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Structural and functional probing of the biogenic amine transporters by fluorescence spectroscopy

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Abstract

Fluorescence spectroscopy techniques have proven extremely powerful for probing the molecular structure and function of membrane proteins. In this review, it will be described how we have applied a series of these techniques to the biogenic amine transporters, which are responsible for the clearance of dopamine, norepinephrine, and serotonin from the synaptic cleft. In our studies, we have focused on the serotonin transporter (SERT) for which we have established a purification procedure upon expression of the transporter in Sf-9 insect cells. Importantly, the purified transporter displays pharmacological properties in detergent micelles similar to that observed in membranes suggesting that the overall tertiary structure is preserved upon purification. Using this purified SERT preparation and the fluorescent cocaine analogue RTI-233 as a molecular reporter, we have been able to characterize the microenvironment of the cocaine-binding pocket. In current follow-up studies, we are attempting to map the relative position of this binding pocket using fluorescence resonance energy transfer (FRET) between RTI-233 and an acceptor fluorophore covalently attached to endogenous cysteines in the transporter. Finally, it will be described how we recently initiated the implementation of single-molecule confocal fluorescence spectroscopy techniques in our studies of the SERT.

Keywords: Structural probing; Functional probing; Biogenic amine; Flourescence spectroscopy

1. Introduction

The clearance of biogenic amine neurotransmitters from the synaptic cleft occurs via three distinct transporters, the serotonin transporter (SERT), the dopamine transporter (DAT), and the norepinephrine transporter (NET). These three highly homologous transporters belong to the family of Na^+/Cl^--coupled transporters that are believed to share both a common overall tertiary structure and evolutionarily conserved mechanism of substrate translocation. It is the general perception that the substrate translocation process is best explained by an alternate access model (Rudnick, 1997), although an increasing amount of data suggest that this might represent a clear oversimplification. Following binding of extracellular substrate and electrolytes to an 'outward-facing' conformation, the transporter alternates to an 'inward-facing' conformation powered by the inwardly directed electrochemical potential for Na^+. Upon release of the substances to the cytosol, the transporter reorients to the 'outward-facing' conformation. In this process, several transporter domains are thought to undergo large-scale conformational changes. Biochemical and electrophysiological approaches have been applied in an attempt to understand these structural changes (Chen and Reith, 2000; Norregaard and Gether, 2001; Torres et al., 2003); however, a greater understanding of the finely orchestrated structural changes has not yet been achieved both due to the lack of a high-resolution transporter structure and because methods allowing time-resolved assessment of conformational changes have been applied to this family of transporters to a very limited extent for mostly technical reasons.

The absence of high-resolution structural information has also hindered detailed insight into the binding site for inhibitors of the biogenic amine transporters including drugs of abuse such as cocaine and antidepressants such as the

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selective serotonin reuptake inhibitors. In the case of cocaine, several residues have been identified that, upon mutation, alter the apparent affinity of cocaine (Itokawa et al., 2000; Lin et al., 2000a,b; Lin and Uhl, 2002; Wu and Gu, 2003; Kitayama et al., 1992); however, it is unclear whether any of these interact directly with cocaine. For example, although mutation of the intracellular Tyr^{335} to alanine decreases the apparent affinity 150-fold, this is most likely a consequence of an altered equilibrium between distinct states in the transport cycle rather than due to disruption of a specific molecular interaction (Loland et al., 2002). Accordingly, there has been an obvious need to complement mutational studies with alternative approaches such as, for example, photolabeling strategies or fluorescent spectroscopy.

The use of fluorescence techniques to probe the structure and function of membrane proteins has proven to be extremely powerful. It embraces numerous applications in numerous membrane proteins ranging from biophysical characterization of ligand binding sites and direct time-resolved detection of conformational changes to assessment of oligomerization and fluorescent imaging in living cells (Kobilka and Gether, 2002; Schmid et al., 2001). In this review, we will describe how we are currently using fluorescent probes in the study of biogenic amine transporters with particular focus on the purified human SERT (hSERT). Although several of the described approaches are established techniques in several other membrane proteins, the application of fluorescent methods to this particular class of proteins is relatively novel.

2. Fluorescently labeled ligands

Fluorescently labeled high-affinity ligands represent a widely applicable tool. They can be used, for example, to substitute for radioligands in binding assays and to visualize their target protein directly in living cells (Hein et al., 1997). Fluorescently derivatized ligands can also be used as a tool to characterize the biophysical microenvironment of a binding site as well as its accessibility to the aqueous milieu upon which the location of the binding site in the protein structure may be inferred. In this manner, the use of fluorophore-labeled peptide agonists of substance P and the macrophage inflammatory protein-1α (MIP-1α) have confirmed that these large peptide ligands upon binding are strongly exposed to the aqueous environment consistent with a binding site involving the extracellular regions of their respective G-protein coupled receptors (Turcatti et al., 1997; Zoffmann et al., 2002). In contrast, the use of either an intrinsically fluorescent antagonist of the β_2-adrenoceptor carazolol or a fluorescently derivatized nonpeptide antagonist of the substance P receptor showed evidence that these small-molecule ligands bind deep within the G-protein coupled receptor structure completely inaccessible to the aqueous environment (Tota and Strader, 1990; Turcatti et al., 1997).

In transporters, fluorescent substrates are highly attractive allowing for nonradioactive uptake assays. Due to the structural specificity required for substrates and the generally large molecular size of fluorescent moieties, only very few fluorescent molecules are known for the biogenic amine transporters. Recently, the fluorescent substrate ASP(+) [4-(4-(dimethylamino)styrl)-*N*-methylpyridinium] that acts as a substrate at SERT, NET, and DAT was used to explore kinetics of substrate translocation in the NET (Schwartz et al., 2003). Most importantly, the use of ASP(+) allowed the distinguishing of substrate binding from substrate transport. This showed that substrate binding could occur independently of the presence of Na^+, although the transport process is strictly Na^+-dependent (Schwartz et al., 2003).

3. Probing the cocaine binding pocket using fluorescent cocaine analogues

Cocaine binds with high affinity to the SERT, NET, and DAT and is generally believed to act as a competitive blocker of substrate translocation (Povlock and Amara, 1997; Carroll et al., 1997). Attempts to define the cocaine binding site in the transporter structure have been the focus of many studies, including screening for potential coordinating residues by site-directed mutagenesis (Itokawa et al., 2000; Lin et al., 2000a,b; Lin and Uhl, 2002; Wu and Gu, 2003; Kitayama et al., 1992), application of methanethiosulfonate accessibility studies (Chen and Rudnick, 2000; Ferrer and Javitch, 1998), and the use of photoaffinity cocaine analogues (Vaughan et al., 1999, 2001; Zou et al., 2001). However, despite these efforts, the binding site still remains largely uncharacterized.

We decided therefore to explore the biophysical nature of the cocaine binding site by the use of fluorescently derivatized cocaine analogues. The structural requirements for generating a fluorescent inhibitor are less stringent than for a substrate, making it possible to attach convenient fluorescent moieties to the backbone of known inhibitors. A series of fluorescent cocaine analogues has, in this way, been synthesized by Rasmussen et al. (2001) and Carroll and Maresch (unpublished observation). One of the fluorescent analogues, RTI-233, contains the environmentally sensitive fluorescent moiety NBD (7-nitrobenz-2-oxa-1,3-diazol-4-yl) incorporated via a *N*-methylethanolamine linker to a 2β-carboxy group of the tropane backbone of a cocaine analogue with increased binding affinity as compared to that of cocaine itself. Importantly, RTI-233 maintained a high binding affinity displaying a K_i value of ~ 6 nM for the SERT. Unfortunately, due to the hydrophobic nature of RTI-233, it was not possible to conduct experiments on membrane preparations from cells expressing SERT, as fluorescence from nonspecific binding to the membranes overwhelmed the fluorescence from specific binding of SERT. Similarly, the hydrophobic properties of RTI-233 prevented us from conducting experiments in

living cells because of nonspecific association of RTI-233 to the lipid membranes (Loland et al., unpublished observation). However, this fluorescence 'noise' was eliminated upon purification of the SERT. The SERT was expressed in Sf-9 cells and purified by solubilization in digitonin followed by nickel affinity and concanavalin A chromatography (Rasmussen et al., 2001). The purified SERT displayed a similar pharmacological profile to Sf-9 membrane-expressed SERT, supporting the contention that purified SERT in detergent micelles could be used as a model system for exploring the biophysical characteristics of the binding site for cocaine-like blockers (Rasmussen et al., 2001).

The high sensitivity of the NBD moiety to the polarity of the environment is illustrated in Fig. 1A by the emission spectra for RTI-233 in dioxane/water mixtures. Decreasing the polarity of the solvent by adding increasing concentration of dioxane caused a significant blue shift in λ_{Max} (wavelength at which maximum emission occurs) and a concomitantly dramatic increase in the fluorescence quantum yield from RTI-233. The strong dependence of the fluorescence of RTI-233 on the polarity of the surrounding solvent corroborated the possibility of using RTI-233 as a molecular reporter of the microenvironment in the cocaine-binding crevice of the SERT.

The highly hydrophobic microenvironment of the cocaine binding site was evidenced by recording the emission spectrum of the fluorescent cocaine analogue bound to the purified SERT. This displayed a λ_{Max} of 532 nm corresponding to the λ_{Max} of RTI-233 in 80% dioxane (Fig. 1B). Specific binding of RTI-233 to the SERT was demonstrated by the competitive inhibition of fluorescence using excess serotonin, citalopram, or RTI-55. Moreover, specific binding was evidenced by measurement of steady-state fluorescence anisotropy showing constrained mobility of bound RTI-233 relative to RTI-233 free in solution (Rasmussen et al., 2001).

The accessibility of bound RTI-233, as compared to free RTI-233, was evaluated in collisional quenching experiments using the aqueous quencher iodide (I^-). Collisional quenching requires a bimolecular interaction between the quencher and the fluorophore, and therefore such experiments can determine the 'availability' of the fluorophore to the surrounding solvent (Lakovicz, 1999). The Stern–Volmer plots in Fig. 1C and D in which F_0/F is plotted against the potassium iodide or 2,2,6,6-tetrame-

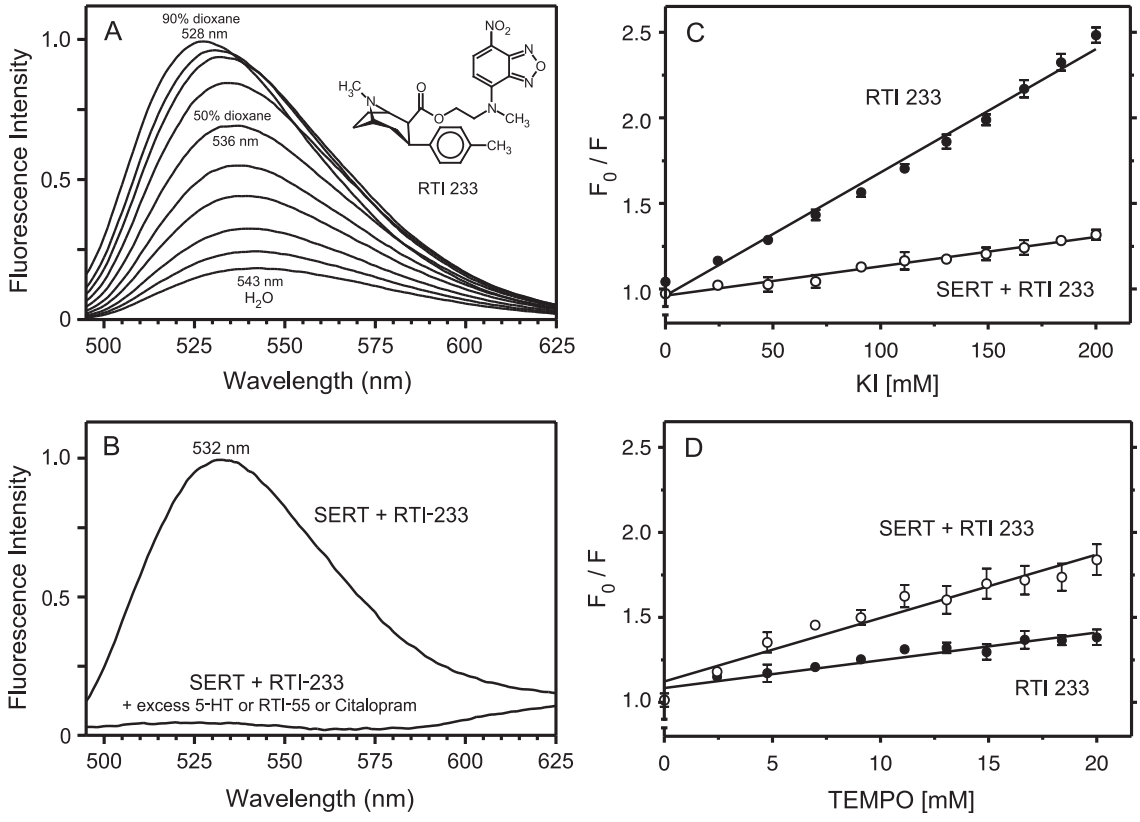

Fig. 1. Fluorescent properties of RTI-233 free in solution and bound to the purified SERT. (A) Emission spectra of free RTI-233 in dioxane/H_2O. The fraction of dioxane ranges from 0% to 90% (v/v) with 10% intervals. (B) Emission spectrum of RTI-233 bound to SERT (λ_{Max} = 532 nm) with control emission spectra obtained by preincubation with an excess of 5-HT, citalopram, or RTI-55. The spectra shown are representative of nine experiments. In all experiments, the excitation wavelength was 480 nm with a 5-nm bandpass for both excitation and emission. (C) Stern–Volmer plots of collisional quenching of free RTI-233 and RTI-233 bound to SERT by the aqueous quencher potassium iodide (KI). (D) Quenching of RTI-233 fluorescence by the lipid-soluble quencher TEMPO. Open circles: RTI-233 bound to rSERT; closed circles: free RTI-233. (Figure reproduced from Rasmussen et al., 2001).

thylpiperidine-*N*-oxyl (TEMPO) concentration show that iodide was found to be a strong quencher of the fluorescence of RTI-233 free in buffer. Quenching of bound RTI-233 was substantially smaller; however, despite the hydrophobic nature of the binding crevice, it was still apparent. This finding suggests a partially exposed binding site. The findings are in contrast to the observations for small-molecule ligand binding sites in other membrane proteins, such as G-protein coupled receptors as mentioned above, where the binding sites for small-molecule ligands are known to be deeply embedded in the transmembrane core of the molecule and entirely inaccessible to aqueous quenching (Tota and Strader, 1990; Turcatti et al., 1997).

4. Analyzing the cocaine binding pocket by fluorescence resonance energy transfer

Using RTI-233 we achieved insights into the biophysical microenvironment of the cocaine binding site, but a precise location of the site could not be inferred from the results. We are now addressing this problem from a different point by performing fluorescent resonance energy transfer (FRET) measurements in the purified human SERT using endogenous cysteines labeled with the long-wavelength fluorophore Texas Red as acceptor and the fluorescent cocaine analogue RTI-233 as donor (Fig. 2). This donor–acceptor pair was chosen for their appropriate spectral overlap as illustrated in Fig. 2A by the RTI-233 emission and Texas Red excitation spectra (Fig. 2A, dark blue and orange spectra).

Labeling the purified SERT with Texas Red bromoacetamide is well tolerated and results only in a minor decrease in B_{max} and virtually unaltered binding affinities of several ligands, suggesting a preserved overall transporter structure (Rasmussen and Gether, unpublished observation). With the fluorescent cocaine analogue RTI-233 bound to the Texas Red labeled SERT, the emission from Texas Red fluorophore(s) increased significantly in intensity as compared to Texas Red-SERT with no donor fluorescence bound (Fig. 2B, red solid and broken line spectra). This increase in acceptor fluorescence (Fig. 2B, red spectra) was associated with a decreased emission from RTI-233 bound to Texas Red-hSERT as compared to the emission of RTI-233 bound to unlabeled SERT (Fig. 2B, blue and red solid line spectra), further supporting the observed energy transfer from RTI-233 to Texas Red fluorophore(s). To identify the labeled site(s) responsible for the observed FRET, we have generated a large series of SERT mutants with 1–5 endogenous cysteines substituted at a time.

Interestingly, we have identified a mutant in which a substantial loss in FRET between RTI-233 and the Texas Red-labeled SERT was observed upon substitution of five endogenous cysteines (Rasmussen and Gether, unpublished observation). This supports the hypothesis that the major FRET-contributing sites can be identified and hence that distances from the cocaine binding site to labeled endogenous cysteine(s) might be estimated.

5. Assessing conformational changes by fluorescence labeling of the transporter

Many different techniques have been applied to membrane proteins to identify and characterize conformational changes critical for their proper function. In biogenic

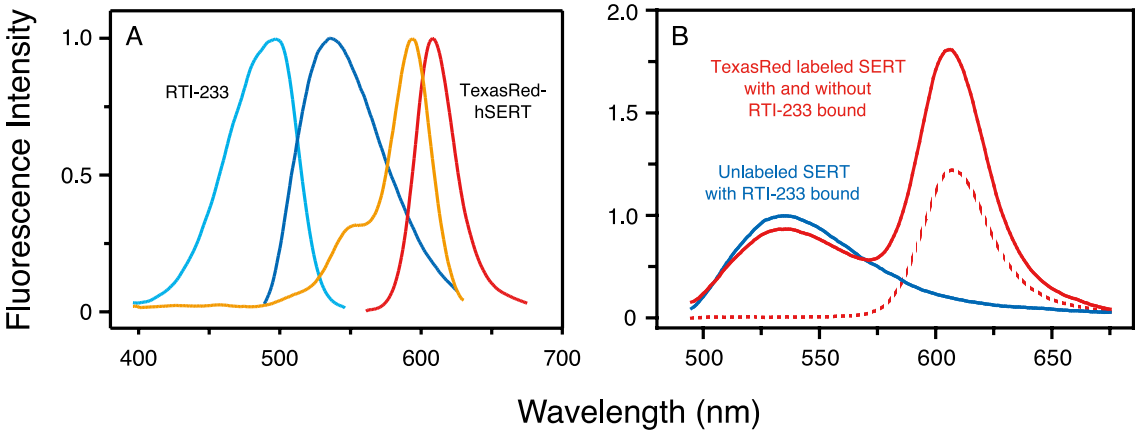

Fig. 2. Fluorescence resonance energy transfer within the RTI-233 bound and Texas Red-labeled SERT. (A) Spectral properties of the donor–acceptor pair for fluorescence resonance energy transfer. The excitation spectra of RTI-233 and Texas Red-labeled SERT measured separately are shown in blue and orange, respectively. The emission spectra of RTI-233 and Texas Red-labeled SERT measured separately are shown in dark blue and red, respectively. (B) Emission spectra showing energy transfer from RTI-233 in the cocaine binding site to the Texas Red fluorophores labeled in the SERT. The emission of RTI-233 bound to the unlabeled SERT are shown in blue, whereas the red spectra shows the Texas Red-labeled transporter with (solid red line) or without (broken red line) RTI-233 bound. Excitation was set at 480 nm.

amine transporters, conformational changes has been indirectly assessed by the use of the substituted cysteine accessibility method (SCAM) (Chen and Rudnick, 2000; Ferrer and Javitch, 1998; Norregaard et al., 2003) or the use of engineered Zn^{2+} binding sites (Norregaard et al., 1998, 2000; Loland et al., 1999). Application of SCAM has provided strong evidence that inhibitors such as cocaine can promote significant conformational changes in several parts of the transporter molecule. This conclusion was based on the finding that cocaine could alter significantly the reactivity of several endogenous cysteines in both DAT and SERT (Ferrer and Javitch, 1998; Chen and Rudnick, 2000). Evidence for substrate and/or Na^+-dependent conformational changes has also been obtained in SERT, NET, and DAT by the use of SCAM. The accessibility of cysteines introduced in transmembrane segment 3 of SERT and NET (Chen and Rudnick, 2000), in the intracellular loops of the SERT (Androutsellis-Theotokis and Rudnick, 2002), or at the top of transmembrane segment 7 in the DAT has, for example, been shown to be highly sensitive to the presence of substrate and/or Na^+ (Norregaard et al., 2003). The occurrence of conformational changes at the extracellular ends of transmembrane segments 7 and 8 has been additionally supported by the observation that binding of Zn^{2+} between transmembrane segments 7 and 8 results in potent and noncompetitive inhibition of the translocation process consistent with the ability of Zn^{2+} to constrain critical movement between these two domains (Loland et al., 1999; Norregaard et al., 2000). Nevertheless, although both SCAM and Zn^{2+} site engineering are powerful tools, they are indirect methods that do not allow for direct assessment of conformational changes and thus for kinetic measurements. Moreover, the use of SCAM is critically dependent on the ability of cysteine-reactive compounds to perturb the functional properties of the protein upon reaction with a given cysteine. Similarly, the use of Zn^{2+} site engineering is critically dependent on the ability of Zn^{2+} to perturb protein function by binding to the engineered site.

In contrast to SCAM and Zn^{2+} site engineering, conformational readouts based on fluorescent labeling of the protein of interest represent a direct means of reporting conformational changes that allows for fast real-time kinetic measurements. Conformational movements can be detected by exploiting several different photophysical phenomena, such as the inherent sensitivity of many fluorophores to the polarity of the surrounding microenvironment (Kobilka and Gether, 2002), collisional quenching by aqueous and nonaqueous quenchers (Jensen et al., 2001) as well as FRET, if two distinct sites can be simultaneously labeled with an appropriate pair of donor and acceptor fluorophores (Cha et al., 1999). A major challenge is nonetheless how to achieve site-specific chemical labeling of the protein of interest with the desired conformationally sensitive reporter fluorophore. Chemical labeling of proteins can quite easily be carried out by taking advantage of the reactivity of cysteines or, alternatively, lysines; however, to minimize labeling of irrelevant proteins, the protein must either be isolated by purification or an expression system should be used in which the heterologously expressed protein predominates compared to other proteins. Interestingly, the latter is a feasible approach using Xenopus laevis oocytes as an expression system. Thus, the expression levels that can be achieved in oocytes are so high that the majority of protein expressed in the membrane is protein-encoded by the injected cRNA. To perform the experiments, the membrane protein of interest is expressed in the oocytes and subsequently directly labeled in situ with sulfhydryl-reactive fluorophores and the fluorescence measured using a fluorescence microscope setup. Importantly, this method allows for simultaneous two-electrode voltage clamp measurements and thus simultaneous evaluation of protein function. This technology has successfully been applied to the γ-aminobutyric acid $(GABA)_A$ receptor (Chang and Weiss, 2002), the shaker potassium channel (Mannuzzu et al., 1996; Cha and Bezanilla, 1997; Cha et al., 1999), and the Na^+-glucose transporter-1 (SGLT-1) (Meinild et al., 2002). Recently, it has also been applied to the GABA-transporter-1 (GAT-1) and to the SERT where labeling with tetramethylrhodamine-5-maleimide and sulforhodamine-methanethiosulfonate, respectively, enabled detection of discrete conformational changes in these transporter protein (Li et al., 2000; Li and Lester, 2002). In the SERT, it was proposed that the first extracellular loop forms a part of a conformationally active external gate (Li and Lester, 2002).

Unfortunately, the fluorescent-labeling studies in oocytes are often hampered by a poor signal-to-noise ratio due to nonspecific labeling of other proteins. In addition, it is necessary to avoid labeling of intracellular proteins, and thus it is only possible to label the protein on its extracellular side using fluorophores that cannot penetrate the membrane. The alternative is to use a purified protein preparation for fluorescent labeling. In the case of the β_2-adrenoceptor, for example, a purified preparation of the receptor in detergent micelles was successfully used in fluorescent-labeling studies aimed at mapping conformational changes critical for receptor activation (Gether, 2000). By covalent incorporation of the environmentally sensitive and sulfhydryl-reactive fluorophore IANBD probe [*N*,*N*′-dimethyl-*N*-(iodoacetyl)-*N*′-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) ethylene-diamine] into the purified β_2-adrenoceptor, it was possible to map agonist-specific conformational changes critical for receptor activation. First, labeling of endogenous cysteines with IANBD provided evidence for the agonist-induced conformational changes of specific transmembrane segments (transmembrane segments 3 and 6) (Gether et al., 1995, 1997). Next, the analysis of a series of mutants with single cysteine substitutions at the intracellular side of transmembrane segment 6 provided insight into the character of the transmembrane segment 6 move-

ment likely involving a major movement of transmembrane segment 6 away from the receptor core (Jensen et al., 2001).

A similar insight has thus far not been reached in biogenic amine transporters, although our purified preparation of the SERT corresponds well to the purity that we obtained for the β_2-adrenoceptor. However, several critical requirements for performing similar studies in SERT have been established. It is possible to label the SERT with the environmentally sensitive and cysteine-specific IANBD probe at concentrations up to 100 μM with no significant reduction in the B_{max} or effect on binding affinities of 5-hydroxytryptamine (5-HT) and inhibitors of the transporter. In addition, we have obtained evidence that certain sites might be preferentially labeled compared to others. This is important given that the SERT contains a total number of 18 cysteines of which 16 are believed to be available for chemical derivatization. The evidence was obtained by performing collisional quenching experiments using the aqueous quencher iodide on IANBD-labeled wild-type SERT and on a mutant SERT in which five cysteines were substituted. These experiments showed a marked quenching of fluorescence in the presence of iodide in wild type; however, in the mutant, quenching by iodide was reduced substantially (Rasmussen and Gether, unpublished observation). This suggested that one or more of the mutated sites accounted for the wild-type phenotype. Interestingly, we observed that mutating only Cys^{109}, situated in the short first extracellular loop connecting transmembrane segments 1 and 2, resulted in the same phenotype as that observed in a mutant with five cysteines knocked out simultaneously. These results suggest that Cys^{109} is a major labeling site for IANBD (Rasmussen and Gether, unpublished observation). Furthermore, the data suggest that the environmentally sensitive fluorophore is buried in a mixed hydrophilic/hydrophobic environment at this site.

Nonetheless, we have not yet been able to detect conformational changes in the IANBD-labeled SERT, suggesting that the labeled Cys^{109} and other unidentified labeling sites either do not move upon substrate/inhibitor/ion binding or, more likely, that they move but not in a way that significantly alters the polarity of the environment surrounding the attached fluorophore. The absence of measurable fluorescent changes may also be caused by labeling a large fraction of the 18 endogenous cysteines that would mask any emission changes at a single site. Systematic cysteine knockout mutagenesis might alleviate such problems. It should be noted that we can detect distinct ligand-stabilized conformational states of the SERT purified in detergent micelles using a biochemical assay. Chemical cleavage of purified SERT using activated Cu^{2+}/1,10-phenanthroline results in distinct cleavage patterns depending on the ligand bound (Rasmussen and Gether, unpublished observation). These results support the contention that IANBD-labeled SERT with a minimized number of cysteines at some point can be established as a model system for uncovering conformational changes in the biogenic amine transporters.

6. Implementation of single molecule confocal fluorescence spectroscopy

Confocal fluorescence correlation spectroscopy (confocal FCS) is a powerful single-molecule fluorescence technology that can be used to explore protein structure and protein function in vitro as well as in living cells. In FCS, the movement of a fluorescent particle through a defined space is recorded by the fluctuations in fluorescence intensity as the particle moves through that volume. This technique was originally developed in 1972 (Magde et al., 1972), although it only became generally applicable 20 years later when the definition of a femtoliter observation volume became a possibility by use of a confocal microscope, allowing for optimal analysis of fluorescence fluctuations (Rigler et al., 1993). The intensity fluctuations are detected by an avalanche photodiode rather than the usual photomultiplier tube used in confocal microscopy; this allows for the detection of events on the scale of single photons. Importantly, the small volume and the ultrasensitive detection allow for the investigation of literally single molecules. The information from the intensity fluctuations over time are converted by an integrated correlator to an autocorrelation function which describes both the diffusion time and the concentration of the fluorescent particles in the volume. Although many investigators use self-built FCS systems, Zeiss has a commercially available combined confocal microscope and correlator which is nicely suited for the biologist.

From the information contained in the autocorrelation, many properties of the fluorescent molecule can be determined. FCS has been used to investigate binding properties for a number of systems, including ligand binding studies of the 5-HT_3 receptor (Wohland et al., 1999), insulin receptor (Rigler et al., 1999), and colchicine binding to tubulin (Van Craenenbroeck and Engelborghs, 1999), as well as protein–protein interactions such as the ATP synthase complex (Hasler et al., 1999). Investigation of protein–protein interactions has also helped to elucidate the oligomerization state of molecules such as the somatostatin receptors (Patel et al., 2002) and E-cadherin (Iino et al., 2001). Currently, we are exploring the possibility of assessing the oligomerization stoichiometry of a bacterial homolog of the mammalian biogenic amine transporters. Furthermore, conformational changes of DNA (Bonnet et al., 1998; Eggeling et al., 1998) and both cytoplasmic (Chattopadhyay et al., 2002) and integral membrane proteins (Kastner et al., 2003) have been probed with the use of FCS. Yet another application for FCS is its use in high throughput screening, as was demonstrated by Pitschke et al. (1998) in their detection of aggregated amyloid β-protein in the cerebrospinal fluid of Alzheimer's patients. Several in-depth reviews on the technique and

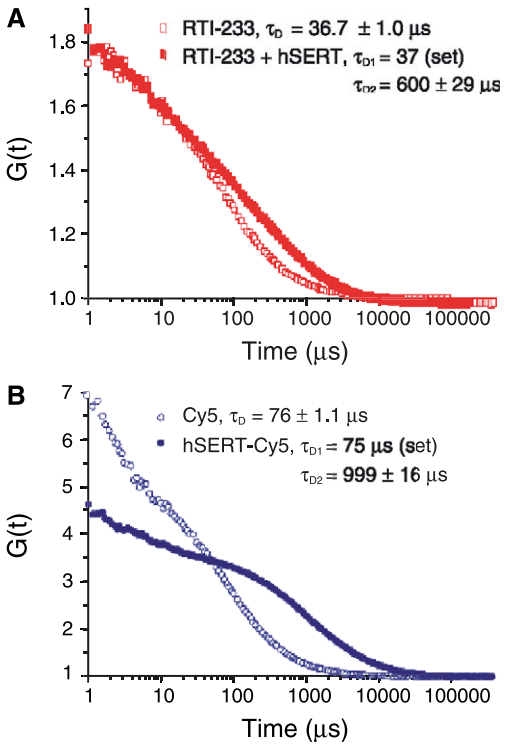

Fig. 3. Autocorrelation curves for hSERT with bound and free fluorophore. (A) The cocaine analogue RTI-233 shown free in 0.1% digitonin buffer (open squares) and bound to unlabeled hSERT (closed squares). The binding was performed in 0.1% digitonin buffer at room temperature for 30 min, with 5 nM of RTI-233 and approximately 100 nM of unlabeled hSERT. No significant autofluorescence from the unlabeled hSERT was observed. (B) The fluorescent Cy5 maleimide free in solution (open circles) and covalently attached to hSERT (closed circles). The presence of two components, a fast one with a diffusion time similar to that observed with the free dye and a slow component comprising the labeled hSERT, is detected despite affinity column separation of Cy5-hSERT from free Cy5 maleimide.

applications of FCS have recently been published (Rigler and Elson, 2001; Hess et al., 2002; Haustein and Schwille, 2003; Bacia and Schwille, 2003).

The advantages of FCS include that of needing only very small concentrations (1 nM) of fluorescent molecule, examining particles as small as a dye molecule or as large as a vesicle (100–200 nm diameter), and perhaps most interestingly, examining kinetic events such as binding, as well as the movement of a fluorescent molecule, in real time. Furthermore, FCS possesses the additional advantage of preserving the sample. In techniques such as fluorescent recovery after photobleaching (FRAP) and FRET, the fluorescent molecules of interest are bleached on a large scale. With FCS, only the molecules passing through the confocal volume are affected, and the laser intensity used for illumination is often so low that the particle does not become bleached at all. FCS can be achieved in solution or in the cellular environment, utilizing fluorescent dyes or intrinsically fluorescent protein tags, such as variants of the green fluorescent protein (GFP) family.

7. FCS measurements on purified SERT

Our initial FCS studies have been performed in solution using the purified SERT and the fluorescently labeled cocaine analogue RTI-233 (Rasmussen et al., 2001). Initial investigation of the diffusional characteristics of the digitonin-solubilized SERT was determined with protein covalently labeled with the sulfhydryl-reactive fluorophores iodoacetemide NBD, Texas Red bromoacetamide, or Cy5 maleimide. As would be expected, the results show that the labeled protein possesses a much longer diffusion time than the free dye alone (Fig. 3). This was also observed for SERT labeled with dyes excitable at other wavelengths such as, e.g., NBD (488 nm) and Texas Red (543 nm) (Table 1). The diffusion time of the fluorescent molecule may be used to estimate the approximate molecular weight by using the Stokes–Einstein equation and the equations for calculating the hydrodynamic radius of a molecule and the diffusion coefficient. Using the diffusion times obtained for the free dyes, the agreement with the known molecular weight was good, and the fit of the autocorrelation curve indicated the presence of only one component. In contrast, the dye-labeled transporter appears to contain two components, a small one corresponding to the diffusion time of the free dye and a much larger component. Upon inspection of the large component, it appeared that the sizes of the purified SERT based on the FCS measurements far exceeded the expectations of even a tetrameric complex of SERT in a digitonin micelle. There are, however, several explanations for an overestimate in size. Any fluorescent aggregates existing in the solution will skew the autocorrelation to emphasize the very large, very bright particles traveling through the confocal volume, resulting in an overestimate of diffusion time. In addition, heterogenous labeling of SERT by the fluorescent probe could confound the diffusional results, resulting in much brighter particles dominating the autocorrelation. As there are 18 cysteines in the SERT, it is likely that labeling is heterogenous. Furthermore, it must be taken into consideration that the digitonin micelle itself is very

Table 1
The fluorescently labeled hSERT diffuses as a very large complex

Fluorophore	Laser line (nm)	τ_D free (µs)	τ_D bound (µs)
NBD	488	38.5 ± 5.5	1280 ± 110
Texas Red	543	50.0 ± 1.0	925 ± 22
Cy5	633	76.0 ± 1.1	999 ± 16
RTI-233	488	36.7 ± 1.0	600 ± 29

FCS studies were performed using a standard LSM 510-ConfoCor II system from Zeiss. The free dyes and labeled hSERT were diluted to single molecules (~ 1 nM) in 0.1% digitonin buffer. All were examined at low laser intensities (10% or less), and data shown represent the results of several experiments, each conducted 10 times for 20–30 s. The diffusion times represent the dye and fluorescent ligand alone, and covalently linked to hSERT or for the RTI-233, in the presence of unlabeled hSERT. The bound components are the larger component from a two-component fit, with the free dye diffusion time set as the smaller component, as shown in Fig. 3. The fits for the bound fluorophores (not shown) were very obviously two components, as concluded from the residuals.

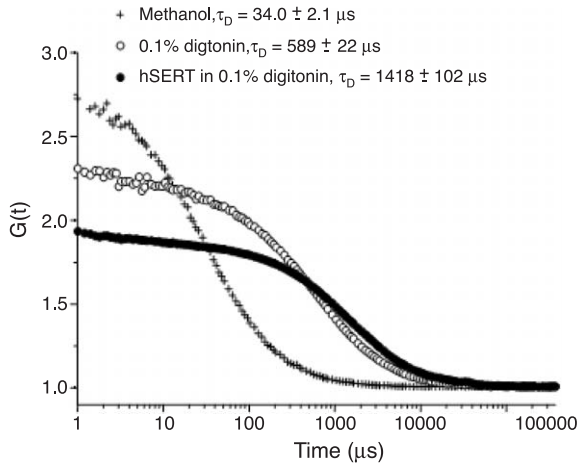

Fig. 4. Cholesteryl BODIPY incorporated into digitonin micelles and the hSERT complex. Cholesteryl BODIPY was diluted in methanol, 0.1% digitonin, or hSERT in 0.1% digitonin. The diffusion time of the lipid probe increased dramatically upon dilution into the digitonin buffer and the hSERT in digitonin buffer. Due to the extreme hydrophobicity of the fluorescent lipid, only one component was detected in the fits for digitonin and hSERT. Measurements were performed as described above.

large compared to most detergent micelles, approximately 200 kDa (Haga et al., 1990; Adkins, unpublished observations). The size of a micelle incorporating multiple SERT molecules is unknown, although examination of the micelles themselves using a fluorescent lipid probe indicates that they are larger when determined by FCS than by differential centrifugation (1118 kDa vs. 200 kDa), although this, in turn, is smaller than the digitonin complex containing hSERT (Fig. 4). Importantly, the diffusional characteristics of the digitonin-solubilized SERT were unaffected by alkylating cysteines with *N*-ethyl-maleimide at the time of solubilization, excluding that disulfide-mediated aggregation was causing the apparent size overestimation (data not shown). Note that in the experiments with *N*-ethyl-maleimide, the diffusional characteristics of the digitonin-solubilized SERT were assessed using the fluorescent cocaine analogue RTI-233 (see below). Altogether, the data strongly suggest that the SERT is purified as an oligomeric complex, and the large size is not due to aggregation, but likely to a combination of the large micelle size, a hSERT oligomeric complex, and an intrinsic overestimate of size by FCS due to multiple cysteine labeling of the protein. The use of the fluorescent cocaine analogue (RTI-233) for exploration of the cocaine binding pocket has been described above. We have also used RTI-233 to assess binding to purified SERT using FCS. The NBD fluorophore in RTI-233 is, however, not optimal for FCS studies due to a low quantum yield, and thus reliable data were rather difficult to obtain. Nevertheless, we did obtain clear evidence that in the presence of unlabeled SERT, RTI-233 displays a substantial increase in diffusion time and that this diffusion time for RTI-233 bound to SERT is on the same order of magnitude as the dye-labeled hSERT (Table 1; Fig. 3B). Other fluorescent cocaine analogues (RTI-587 and RTI-592) also display specific binding to the unlabeled hSERT (Adkins and Gether, unpublished observation).

Altogether, these initial experiments have demonstrated some of important potentials of confocal single-molecule fluorescence techniques. First, we have been able to estimate directly the approximate size of a purified transporter complex. The fact that the SERT is purified as an oligomeric complex provides additional support for the contention that the biogenic amine transporters form oligomeric complexes (Schmid et al., 2000; Hastrup et al., 2001; Kilic and Rudnick, 2000; Norgaard-Nielsen et al., 2002). Next, we have been able to demonstrate that FCS can be used for rapid detection of direct binding to the purified SERT without the need for separating bound from unbound and using extremely small amounts of both ligand and transporter. An even greater potential might be cellular FCS using transporters that are N-terminally tagged with green fluorescent proteins. This allows for the direct assessment of mobility of the transporter in the plasma membrane and thereby real time assessment of, for example, cellular microdomain association under different functional conditions.

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