra- or extracellular site. A variety of surface modification strategies have been developed to enable the conjugation of ligands such as peptides and large proteins, including antibodies, with CdSe-based qdots. For example, Mattoussi *et al.* (5), Goldman *et al.* (6) and Aneekava *et al.* (7) conjugated proteins to qdots using an electrostatic-based method, involving binding of a chimeric fusion protein to the oppositely-charged capped qdots. Other reported modification strategies have included 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) coupling between amino-terminated peptides and carboxylic acid residues on the qdot surface (8,9), the covalent attachment of proteins through thiol-maleimide conjugation [e.g. antibody conjugates (10)], the use of pyridine-capped qdots (11), and the conjugation of biotinylated proteins with streptavidin-coated qdots (12–14).

Relatively few imaging applications of small-molecule qdot conjugates have been investigated, despite the potential of these conjugates to target membrane-bound proteins other than those for which target-specific antibodies or peptides are available. Ligands derived from small molecules may be readily synthesized using conventional synthetic organic techniques enabling the development of a wider array quantum dot probes that may be useful in studies of fundamental biological processes. For example, Rosenthal *et al.* (15) used qdots functionalized with serotonin to target human and *Drosophila* serotonin transporters (hSERT, dSERT) expressed in HeLa and HEK-293 cells, and serotonin- 5HT₃ receptors expressed in *Xenopus* oocytes.

GABA receptors are multi-subunit, transmembrane receptor proteins that *in vivo* respond to the neurotransmitter γ -aminobutyric acid and mediate inhibitory synaptic transmission in the nervous system. One member of this family of receptors, known as the $\rho 1$ GABA_C receptor, is a homopentameric ligand-gated ion channel that exhibits full functionality when expressed in the *Xenopus* oocyte system (16–19). In a study of $\rho 1$ GABA_C receptors expressed in *Xenopus* oocytes, Gussin *et al.* (20) found that these receptors exhibit robust binding of a CdSe/ZnS qdot conjugate containing numerous (~150–200) copies of the small molecular weight compound muscimol, a known GABA_C agonist. This structure, here termed M-PEG-qdot, was thus highly multivalent, with many copies of muscimol per qdot available for interaction with the receptor.

The evident binding activity of M-PEG-qdot reported by Gussin *et al.* (20) raises the interesting question as to the extent to which this activity depends on the number of copies of qdot-bound muscimol. The dependence of the reactivity of qdot and other supramolecular conjugates on the valency (i.e., population) of targeting moieties is a topic of substantial interest and one that, to our knowledge, has not been extensively investigated. For example, if multivalency enhanced binding of a qdot conjugate to its target receptor, one might expect an improved ability to dynamically track the receptor. We reasoned that, in addition to its significance for elucidating properties of the GABA_C receptor, determining how differential muscimol loading affects the M-PEG-qdot/GABA_C interaction might prove useful as a model system in studies of multivalency in qdot-based conjugates. Here we have used quantitative fluorescence imaging of GABA_C-expressing *Xenopus* oocytes to investigate

how relative muscimol valency affects GABA_C receptor binding of M-PEG-qdot conjugates. Preliminary results have been reported (21).

EXPERIMENTAL PROCEDURES

Preparation of muscimol and methylamine capped PEG ligands

The conjugates used in this study consisted of AMP®-coated CdSe/ZnS core-shell nanocrystals with differing ratios of PEG-based ligands covalently attached to the AMP coating. Polydisperse PEG3400 (approximately 78 PEG units) was employed as a linker in these conjugates, as previous studies have shown that long-chain PEGs reduce non-specific binding of quantum dots to cellular membranes (22). The distal ends of these ligands were terminated with either a muscimol (5-aminomethyl 3-hydroxyisoxazole) group linked via an alkyl spacer (20), or, as a control, a methylamine group (Fig. 1). The muscimol ligand (abbreviated as M-PEG) was synthesized as previously described (20,23), and a modification of this procedure was used to synthesize the methylamine-capped ligand (termed methylamine-PEG) (see Supplemental Material).

Preparation of qdot conjugates

AMP®-coated quantum dots with a maximum fluorescence emission of 605 nm were obtained from Invitrogen Corporation (Carlsbad, CA) and used as supplied. All conjugates were synthesized from the same batch of AMP dots to eliminate variability in fluorescence quantum yields. Surface conjugation of the AMP dots with M-PEG and methylamine-PEG ligands was achieved using EDC coupling and was performed as previously described (20). The terminating amino group of both ligands served as the point of attachment to the carboxylic acid functionalities of the AMP coating. The conjugation was carried out in borate buffer, pH 8.5 (Polysciences, Inc., Warrington, PA) and involved the following steps. First, 100µL of a solution containing 8×10^{-7} moles of ligand (M-PEG, methylamine-PEG, or a mixture of M-PEG and methylamine-PEG) dissolved in borate was mixed with a 100µL solution of AMP dots at a concentration of 8µM (resulting in a ligand: AMP dot ratio = 1000:1). After stirring for 10 minutes, 100µL of borate containing N-hydroxy succinimide (6×10^{-7} moles) and 100µL of borate containing EDC (6×10^{-7} moles) were added. The resulting solution was stirred at ambient temperature for 1 hour, and each conjugate was then purified via size exclusion chromatography on Sephadex G50 (borate elution). Ligand loading was varied by mixing M-PEG and methylamine-PEG in differing ratios, and conjugating the mixture to the AMP dot surface. The methylamine-capped and muscimol-capped ligands were assumed to have equal reactivity with the AMP dots, since (i) for both ligands, the terminating moiety (muscimol or methylamine) is distant (about 78-PEG units) from the functional group that reacts with the surface of the AMP-dot, and (ii) the PEG3400 chain is expected to dominate the overall steric bulk of both ligands. Furthermore, as the mass of the PEG3400 chain far exceeds those of the terminating methylamine or muscimol, we assumed a mass of 3400 for both ligands in calculating quantities of the two ligands used in the conjugation. Prior to conjugation to the AMP dot surface, the two ligands were mixed in molar ratios (M-PEG: methylamine-PEG) of 1:0, 3:1, 1:1, 1:3 and 0:1. Throughout this paper, preparations resulting from the conjugation of these ratios are termed M-PEG-qdot100, M-PEG-qdot75, M-PEG-qdot50, M-PEG-qdot25 and M-PEG-qdot0, respectively. The ratios of ligands used in this study are described in Table 1 and referred to as “muscimol valency”. All conjugates were characterized by UV-visible spectrophotometry using an extinction coefficient of $650,000 \text{ M}^{-1} \text{ cm}^{-1}$ at 598 nm (first absorption feature of the quantum dot), and by gel electrophoresis on 1% agarose as described (20). The fluorescent properties of the conjugates at a fixed concentration (7 nM) were determined using an ISS PCI spectrofluorometer (quartz cell, 10-mm path length; excitation at 400 nm), and

compared with properties of an equimolar sample of unconjugated AMP-coated qdots. Figure 1 shows a schematic representation of the resulting conjugates.

Oocyte preparation and GABA_C receptor expression

All animal procedures adhered to institutional policies and to the Statement for the Use of Animals in Ophthalmic and Vision Research adopted by the Association for Research in Vision and Ophthalmology (ARVO). Oocytes were obtained from *Xenopus laevis* toads (Xenopus One, Ann Arbor, MI), and human $\rho 1$ GABA_C receptors were expressed in the oocytes through injection of RNA synthesized *in vitro* (mMessage mMachine, Ambion / Applied Biosystems, Austin, TX) to express the homopentameric human $\rho 1$ GABA_C receptor (18,19). Oocytes that were not injected with RNA, i.e., did not express the GABA_C receptors, were used as controls.

Confocal microscopy and image analysis

Visualization and quantification procedures relevant to the fluorescence analysis of oocytes followed methods similar to those previously described (20). Unless otherwise noted, images were obtained in experiments that involved static (i.e., non-perfusing) incubation of the oocyte. Specifically, fluorescence and bright-field images were obtained from oocytes positioned in glass-bottom dishes (MatTek Corp., Ashland, MA) and incubated for defined periods in Ringer solution (100 mM NaCl, 2 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, and 10 mM glucose, pH 7.2–7.4) supplemented with the test components. The bright-field images illustrate the focus of the (opaque) oocyte. Fluorescence was measured using a confocal microscope (Leica model DM-IRE2 with 20 $\times$ objective; λ excitation = 476 nm; λ emission = 580–620 nm to include the qdot emission peak at λ = 600–605 nm). Microscope settings relevant to excitation illumination and detection of fluorescence emission were established at the beginning of each series of experiments and maintained without change for the entire group of measurements. Experiments on a given day were performed on a single batch of oocytes and employed a single preparation of test solutions. Each fluorescence image was quantitatively analyzed for fluorescence of the oocyte surface membrane and surrounding medium (background) using MetaMorph version 7.0r4 software (Universal Imaging Corp., Downingtown, PA), as previously described (20). Briefly, the cursor was used to trace the arc-like border of the oocyte as a series of 15–25 straight-line segments that spanned the entire field of view of the border, and included 500–725 pixels (i.e., data points). For determination of background fluorescence (fluorescence of the extracellular medium bathing the oocyte), the multi-segment line used to determine the border fluorescence was copied and replicated to cover a representative region in the medium. The intensities of pixels covered by these segmented lines were then obtained. (Fig. 2). Net adjusted fluorescence for each halo pixel was calculated as

$$(F_{\text{halo}} - F_{\text{background}})/\text{SFR} \quad (1)$$

where F_{halo} is the raw fluorescence value of the pixel, $F_{\text{background}}$ is the median value of raw fluorescence for all measured background pixels, and SFR is the “specific fluorescence ratio” corresponding with each M-PEG-qdot valency (see Results). In some cases when multiple oocytes were present in the same image, a single determination of background value was used for analysis of all of the oocytes in the image. Because of hard limits on the range of measured values (data could only range from 0 to 255), data from each experiment were submitted to a Kolmogorov-Smirnov test to assess for normal distribution and spread. Testing for normal distribution was performed on all of the reported data; sets of data that passed the test for normal distribution were analyzed using parametric statistics (means, standard errors of means, analysis of variance, and *t*-tests). For data sets that were not

normally distributed, non-parametric statistics were used for analysis [median, confidence interval (CI), and chi-square (χ^2)]. For parametric analyses, data were subjected to 1- and 2-way analysis of variance (ANOVA) for determinations of differential changes in median intensities across images. Post-hoc tests of significant ANOVAs were conducted using the Bonferroni correction. For non-parametric data, analysis of histograms of image intensity was conducted, and a set bin-width of 10 units was applied.

Flow chamber

The kinetics of removal (i.e., wash-out) of M-PEG-qdot preparations from interaction with the oocyte were investigated in experiments that involved superfusion of the oocyte. These experiments employed a customized flow chamber that was compatible with the confocal microscope and maintained the oocyte in a stable position to permit serial image capture (Fig. 3). This chamber utilized an oocyte perfusion chamber designed for inverted microscopy (Model OPC-4; Bioscience Tools, San Diego, CA). The base of the commercially obtained chamber assembly, which contained a glass bottom at its center and a surrounding well on which the oocyte chamber component was mounted, was reshaped to interface with the confocal microscope's mounting tray positioned above the objective lens. Additionally, safety reservoirs for capturing fluid overflow were added to the base by creating wells around the oocyte chamber; a tunnel designed for an agar bridge in the commercial device was sealed with wax; and the chamber wall near its downstream end was modified to contain a small notch that served to position the oocyte. A gravity-flow perfusion channel system consisting of a 7-channel perfusion manifold (~40 cm tubing length) was used to allow the smooth exchange of test solutions at a flow rate of approximately 0.3 mL/min. The orifice of a vacuum line was positioned at the surface of the fluid in the chamber to minimize rippling.

RESULTS

Electrophoretic and spectral properties

Coupling of the PEG ligands (muscimol-terminated and methylamine-terminated) to the surfaces of the AMP-coated quantum dots was assessed by agarose gel electrophoresis. The results, shown in Fig. 4A, indicate that the M-PEG-qdot0, M-PEG-qdot25, M-PEG-qdot50, M-PEG-qdot75 and M-PEG-qdot100 preparations (lanes 2–6) exhibited fluorescence spots that were elongated by comparison with that due to unconjugated qdots (lane 1). The elongated shape presumably reflected both the heterodispersity of the PEG3400 chain, and stochastic variation in loading of a given qdot with methylamine- vs. muscimol-terminated chains. The Fig. 4A results furthermore indicate a progressive decrease in electrophoretic mobility with increased muscimol loading. Such decrease is generally consistent with previous results obtained with coupling of methylamine-capped PEG2000 ligand to AMP-qdots (22). For the present case of muscimol vs. methylamine terminated chains, the total mass of the PEG3400 chains contained in the conjugate far exceeds the overall mass contribution of either aminohexanoyl muscimol (~227 Da) or methylamine (~32 Da). Thus, the presently observed decrease in electrophoretic mobility with increasing muscimol valency cannot be attributed solely to mass differences among the conjugates. Changes in hydrodynamic properties may also influence the observed electrophoretic behavior (see Discussion).

The fluorescence spectra of the investigated batches of M-PEG-qdot100, M-PEG-qdot 50, M-PEG-qdot 25 and M-PEG-qdot0 were obtained, and the peak fluorescence (at γ ~602 nm) determined (Fig. 4B). Because the spectral properties of the conjugates differed (peak fluorescence intensity in arbitrary units was 34,483.4 for M-PEG-qdot100, 47,971.1 for M-PEG-qdot50, 62,204.9 for M-PEG-qdot25 and 67,257.1 for M-PEG-qdot0), the raw data

obtained from fluorescence images were adjusted to reflect the specific fluorescence of each conjugate relative to the M-PEG-qdot100 conjugates. This adjustment involved division of the raw fluorescence values by the specific fluorescence ratio SFR (see eq. 1), where the SFR was defined as the ratio of the specific fluorescence of a given M-PEG-qdot divided by the specific fluorescence of M-PEG-qdot100. The SFR values determined for M-PEG-qdot50, M-PEG-qdot25 and M-PEG-qdot0 were, respectively, 1.3911, 1.8039 and 1.9504.

Oocyte surface membrane binding

Fluorescence intensities in relation to muscimol valency—To investigate the effect of muscimol valency on the binding of the conjugate to the GABA_C receptor, oocytes were incubated with M-PEG-qdot preparations containing differing proportions of muscimol-terminated and methylamine-terminated chains. Figures 5 and 6A show images and histographic data obtained in single representative experiments; results obtained from these and other oocytes are shown in Supplemental Material Table S1. Upon completion of a 10–15 min incubation of 30–34 nM of the M-PEG-qdot compounds, as a static bath, with the oocytes, fluorescence and bright-field images of the oocytes were obtained. Figure 5 shows fluorescence and bright-field images obtained in representative experiments that involved the incubation of $\rho 1$ GABA_C-expressing oocytes with M-PEG-qdot0, M-PEG-qdot50, and M-PEG-qdot100, and of non-expressing (control) oocytes with M-PEG-qdot100. The fluorescence images obtained following incubation with M-PEG-qdot100 and M-PEG-qdot50 with the GABA_C expressing oocytes show marked fluorescence halos at the oocyte surface membrane. By contrast, the non-expressing control oocyte lacked significant border fluorescence on similar incubation with M-PEG-qdot100. Thus, binding of the M-PEG-qdot conjugate was specific for cells expressing the $\rho 1$ receptor. Fluorescence intensities of the halo and of the surrounding medium (background) were quantified as described above (Experimental Procedures). Fluorescence intensities measured for the $\rho 1$ -expressing oocytes shown in Figure 5 are reported in Table S1, lines 15 and 16 for M-PEG-qdot50, **and lines 19 and 20** for M-PEG-qdot100. For the non-expressing oocyte (negative control) incubated with M-PEG-qdot100, fluorescence intensities of the border and background were similar and yielded a null value for net fluorescence (Table S1, line 25), consistent with the absence of a visible surface halo. Incubation of either GABA_C-expressing or non-expressing control oocytes with M-PEG-qdot0, i.e., a compound that lacked muscimol moieties, did not yield a visible halo, as shown on the left-hand side images. Quantitative analyses of these GABA_C-expressing oocytes confirmed the absence of significant fluorescence for M-PEG-qdot0 (Table S1, lines 13 and 14).

We examined the distribution of fluorescence intensities to determine whether this distribution is correlated with the valencies of the tested M-PEG-qdot preparations. Figure 6A shows results from a representative set of experiments involving GABA_C expressing oocytes that included M-PEG-qdot100, -50 and -25. The histograms show net adjusted fluorescence intensity distributions obtained with M-PEG-qdot compounds of different valencies. Within each panel, the illustrated distribution indicates the frequency of pixel value of the border from which the median background value was subtracted, and then multiplied by the specific fluorescence ratio. Distributions for the border regions were essentially unimodal, with an exhibited increase in fluorescence with increasing muscimol valency, consistent with the trend in mean fluorescence intensity observed as a rightward shift in distribution with increasing valency. Median values were 17.94 for M-PEG-qdot0, 102.56 for M-PEG-qdot25, 83.39 for M-PEG-qdot50, and 135.00 for M-PEG-qdot100. Additional sets of experimental data were obtained, that included M-PEG-qdot75 preparations (not illustrated), and showed similar distribution patterns.

Aggregate determinations of binding extent and time course—Data obtained from multiple oocytes ($n=5$ for each valency, over two different experimental days) were pooled, and the distribution of fluorescence intensity analyzed, for M-PEG-qdot preparations of different valencies. Figure 6B illustrates the aggregate normalized distribution of net adjusted fluorescence. For each valency, fluorescence data were pooled across oocytes and across experiment dates, and the net fluorescence intensity obtained for each data point was adjusted using the specific fluorescence ratio. The intensity determined for each bin (10 fluorescence units) was then normalized to the overall number of observations. Consistent with the pattern observed in Fig. 6A, the cumulative distribution for the border regions exhibited a trend toward increased fluorescence with increasing muscimol valency. However, the distribution patterns observed in the aggregate histograms appeared bi- or tri-modal, presumably due to differences in fluorescence intensity among the group of oocytes analyzed. For completeness, Table S1, Lines 21–24 present both the mean and median aggregate values of fluorescence intensity. The mean and median values indicated a similar trend among preparations of differing valency. That is, net adjusted fluorescence values for the M-PEG-qdot0 preparation are lower than those for the M-PEG-qdot25, M-PEG-qdot50, and M-PEG-qdot100 preparations, and those for the M-PEG-qdot100 preparation are higher than those of the M-PEG-qdot25 and M-PEG-qdot50 preparations. Furthermore, values determined for the M-PEG-qdot25 and M-PEG-qdot50 preparations are close, although the means indicate a higher fluorescence for the M-PEG-qdot50 preparation while the medians indicate higher fluorescence for the M-PEG-qdot25 preparation.

Figure 7 illustrates overall results obtained from groups of oocytes incubated with the M-PEG-qdot100, -50, -25 and -0 preparations, as described in the preceding paragraph, by considering the relationship between muscimol valency and the fluorescence signal exhibited by the oocyte surface membrane as a function of incubation period. In both Panel A and Panel B of the Figure, comparisons of aggregate adjusted fluorescence intensities are based on median values (rather than averages) because of the non-normal distribution of pixel data as shown in Fig. 6. Fig. 7A indicates median adjusted fluorescence data (with error bars corresponding to a 99% confidence interval). These data were submitted to a 2-way ANOVA with valency as a between-samples variable and time as a within-samples variable. There was an interaction between valency and time [$F(15, 49272)=79.94$, $p<0.0001$]. At all time points, the fluorescence values for M-PEG-qdot100 were higher than those obtained with M-PEG-qdot50 [at $t = 2$ min, $F(1,3374) = 1646.99$, $p<0.0001$; at $t = 3$ min, $F(1,4356) = 1280.90$, $p<0.0001$; at $t = 5$ min, $F(1,4351) = 1530.02$, $p<0.0001$; at $t = 10$ min, $F(1,4296) = 1125.66$, $p<0.0001$; at $t = 15$ min, $F(1,4452) = 764.93$, $p<0.0001$; and at $t = 20$ min, $F(1,4480) = 2073.13$, $p<0.0001$] and with M-PEG-qdot25 [at $t = 2$ min, $F(1,3388) = 2952.15$, $p<0.0001$; at $t = 3$ min, $F(1,4323) = 1749.74$, $p<0.0001$; at $t = 5$ min, $F(1,4341) = 1676.36$, $p<0.0001$; at $t = 10$ min, $F(1,4444) = 1188.04$, $p<0.0001$; at $t = 15$ min, $F(1,4450) = 1205.45$, $p<0.0001$; and at $t = 20$ min, $F(1,3833) = 2667.31$, $p<0.0001$]. For all tested incubation periods, median fluorescence intensity values for M-PEG-qdot0 were significantly lower than those obtained with compounds containing muscimol moieties [$F(3, 8078) = 3195.36$, $p<0.0001$; Bonferroni paired comparisons for all, $p<0.0001$]. In particular, at each time point over the period $t = 5$ –20 min (i.e., excluding the initial determinations), values of net fluorescence signal obtained with M-PEG-qdot25 exceeded, by ~6-fold, those determined with M-PEG-qdot0. The net fluorescence signals obtained with M-PEG-qdot50 over this same interval were generally similar to those obtained with M-PEG-qdot25, and those obtained with M-PEG-qdot100 exceeded, by ~30–50%, those determined for M-PEG-qdot25 and M-PEG-qdot50.

Fig. 7B shows aggregate data collapsed across time-points over the interval of 4–20 min. The median net adjusted fluorescence intensities ($\pm$ CI) observed across time were 139.88 ± 2.02 for M-PEG-qdot100, 76.82 ± 1.76 for M-PEG-qdot50, 80.78 ± 1.81 for M-PEG-qdot25

and 12.87 ± 2.46 , for M-PEG-qdot0 preparations. These data indicate that the median fluorescence intensity, which reflects binding of the M-PEG-qdot preparations to oocyte expressing $\rho 1$ GABA_C receptor, increased with muscimol valency [$F(3, 8610) = 2818.82$, $p < 0.0001$; Bonferroni comparisons between valencies yielded $p < 0.0001$, except for M-PEG-qdot50 compared with M-PEG-qdot25, for which $p = 0.004$]. Moreover, the low level of binding to the GABA_C-expressing oocytes exhibited by M-PEG-qdot0, and the low binding to non-expressing oocytes by the M-PEG-qdot100, provide evidence for low non-specific binding of the investigated conjugates to oocyte membranes (22).

To further examine the relative timing of the approach of the fluorescence signal to near-plateau values with the compounds of different valencies, net fluorescence data obtained in each of the Fig. 7A group of experiments were converted to standardized fluorescence values using a z-score transformation

$$z = (\chi - \mu) / \sigma \quad (2)$$

where χ is the net fluorescence value determined at a given time t , μ is the median of the fluorescence data obtained at the final time point (t_{final} : 20 min), and σ is the standard deviation of the fluorescence data obtained at t_{final} . This transformation was conducted for each experiment. The transformed data were then averaged across experimental conditions to yield the aggregate results illustrated in the Figure. Fig. 7C illustrates the resulting z-score values determined in the experiments with M-PEG-qdot100, M-PEG-qdot50, and M-PEG-qdot25. (Note here that $z = 0$ corresponds with a net fluorescence equal to the median of the t_{final} data, and $z = -1$ corresponds with a net fluorescence equal to one standard deviation below the median determined at t_{final} .) The z-transformed data of Fig. 7C demonstrate that there is no significant interaction between valency and time, i.e., binding rates of the M-PEG-qdot conjugates appear not to depend on the valency of the preparations.

Effects of conjugate concentration on time course of binding—In an additional series of experiments, we examined the time-dependence of binding of differing concentrations of a single muscimol valency (M-PEG-qdot100). In all cases, these experiments involved the measurement of fluorescence intensity at defined times over an approximately 15–20 min period following the initiation of incubation with the M-PEG-qdot conjugate. Fig. 8 shows representative images obtained at two fixed times (2 min and 15 min) in experiments that involved incubation of the oocyte with 30 nM, 10 nM and 3 nM of M-PEG-qdot100. These images illustrate the increase in the visible fluorescence intensity of the halo at the oocyte surface membrane. The increase is particularly noticeable when the oocyte was incubated with 30 nM of M-PEG-qdot100. In order to quantify the extent of the rise in intensity, the fluorescence from the images obtained in the experiments described in Fig. 8 and in others of similar design was quantified, and the results were analyzed to obtain values of median net fluorescence (median difference in fluorescence of the border halo vs. surrounding medium) and 99% confidence intervals (CIs) over the investigated periods of incubation with M-PEG-qdot100 at 30 nM, 10 nM and 3 nM. As illustrated in Fig. 9A, the final determination (i.e., the determination at $t = 15$ or $t = 20$ min) varied with M-PEG-qdot100 concentration (78.34 ± 3.82 , 27.87 ± 9.22 , and 17.64 ± 6.90 for 30 nM, 10 nM and 3 nM respectively) and, consistent with previous results (20), the data obtained with M-PEG-qdot100 exhibited a median net intensity that, at $t = 5$ min, represented ~75–80% of the near-plateau values determined at $t = 15$ –20 min. Values of median net fluorescence obtained with 10 nM of the conjugate, in addition to being considerably smaller than those obtained with the 30 nM concentration, approached a near-plateau level on a relatively long time scale (compare red and green symbols in Fig. 9).

The relative timing of the approach of the fluorescence signal to near-plateau values at 30, 10, and 3 nM was further examined by standardizing the raw data obtained in each experiment via a z-transformation (cf. eq.2), using the median net fluorescence determined at the final time point (t_{final}) of the incubation as the normalizing value ($t_{\text{final}} = 20$ min for 3 and 10 nM, and 15 min for 30 nM). The resulting z-transformed data, shown in Fig. 9B, indicate that, upon control for the effect of M-PEG-qdot100 concentration, there was relatively little difference in the pattern of data obtained at a given concentration, and the binding kinetics did not significantly depend on concentration, for incubation times >5 min.

Wash-out kinetics—Investigation of the off-kinetics of the M-PEG-qdot preparations of different valencies employed the flow chamber described in the Experimental Procedures section. In these experiments, GABA_C-expressing oocytes were incubated for 10 min with a preparation of 30–34 nM M-PEG-qdot (of a given valency). Following this incubation, Ringer solution was passed through the chamber for approximately 5 s to remove excess conjugate, and an image designated $t = 0$ was immediately collected. The flow of a wash-out solution consisting of Ringer, or of Ringer supplemented with 1 mM muscimol was then initiated at a rate of 0.3 mL/min, and images were repeatedly collected over a 45–50 min period. Fig. 10 shows representative images obtained in wash-out experiments with M-PEG-qdots of differing valencies, at $t = 0$ and $t = 30$ min. The decrease in oocyte surface fluorescence exhibited by the $t = 30$ vs. $t = 0$ min image for each of the investigated valences provides evidence for a release of these M-PEG-qdot preparations from the membrane over this 30-min period.

Figure 11 shows z-transformed data for the net difference in fluorescence intensity (border minus background) determined in these wash-out experiments with unsupplemented Ringer (**panel A**) and with 1 mM muscimol (**B**). For these analyses, the fluorescence value obtained at the conclusion of the initial wash (i.e., at $t = 0$) was used for the z-transformation. For each of the valencies examined in the Fig. 11A experiments, we specifically compared the normalized values obtained at $t = 10$ min with those obtained at $t = 40$ min, and determined that the decrease was significant ($p < 0.0001$) in all cases. However, for all valencies, the observed decline represented an incomplete loss of fluorescence signal, suggesting the occurrence of a persisting component of the M-PEG-qdot binding with the oocytes. Furthermore, the relative extent of decline differed among the tested valencies; the declines in fluorescence determined with M-PEG-qdot25 and M-PEG-qdot75 proceeded more gradually than those determined with M-PEG-qdot50 and M-PEG-qdot100 (using only $t = 10$ and $t = 40$; $p < 0.0001$ for M-PEG-qdot75 vs M-PEG-qdot50 and M-PEG-qdot100; $p < 0.001$ for M-PEG-qdot25 vs M-PEG-qdot50 and M-PEG-qdot100). For each M-PEG-qdot preparation, comparison of untransformed fluorescence intensities obtained at different time points showed a roughly 40–70% decrease in normalized fluorescence intensity over the wash-out period, i.e., a decrease of order similar to that previously reported for M-PEG-qdot100 (20). A roughly similar pattern of data was observed when 1 mM muscimol was included in the wash-out medium (Fig. 11B). Here, the comparison of untransformed fluorescence intensities indicated a roughly 45–80% decrease over the washout period, and the decrease occurring over the period $t = 10$ min to $t = 40$ min was significant in all cases ($p < 0.0001$).

DISCUSSION

Using quantitative fluorescence imaging of GABA_C-expressing *Xenopus* oocytes, we have compared the GABA_C binding properties of qdot conjugates that differ with respect to their abundance of muscimol, a known GABA_C ligand. The use of these muscimol-containing conjugates of different muscimol valencies extends earlier work that established the GABA_C binding activity of conjugates containing only muscimol-terminated chains (20). The main

finding of the present study is the dependence of binding on the composition of the linear chain mixture (muscimol-terminated vs. methylamine-terminated chains) joined to the qdot. That is, increasing the muscimol valency from M-PEG-qdot25 (approximately 37–50 muscimol/qdot) to M-PEG-qdot100 (approximately 150–200 muscimol/qdot) leads to an increase in fluorescence intensity of the surface of the GABA_C-expressing oocyte (Figs. 5–7). For example, binding of the M-PEG-qdot100 preparation exceeded by ~45 % that of the M-PEG-qdot25 preparation, when fluorescence intensity data adjusted for the specific fluorescence of each compound was considered. Accompanying this increase was a shift in the distribution of fluorescence measured with this visualization technique (Fig. 6). These findings, which indicate a correlation of receptor binding with the population of qdot-attached ligands, represent, to our knowledge, the first demonstration of differential binding properties of receptor-targeted qdots by variation of the population of an attached receptor ligand.

The precise interplay of differences in avidity vs. affinity of the investigated multivalent conjugates remains to be determined. For example, it is possible that higher muscimol valencies, in addition to increasing the avidity of the conjugate, may be associated with a higher binding affinity of a given muscimol moiety within the conjugate. This could reflect differences in the microenvironment of a given muscimol that result from the differing proportions of muscimol- vs. methylamine-capped chains. A further point of interest for future investigation is the evident significant increase in binding that occurs over the range of zero to 25% muscimol loading (Figs. 5, 6, 7). Conceivably, the contribution of avidity- vs. affinity-dependent processes to changes in binding with increased muscimol valency might differ from the relationship that prevails at higher valencies.

Electrophoretic analysis of the present qdot conjugates indicated a progressively decreased mobility with increasing muscimol valency (Fig. 4A). As noted above (Results), this behavior is unlikely to be due solely to variation in masses of the conjugates. Furthermore, increasing muscimol valency might be expected to increase the net negative charge of the conjugate (due to deprotonation of muscimol under the pH conditions used for electrophoresis). However, an increase in negative charge would be expected, in itself, to increase rather than decrease electrophoretic mobility. Conceivably, the decreased mobility seen with increasing muscimol valency could be due to mechanisms that, as a function of the proportion of muscimol- vs. methylamine-terminated chains (including, perhaps, an effect of muscimol deprotonation), alter the hydrodynamic radius of the conjugate. One possibility is that, with increasing proportion of deprotonated muscimol chains, the hydrodynamic radius of the conjugate might increase as a consequence of electrostatic repulsion among chains. In particular, the relatively large reduction in mobility exhibited by M-PEG-qdot100 vs. M-PEG-qdot75 (lanes 5–6 in Fig. 4A) could reflect, e.g., a reduced extent of muscimol deprotonation (and thus reduced overall charge) when the conjugate contains only muscimol-terminated chains. Alternatively, the relatively low M-PEG-qdot100 mobility might reflect reduced inter-chain folding (leading to greater hydrodynamic radius) in the absence of methylamine-terminated chains.

In separate electrophysiology experiments (not illustrated), we tested whether M-PEG-qdot100 elicits an electrophysiological response from GABA_C-expressing oocytes, and found no detectable response. This absence of activity could reflect an inability of the receptor to transition to its open state following binding of the M-PEG-qdot structure. Alternatively, the M-PEG-qdots may bind to only a small fraction of the receptor population, one sufficient to produce a robust fluorescence signal but insufficient to be measurable electrophysiologically. It is also possible that binding occurs with an occupancy (by a muscimol moiety of the M-PEG-qdot structure) of as little as one of the five ligand

binding sites of the pentameric receptor, an event that could lead to a sufficient visualization signal but be insufficient for receptor activation (16).

Despite the evident dependence of the normalized M-PEG-qdot binding kinetics on muscimol valency (Figs. 5–7), we found no significant dependence of the normalized wash-out kinetics on muscimol valency (Figs. 10 and 11). The finding that the off-kinetics do not vary as a function of muscimol valency could reflect an intrinsic similarity across valencies in the mechanism by which a single qdot dissociates from the oocyte surface. Alternatively, it is possible that the observed similarity reflects occurrence of a bulk property of the qdot population that is bound to the oocyte surface at the beginning of the wash-out assay. For example, it is conceivable that, essentially independent of muscimol valency, qdots bound to the oocyte surface at $t=0$ in the wash-out experiments undergo aggregation, and as a consequence exhibit a similar retardation of wash-out. However, the clear dependence of binding on valency suggests that such an aggregation effect occurs only upon localization of the M-PEG-qdot structure at the receptor (i.e., receptor binding) and cannot account for the initial muscimol-GABA_C interaction. The wash-out data obtained with presentation of competing muscimol do suggest that the presence of this competing agonist has a modest effect on wash-out kinetics, for at least certain valencies of M-PEG-qdot preparations (Fig. 11, M-PEG-qdot75 and M-PEG-qdot25). However, the absence of a wash-out promoting effect among the data collected across all muscimol valencies suggests that wash-out kinetics are governed by multiple factors. These could include, for example, the local density of muscimol (including the effect of variable extension of the PEG-chains, leading to variability in availability of the muscimol) and different mechanisms of removal.

The presence of five potential binding sites for GABA at $\rho 1$ GABA_C receptors (16) emphasizes the possibility that the relatively strong binding observed with conjugates of high muscimol valency reflects binding of the conjugate at multiple GABA-binding sites of a given receptor, yielding a polydentate interaction [cf. e.g. (24)]. An alternative (or perhaps additional) possible mode of interaction relates to the distance potentially spanned by the muscimol-terminated PEG chains of a given M-PEG-qdot. That is, for the present case of muscimol-terminated chains containing PEG3400 (77 ethylene glycol units, on average), and with the PEG chains of a given pair of muscimols in a conformation representing ~80% full extension (25), the maximum distance spanned by the two muscimols would be ~40 nm, a length considerably greater than the diameter of a given pentameric $\rho 1$ GABA_C receptor, about 8 nm (26). Thus, high muscimol valency could favor the binding of a single conjugate to neighboring receptors, and in turn promote an interplay between binding and receptor clustering. Evidence for a close interrelationship between conjugate multivalency and receptor clustering comes from a study by Gestwicki *et al.* (27). These investigators examined the effects of structural parameters such as scaffold shape, size, and density of binding elements of multivalent conjugates on the inhibition and clustering of concanavalin A (Con A). Gestwicki *et al.* (27) found, for example, that structural properties of linear oligomeric ligands favored clustering; and that the shape of a multivalent ligand influenced receptor clustering properties, such as clustering rate, number of receptor in the cluster, and inter-receptor distance. It will be of interest in future studies to examine further the relationship between receptor clustering and the “reach” of (i.e., maximal distance spanned by) multiple targeting ligands conjugated to qdot platforms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Schematic representation of conjugated qdot structures. The central sphere is the CdSe/ZnS core-shell qdot, functionalized by an AMP® coating. Attached to the qdot are long-chain PEG linkers (PEG3400) (~150–200 chains per qdot), each of which is terminated by either muscimol or a methylamine group.

Figure 2.

Example of the method used to determine intensities of the fluorescence images obtained by confocal microscopy, using MetaMorph version 7.0r4 software. The left-hand panel shows the fluorescence image of an oocyte exhibiting a surface halo. The intensity of the halo was determined as follows. First, the cursor was used to trace the entire arc-like border of the oocyte surface membrane as a series of straight-line segments. The right-hand panel reproduces the image at the left, and the segmented line just described is shown as an overlaid green line. This multi-segment line was then copied and replicated to cover a representative region in the surrounding medium (i.e., background). This background multi-segment line, shown in blue in the right-hand panel, contained the same number of pixels (data points) as the multi-segment green line describing the oocyte border. Intensities of pixels covered by the border and background multi-segment lines were then obtained. Right panel: Same image, with multi-segment lines the fluorescence showing how fluorescence intensity for the halo of one oocyte (green line) and the background (blue line) were determined.

Figure 3. Top view of the base-chamber assembly (Panel A) and of the oocyte flow chamber (B) used to examine wash-out kinetics of qdot conjugates.

Figure 4.

A: Agarose (1%) gel of the unconjugated qdots and of the qdots conjugated with different ratios of muscimol / non-muscimol-terminated chains. (1): unconjugated AMP-coated qdots (400nM); (2): M-PEG-qdot0 (580nM); (3): M-PEG-qdot25 (530 nM); (4): M-PEG-qdot50 (730 nM); (5): M-PEG-qdot75 (730 nM); and (6): M-PEG-qdot100 (630 nM). B: Fluorescence spectra of M-PEG-qdot conjugates of different valencies. The concentration of the conjugates used to obtain the spectra was 7 nM for all M-PEG-qdot preparations and values of fluorescence are plotted relative to the obtained for a 7 nM solution of AMP dots.

Figure 5. Representative experiments involving 15-min incubation of GABAc-expressing oocytes with 34 nM M-PEG-qdot0, 34 nM M-PEG-qdot25, and 34 nM of M-PEG-qdot100 (right-hand side). For each condition are shown a fluorescence image (top row), and the corresponding bright-field image of the (opaque) oocytes that illustrates the plane of focus used for fluorescence imaging (bottom row). Representative images obtained from a non-expressing (control) oocyte incubated for 15-min with M-PEG-qdot100 are shown on the right hand panel.

Figure 6.

A: Distributions of net adjusted fluorescence intensity for the border and background regions of representative experiments involving GABAC-expressing oocytes incubated with M-PEG-qdots of different muscimol valencies. Distributions are plotted with fixed bin widths; each bin spans 20 gray scale units. B: Cumulative normalized frequency of fluorescence intensity, based on total percentage per bin for the adjusted net data.

Figure 7.

A: Median net fluorescence intensities obtained with GABAC-expressing oocytes incubated for 2–20 min with M-PEG-qdot100, -50, -25 and -0. Each data point reflects the median fluorescence of $n=5$ oocytes, measured on two different experiment days, using the same batches of M-PEG-qdot preparations. Values obtained for M-PEG-qdot50, M-PEG-qdot25 and M-PEG-qdot0 were adjusted for the specific fluorescence of the compounds relative to that of M-PEG-qdot100. Error bars indicate a 99% confidence interval (99 % CI). B: Histogram representing median adjusted fluorescence intensities ($\pm$ 99% CI), collapsed across time (between $t = 4$ min and $t = 20$ min), as a function of muscimol valency. C: Analysis, by z-transformation, of the Panel A data for binding kinetics M-PEG-qdot25, M-PEG-50 and M-PEG-qdot100. The illustrated data are median values $\pm$ 99% CI. See text for further details.

Figure 8. Representative images obtained in binding kinetics experiments with varying concentrations of M-PEG-qdot100 (images obtained at t = 2 min and t = 15 min).

Figure 9.

Binding kinetics of M-PEG-qdot100. The illustrated data show non-normalized (Panel A) and normalized (B) values for median net fluorescence intensity. Concentrations of the M-PEG-qdot 100 were 3 nM (blue symbols), 10 nM (green symbols) and 30 nM (red symbols). In each experiment, fluorescence values were measured over a period of 15–20 min following the initiation of incubation with the M-PEG-qdots (time zero). For each concentration, the median net difference in fluorescence intensity obtained at a given time was normalized to the median net intensity obtained at the end of the incubation period, to yield the normalized median fluorescence intensity $\pm$ 99% CI.

Figure 10. Representative images obtained during wash-out experiments with M-PEG-qdots of different valencies, at t = 0 and t = 30 min, with a 1 mM muscimol wash-out solution.

Figure 11.

Wash-out of M-PEG-qdots. z-transformed data (median values $\pm$ 99% CI) obtained with unsupplemented Ringer (Panel A), or Ringer supplemented with 1 mM muscimol (B). In each experiment, presentation of the M-PEG-qdot preparation was immediately followed by a rapid ($\sim$ 1 - s) wash with unsupplemented Ringer (time zero in the experiment), and subsequent incubation with the unsupplemented or muscimol-supplemented perfusing medium.

Table 1

Description of the tested M-PEG-qdot conjugates. The quoted approximate number of muscimols tethered to each qdot is based on an assumed equality of the coupling efficiency of muscimol-terminated and methylamine-terminated PEG3400 chains to the AMP-qdots [about 20% (22)].

Structure designation	Respective molar proportion of muscimol- vs. methylamine-terminated ligand in the conjugation reaction	Approximate number of muscimol moieties per qdot
M-PEG-qdot0	0/100	0
M-PEG-qdot25	25/75	37–50
M-PEG-qdot50	50/50	75–100
M-PEG-qdot75	75/25	100–150
M-PEG-qdot100	100/0	150–200