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Reversible Sequential-Binding Probe Receptor–Ligand Interactions in Single Cells

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With the reversible sequential (ReSeq) binding assay,we present a novel approach for the ultrasensitive profiling of receptor function in single living cells. This assay is based on the repetitive application of fluorescent ligands that have fast association–dissociation kinetics. We chose the nicotinic-acetylcholine receptor (nAChR) as a prototypical example and performed ReSeq equilibrium, kinetic, and competition-binding assays using fluorescent derivatives of the antagonist α-conotoxin GI (α-CnTx). Thereby, we determined the binding constants of unlabeled α-CnTx and d-tubocurarine. The high selectivity of α-CnTx for muscle-type nAChR made it possible to observe specific binding even in the presence of other nAChR subtypes. Imaging of individual nAChRs and ligand-binding cycles to single cells in microfluidic devices demonstrated the ultimate miniaturization and accuracy of ReSeq-binding assays even at low receptor-expression levels. We expect our approach to be of generic importance for functional screening of compounds or membrane receptors, and for the detailed characterization of rare primary cells.

Introduction

The advent of proteomics and combinatorial chemistry created a growing need for novel strategies to probe receptor function and screen compound activity efficiently. For instance, biophysical, functional, and pharmacological investigations on cell-surface receptors are often performed with solubilized and purified proteins, or cultured cell lines that overexpress particular receptors that are of importance in cellular signaling and as drug targets.[1] However, it is becoming increasingly apparent that receptor function in such purified systems or in heterologous cell lines might differ from their function in native tissue cells.[2] Because primary cells are often very difficult to obtain and maintain in culture, it is highly desirable to optimize their use by performing series of investigations on the same single cell.

Ligand-binding assays that are presently used are predominantly end-point determinations on either homogenized or solubilized membrane fragments. Furthermore, they require receptor quantities that are equivalent to 10^2 to 10^5 cells per data point. For example, to obtain a complete binding isotherm, many experiments have to be performed in parallel and/or sequentially.

In order to monitor ligand–receptor interactions in single cells with high spatial and temporal resolution, it is essential to take advantage of the high sensitivity of fluorescence techniques that have become available recently.[3,4]

We present a novel method for performing fluorescence binding assays which we refer to as the reversible sequential (ReSeq)-binding assay. With this assay, a series of investigations can be performed on the same cell by repetitively applying specific, fluorescently labeled ligands that have fast association–dissociation kinetics. We demonstrate that complete saturation ligand-binding and competition ligand-binding assays can be performed on a single cell with excellent accuracy. This new approach has several advantages as it i) dramatically reduces the number of cells needed, ii) allows the investigation of cell-to-cell variations because extensive data can be collected with individual cells, and iii) circumvents problems related to low expression levels of receptors and photobleaching of fluorescent ligands, since measurements can be repetitively performed on the same cell to enhance accuracy. Moreover, ReSeq-binding assays can be easily automated and implemented in on-chip analysis which offers a substantial improvement on reliability, efficiency, and reduction of sample consumption.

In order to demonstrate the feasibility of this novel approach, the nicotinic-acetylcholine receptor (nAChR) was chosen as the prototype of a pharmacologically important target. Different subtypes of nAChRs can be found in the postsynaptic membrane of muscle and nerve cells.[5,6] Ligand binding to nAChRs is usually investigated by competition assays that either use radiolabeled or fluorescently labeled α-bungarotoxin (α-BgTx); this is a ligand that binds irreversibly to homopentameric α7, α9, α10, and muscle nAChRs. So far, only a few fluorescent ligands that bind reversibly to nAChRs have been identified.[7–11] These comprise fluorophores that have weak fluorescence properties and are unsuitable for cellular measurements. α-Conotoxins from the venom of marine snails have been shown to target either neuronal- or muscle-type

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nAChR specifically[12–14] and can serve as tools for the elucidation of nAChR structure and function.[15]

Here, we describe fluorescent derivatives of α-conotoxin GI (α-CnTx) which is a small 13-amino-acid-long peptide from the snail *Conus geographicus*.[16] These fluorescent ligands bind with high affinity and strong selectivity to one of the two agonist-binding sites, namely the α/δ site of the muscle-type nAChR.[17] By making use of the reversible binding and fast association–dissociation rates of fluorescently labeled α-CnTx, several kinetic and competitive ReSeq-binding experiments were performed on single cells that demonstrate the full potential of this approach.

Results

Electrophysiological characterization of fluorescently labeled α-CnTx

The binding properties of α-CnTx and its fluorescent variants to nAChR were quantified by whole-cell patch-clamp measurements. The addition of α-CnTx or its fluorescent variants to cells that expressed nAChR gradually decreased the maximum acetylcholine (ACh)-induced current response until a final stable level was reached (Figure 1a). Both the kinetics and the final current levels were dependent on the α-CnTx concentration. The antagonist could bind and be washed off completely within minutes; this allowed repetitive measurements with the same cell. The procedure is only limited by the stability of the patch-clamp seal. The rate constants, $k_{el,on}$ and $k_{el,off}$, of the inhibition kinetics were calculated from single exponential fits to the time dependence of the maximal-current responses. Control experiments with lower pulse rates (4 or 8 min between ACh pulses) yielded identical results; this indicates that binding kinetics were not affected by repetitive ACh pulses. Dissociation kinetics of all fluorescent toxin variants were comparable to the unmodified α-CnTx ($k_{el,off} = 5 \pm 2 \times 10^{-3}$, $6 \pm 4 \times 10^{-3}$, and $3 \pm 2 \times 10^{-3}\ s^{-1}$ for α-CnTx, α-CnTx–Cy5, and α-CnTx–A647, respectively). Association was three to four times slower than with unmodified α-CnTx ($k_{el,on} = 12 \pm 5 \times 10^{4}$, $2 \pm 1 \times 10^{4}$, and $3 \pm 3 \times 10^{4}\ M^{-1}s^{-1}$ for α-CnTx, α-CnTx–Cy5, and α-CnTx–A647, respectively). Accordingly, IC_{50} values calculated for the Cy5, Cy3, and Alexa 647 variants showed five-, twenty-, and eightfold lower affinities than unmodified α-CnTx ($IC_{50} = 20 \pm 2$ nM), respectively (Figure 1b). The Hill coefficients for all toxins were close to unity; this indicates that a similar number of bound toxin molecules were needed to block channel activation. The maximum peak currents in the absence of α-CnTx were usually several nA, which corresponds to only about 10^3 activated channels per cell. Such low numbers of receptors are usually difficult to observe in fluorescence experiments and make standard fluorescence binding assays impossible.

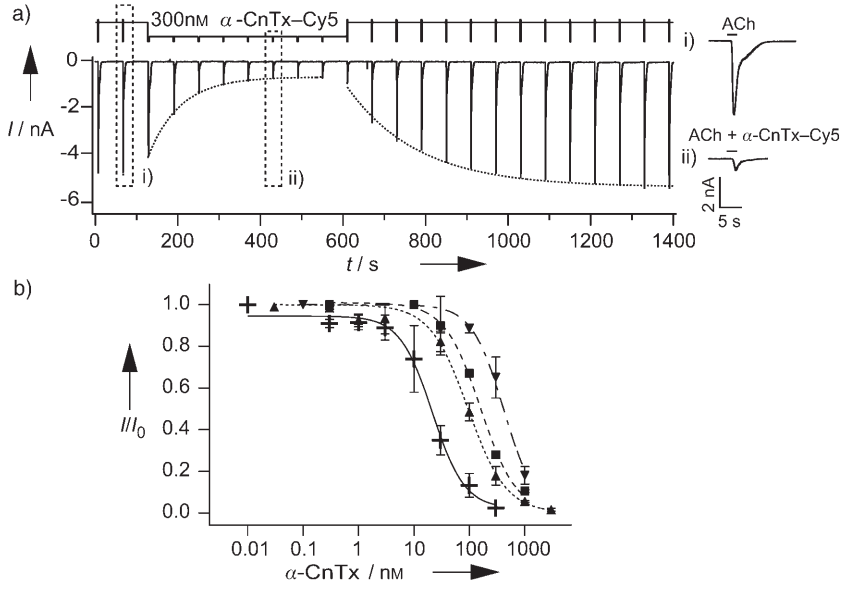

Figure 1. Electrophysiological characterization of α-CnTx and its fluorescent variants. a) Whole-cell current responses from HEK293 cells that expressed nAChR. Upper trace: 2 s pulses of ACh were applied every 60 s (short peaks) and α-CnTx (300 nM) was added continuously for 480 s, as indicated. The lower trace shows the current response, *I*. Peak-current responses were constant in the absence of antagonist ($t = 0$–100 s) while addition of α-CnTx–Cy5 and subsequent washing resulted in decrease ($t = 130$–600 s) followed by increase ($t = 600$–1400 s) of the current peaks, which yielded association- and dissociation-rate constants. Magnifications of the current responses in the absence and presence of α-CnTx–Cy5 are marked by (i) and (ii), respectively. This shows that only the amplitude but not the shape of the response is modified by the antagonist. b) Inhibition of channel currents by α-CnTx and its fluorescent variants. The normalized channel currents I/I_o in the presence of α-CnTx (+), α-CnTx–Cy5 (▲), α-CnTx–Cy3 (▼), and α-CnTx–A647 (■). Fits of the inhibition curves yielded IC_{50} values of 20 nM for unmodified α-CnTx (——), 95 nM for Cy-5 (····), 410 nM for Cy-3 (- - - -), and 159 nM for Alexa 647 (–·–·–). The Hill coefficient was close to unity in all cases.

ReSeq-binding experiments with a single cell

We chose α-CnTx–Cy5 as fluorescent ligand, firstly because it showed the highest affinity and specificity to nAChR, and, secondly, Cy5 has an emission wavelength >600 nm, which is favorable for cellular investigations. Cells that expressed nAChRs were first perfused with α-CnTx–Cy5 for several minutes. During subsequent washing with buffer series of images were recorded (Figure 2a). After complete removal of α-CnTx–Cy5, the measurement could be repeated many times, over hours, with the same cell. In each experiment, the same initial fluorescence intensity was reached within 6% deviation (Figure 2a and b). Ligand interaction with nAChR was specific as α-CnTx–Cy5 did not bind to cells that lacked nAChR.

Binding kinetics and binding constants were evaluated from

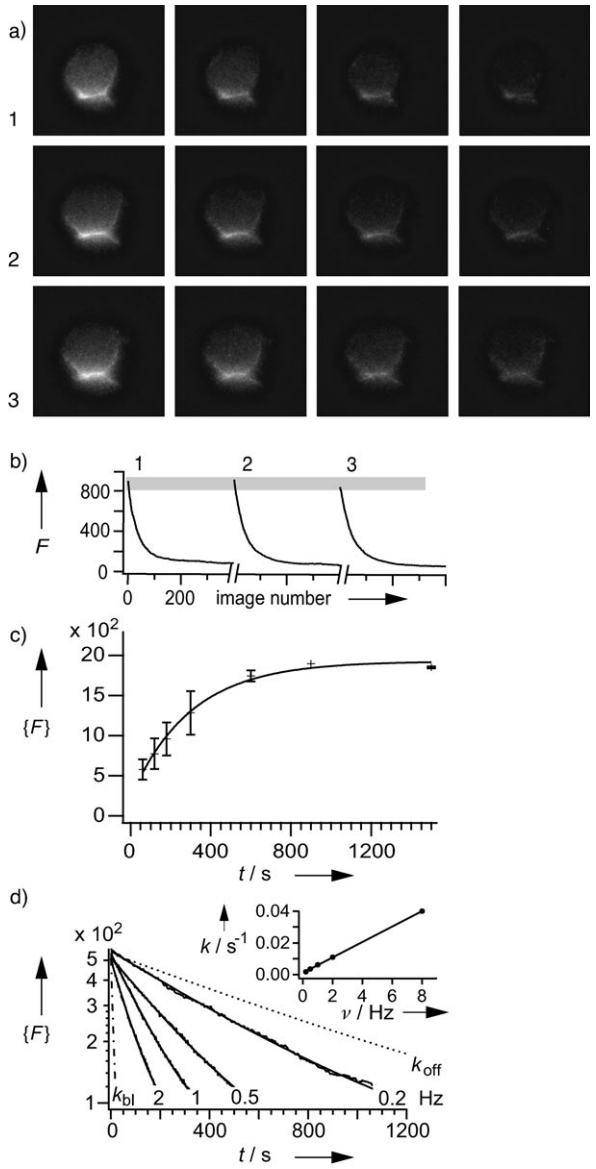

Figure 2. Reversible binding of α-CnTx–Cy5 to nAChRs in HEK293 cells. a) Fluorescence images in each row show (left to right) a single cell during continuous washing after 2 min incubation with α-CnTx–Cy5 (30 nM). After complete dissociation, α-CnTx–Cy5 is bound again to the same cell and washing cycles are repeated. The image series show fluorescence decays after first labeling (1), and repeated labeling 71 min (2) and 85 min (3) later; scale bar = 10 μm. b) Time traces of fluorescence intensity, *F*, integrated over the whole cell during washing. The same absolute fluorescence intensity is reached after each association–dissociation cycle with a deviation of only 6%, as indicated by the grey bar. c) Binding kinetic of α-CnTx–Cy5 (30 nM) to the surface of a single cell. The average fluorescence intensity per pixel, {*F*}, is plotted as a function of incubation time, *t*, and fitted with a single exponential to yield the association-rate constant that was averaged over measurements obtained with three separate cells, $k_{on} = 7 \pm 5 \times 10^4$ mol^{-1} s^{-1}. d) Fluorescence decays recorded at frequencies of 0.2, 0.5, 1, and 2 Hz with the same HEK293 cell that expressed nAChR, after incubation with α-CnTx–Cy5 (30 nM). The apparent fluorescence-decay rates, k_{app}, were evaluated from corresponding single exponential fits (solid curves). The observed fluorescence decay is faster as would be expected from dissociation (dotted line) as a result of photobleaching. Inset: the photobleaching rate, *k*, is directly proportional to the time cells were exposed to light, that is, with the number, ν, of recorded images per second. By plotting k_{app} as a function of the recording frequency, a value for the dissociation-rate constant k_{off} of $1.0 \pm 0.6 \times 10^{-3}$ s^{-1} was obtained from the intercept at zero frequency.

the fluorescence intensities of the image series with a precision that is comparable to patch-clamp experiments. The association-rate constant was determined from repetitive recording of the initial fluorescence intensity after increasing incubation times. This yielded $k_{on} = 7 \pm 5 \times 10^4$ M^{-1}s^{-1} (Figure 2c), which is in good agreement with rate constants obtained from patch-clamp experiments. Ligand dissociation was measured by monitoring fluorescence decay during washing. To distinguish the fluorescence-intensity decrease that is due to ligand dissociation from that of fluorescence bleaching, it was necessary to take repetitive measurements at various image-recording frequencies, as described in the Experimental Section. The resulting $k_{off} = 1.0 \pm 0.6 \times 10^{-3}$ s^{-1} was slightly slower (Figure 2d) than the value observed with patch-clamp experiments. Standard measurements with *only one* association and/or dissociation phase do not yield correct ligand-binding parameters. This becomes especially important when considerable photobleaching is caused by the high excitation intensity needed to detect low nAChR numbers of about 1000 receptors per cell. Such low receptor concentrations are typical for many primary cell types. The complete reversibility of α-CnTx binding allows repeated attachment of fresh α-CnTx–Cy5. Thus, many ReSeq measurements can be performed on the same cell, either under identical conditions so as to obtain better statistics or under varying conditions in order to acquire insight into, for example, the dynamic properties of the system.

Compound screening with single cells

The fast and reversible binding of α-CnTx–Cy5 can be used to rapidly and repetitively measure the binding constants of unlabeled substances in a competition assay. In these experiments we monitored the reduction of initial fluorescence intensities with a single cell that was pre-incubated for 8 min with increasing concentrations of a competing nonfluorescent ligand before the addition of α-CnTx–Cy5 (Figure 3). Longer preincubation times did not influence the results. Competition with unlabeled α-CnTx yielded an IC_{50} (α-CnTx) = 17 ± 4 nM in excellent agreement with $IC_{50} = 20 \pm 2$ nM measured with the patch-clamp technique. This shows that α-CnTx and its Cy5-labeled form compete for the same binding site. Competition experiments with the toxin d-tubocurarine (dTC) gave an IC_{50} (dTC) = 310 ± 60 nM, which corresponds to the affinity of dTC for the α/δ site of muscle-type nAChR.[18] This suggests that both α-CnTx–Cy5 and α-CnTx specifically bind to the α/δ site of nAChR and that Cy5 labeling does not alter the pharmacological properties of the antagonist.

Ligand binding with single cells in microfluidic structures

Assay miniaturization combined with highly sensitive detection techniques opens the possibility of investigating large libraries of receptors and compounds with high efficiency, at massively reduced recording time and sample consumption. We realized an important step towards this goal by using ReSeq-ligand binding with a single cell in microfluidic structures. First, individual HEK293 cells that expressed nAChR were trapped by op-

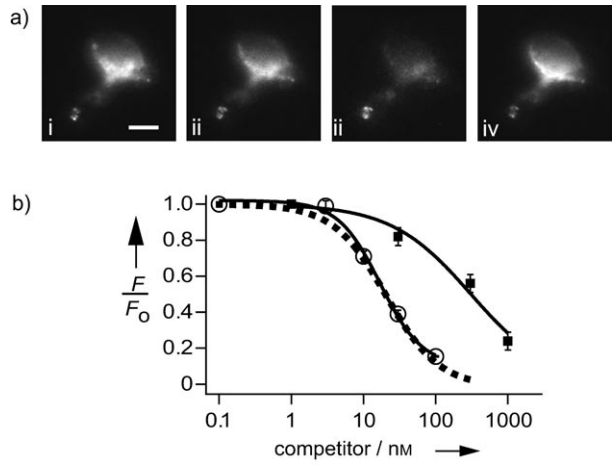

Figure 3. Specific binding of α-CnTx and dTC to the α/δ site of nAChR. Experiments were performed with single cells by fluorescence competition assays. a) Fluorescence images of a cell preincubated for 8 min with either i) no, ii) 10 nM, or iii) 100 nM α-CnTx followed by a 2 min incubation with α-CnTx–Cy5 (30 nM) added. After removal of unlabeled α-CnTx and incubation with α-CnTx–Cy5, the initial fluorescence intensity was recorded (iv); scale bar = 10 μm. b) Competition-binding curve of dTC (■) and α-CnTx (○) compared to patch-clamp data for α-CnTx (-----). Calculated IC_{50} values were 310 ± 60 and 17 ± 4 nM for dTC (——) and unmodified α-CnTx (····), respectively.

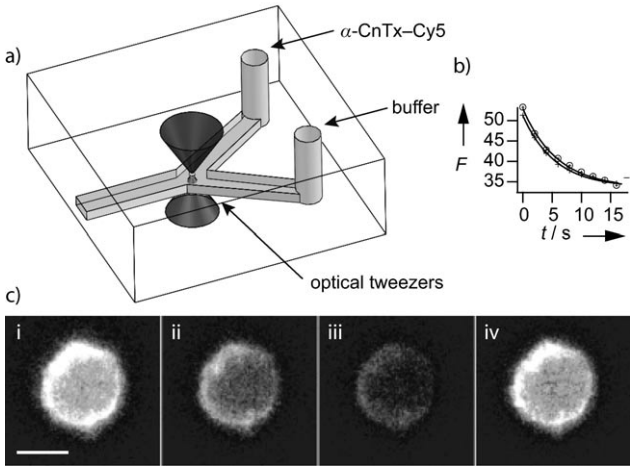

Figure 4. Repetitive and reversible binding of α-CnTx–Cy5 to nAChRs on an optically trapped cell inside a microfluidic channel. a) The microfluidic system comprises two inlet channels for α-CnTx–Cy5 and buffer (arrows), which merge into a central channel where a cell is trapped by optical tweezers. Repetitive binding and washing of α-CnTx–Cy5 was performed by varying the flow speed applied to the two inlet channels. b) Overlay of representative decay curves of fluorescence. Signals were sequentially recorded on a trapped HEK293 cell during washes with buffer. Fits are shown as solid lines and demonstrate that binding and washing are highly reproducible. c) Image series showing a trapped cell directly after incubation with α-CnTx–Cy5 (300 nM) for 1 min (i), subsequent fluorescence decay after 2 s (ii) and 5 s (iii), followed by rebinding of α-CnTx–Cy5 to the same cell (iv); scale bar = 10 μm.

tical tweezers inside a microchannel. An individual trapped cell could be released and a new one captured from a small volume of cell suspension (< 100 nL) that was temporarily pumped across the focus of the laser tweezers. Next, α-CnTx–Cy5 was repeatedly bound to and dissociated from the trapped cell in the same manner as described above (Figure 4). Only 100 nL of α-CnTx–Cy5 (300 nM) were required per cycle of ligand binding and washing. The use of a less sensitive imaging system requires illumination with higher laser intensities compared to the assays described above and results in faster fluorescence signal decay.

Imaging single receptor molecules

When cells were incubated with reduced α-CnTx–Cy5 concentrations for shorter time periods, only about 1–5% of nAChRs on the cell surface bound the toxin. In order to ensure complete removal of free α-CnTx–Cy5 during the wash step, individual α-CnTx–Cy5–receptor complexes could be observed as diffraction-limited fluorescence spots that showed characteristic single-step photobleaching[19–21] (Figure 5). Typically, tens of frames could be recorded before fluorophore photobleaching was observed. Most importantly, because α-CnTx–Cy5 could be washed off completely and added fresh once again, single-molecule experiments could be repeated more than 30 times with one cell, and data with excellent statistics could be collected.[22] Fluorescence was not observed with cells that lacked nAChRs; this showed that binding was specific. Hence, α-CnTx–Cy5 can be used as an excellent, specific ligand for probing nAChR.

nAChR subtypes. High-content assays for profiling

As α-CnTx is known to bind specifically to muscle-type but not neuronal nAChR,[17] we investigated whether this trait was conserved in α-CnTx–Cy5. Consequently, HEK293 cells that coexpressed muscle-type nAChR and cytosolic green fluorescent protein (GFP) were cocultured on glass coverslips with cells that coexpressed neuronal α7/5-HT_3 receptor chimera[23,24] and

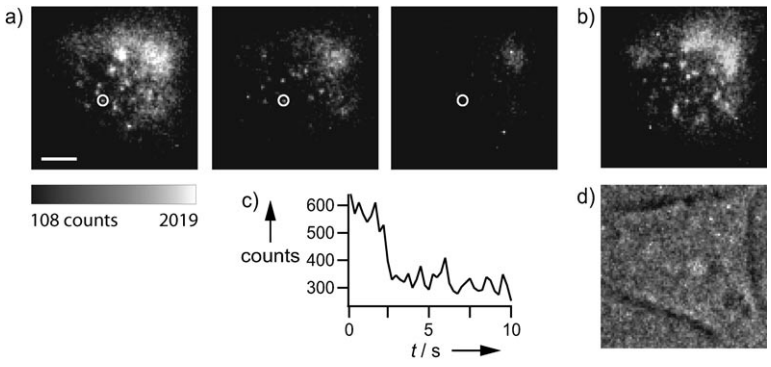

Figure 5. Single-molecule images of α-CnTx–Cy5 bound to nAChRs on a HEK293 cell. Single molecules are recognized as diffraction-limited spots. a) Shows the first, sixth, and seventeenth images (left to right) of a sequence recorded at 4 Hz; scale bar = 5 μm. Cells were incubated with α-CnTx–Cy5 (30 nM) for 10 s and monitored during continuous washing. b) After complete photobleaching, the toxin could be washed off and nAChRs could bind with fresh α-CnTx–Cy5. c) Time trace of the fluorescence from a single molecule, marked with a white circle in a), featuring characteristic single-step photobleaching. d) Transmission image of the cell.

cytosolic cyan fluorescent protein (CFP). While the nonspecific fluorescent antagonist α-BgTx–A647 bound to both receptor types (Figure 6a), α-CnTx–Cy5 bound exclusively to cells that expressed muscle-type nAChR (Figure 6b). This demonstrates that α-CnTx–Cy5 conserves the high receptor subtype specificity of unlabeled α-CnTx.

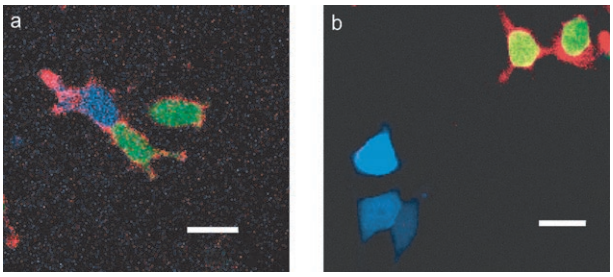

Figure 6. Specific binding of α-CnTx–Cy5 to muscle-type nAChRs. Confocal-microscope images of HEK293 cells that either co-express muscle-type nAChR and cytosolic GFP (green), or neuronal α7/5-HT_3 receptor chimera and cytosolic CFP (blue). a) The nonspecific antagonist α-BgTx–A647 (red) binds to both receptor types. b) α-CnTx–Cy5 (red) binds exclusively to cells that express muscle-type nAChRs. This demonstrates that the high subtype specificity of α-CnTx is conserved even after fluorescent labeling; scale bar = 25 μm.

Discussion

Our results describe the general application of fluorescent antagonists with fast association–dissociation kinetics in functional investigations of membrane receptors. The ReSeq concept consists of cycles of incubation/acquisition that enable numerous experiments to be carried out with the same single cell. This approach offers several advantages. First, single cells that have low-receptor concentrations comparable to cells in native tissue can be investigated. Second, the influence of different chemical stimuli (ligands) can be assessed consecutively on the same individual cell. Third, data with considerably improved statistics can be obtained from a single cell. Thus, by using the ReSeq technique an entire binding assay can be performed on a single cell, whereas with conventional fluorescence- or radioligand-binding assays, at least hundreds of cells are needed for sufficient signal-to-noise ratios. Moreover, adjustment of the amount of cell-surface receptor by up- or down-regulation of gene expression in response to a stimulus (e.g., ligand application), can also be monitored.

We have demonstrated the feasibility of the ReSeq approach by using a fluorescently labeled α-conotoxin that binds with fast kinetics to nAChR. The ligand α-CnTx–Cy5 maintained the attractive properties of conotoxins: high affinity, site specificity (in this case for the α/δ site), and strong selectivity for muscle- and not neuronal-type nAChR. We infer that these fluorescent antagonists might find a broad application in nAChR research, for instance, in investigations with cells that express multiple variants of ACh receptors. Combined with ReSeq-binding assays, fluorescent α-conotoxins can be particularly useful for medical diagnostics with cells from the tissue of patients suffering from congenital diseases, such as myasthenic syndrome, for which it has been shown that alterations in nAChR binding properties are a major source of malfunction.[25]

Our approach can easily be extended to studies with many other membrane receptors. First, conotoxins are a large family of antagonists that are specific for a broad panel of receptors and can be labeled in a similar way as α-CnTx–Cy5. Second, the requirements for ligand properties are relatively modest as dissociation lifetimes of around one minute usually correspond to a K_D value in the order of 100 nM–1 μM. Synthesizing fluorescent ligands that exhibit such affinities should prove easier than for standard fluorescence applications where a K_D in the low nanomolar range is required. In general, ReSeq can be conveniently performed with dissociation and association rate constants between 10^{-3} to 10^{-1} s^{-1} and 10^5 to 10^7 $M^{-1}s^{-1}$, respectively.

A key advantage of our approach is that pharmacological investigations can be performed with single cells that express the physiological amount of receptor, which is usually difficult to realize with other methods due to photobleaching. Conventional measurements require receptor overexpression, which risks the induction of effects that are absent at the low-expression levels found in native cells. For instance, there are indications that the function of recombinant G protein-coupled receptors are modified when expressed in non-native cells.[2] In this context, our approach to investigate receptor function in primary cell lines will be of importance.

Ultimately, single-molecule sensitivity can be achieved as illustrated with nAChR. This opens the possibility of performing functional investigations on tissue cells that express minimal amounts of membrane receptor. In general, repetitive, reversible binding is an ideal approach for monitoring single molecules where photobleaching is usually the limiting factor. By performing repetitive measurements, many image sequences can be acquired to achieve optimal statistics within a single experiment.

Repetitive competition experiments allow the rapid measurement of binding curves of ligands on single cells. This methodology might prove to be very useful for probing receptor function in rare tissue cells. In particular, the repetitive measurement procedure opens the way to automation and miniaturization, for instance by using microfluidic structures. Screening of potential drugs can be performed on a single cell with a rate that only depends on the association–dissociation cycle time. The assay consumes only minimal amounts of test components, and substantially increases the reliability and reproducibility of measurements, especially if weak signals have to be observed. For example, in the case of α-CnTx–Cy5, a measurement currently requires only 30 fmol of toxin and takes about 20 min. It is possible that the use of ligands with faster kinetics could reduce this time span down to less than 5 min. In addition, the use of more complex microstructures could enable complete binding assays with a single cell, or increase the statistical strength of the experiments with parallel approaches since the assay only uses some hundred nanoliters of cell suspension. With this method, automated screening of many ligands with a single cell extracted from animal tissue becomes a realistic goal.

Experimental Section

Fluorescent labeling of α-conotoxin GI: α-CnTx (Bachem, Bubendorf, Switzerland) was labeled with *N*-hydroxysuccinimide (NHS) esters of Alexa 647 (Molecular Probes, Eugene, OR, USA), Cy5, and Cy3 (Amersham, Buckinghamshire, UK) to yield α-CnTx–A647, α-CnTx–Cy5, or α-CnTx–Cy3, respectively. Typically, α-CnTx (1 mg) was dissolved in $NaHCO_3$ (100 mM, 100 μL) at pH 8.5, added to a solution of fluorophore NHS-ester (10 mg mL^{-1}, 70 μL) in dimethylformamide (DMF) and incubated overnight at RT in the dark. After solvent evaporation the residue was dissolved in a minimal amount of methanol, applied to a silica gel G60 preparative thin layer chromatography plate (0.25 mm; Merck, Darmstadt, Germany), dried thoroughly, and developed in MeOH/25% NH_4OH (95:5, *v*/*v*). The product was extracted with methanol and its purity demonstrated by thin-layer chromatography. The identity of the product was established by ESI or MALDI mass spectrometry. α-CnTx–Cy5: calcd $m/z = 2075.8$, measured MALDI $(m+3H^+)/z = 2078.8$, measured ESI $(m+3H^+)/2z = 1038.3$; α-CnTx–Cy3: calcd $m/z = 2049.7$, measured ESI $(m+3H^+)/2z = 1025.3$; α-CnTx–A647: calcd m/z = chemical formula unknown, measured ESI $m/z = 1051.7$. The purity of the products was estimated to be $\geq 95\%$ as no trace of α-CnTx could be detected by either thin-layer chromatography ($R_f = 0.12$ and 0.75–0.92 for unlabeled and labeled α-CnTx, respectively) or mass spectrometry (α-CnTx: calcd $m/z = 1437.8$, measured ESI $(m+3H^+)/3z = 480.3$).

Transient transfection of HEK293 cells: Human embryonic kidney (HEK293) cells were cultured in Dulbecco's modified Eagle medium supplemented with fetal calf serum (2.2%) and incubated in a humidified atmosphere with CO_2 (5%) at 37 °C. Cells were grown (60–80% confluence) either on glass slides (25 mm) in six-well plates or cell-culture dishes (35 mm). Cells were transfected with plasmids containing the coding region of human muscle-type nAChR subunits α (0.08 μg), β (0.04 μg), δ (0.04 μg), and ε (0.04 μg) by using Effectene lipofection (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The plasmids were a kind gift from P.-J. Corringer and J.-P. Changeux (Institut Pasteur, Paris). Cytosolic GFP DNA (0.2 μg; Clontech, Palo Alto, CA, USA) was cotransfected to aid the identification of transfected cells.

Experiments were performed 24–55 h after transfection. Approximately 1000 active receptors were expressed per cell as determined with whole cell patch-clamp currents. The cell-culture medium was replaced prior to fluorescence and patch-clamp experiments with buffer that contained NaCl (147 mM), glucose (12 mM), HEPES (10 mM), KCl (2 mM), $MgCl_2$ (1 mM), and was adjusted to pH 7.4 with NaOH. This buffer was used to dissolve the ligands and to continuously perfuse the cells.

Electrophysiology: Standard patch-clamp measurements were carried out in whole cell configuration by employing an EPC-9 patch-clamp amplifier (HEKA Elektronik Dr. Schulze GmbH, Lambrecht, Germany). The software PULSE 8.3 (HEKA) was used for data acquisition and storage. Borosilicate glass pipettes (resistances 2–5 MΩ) were filled with buffer that contained NaCl (140 mM), EGTA (10 mM), HEPES (10 mM), and was adjusted to pH 7.4 with NaOH. The ground electrode was connected to the bath with a KCl (1 M) agar bridge. All experiments were performed at RT and the membrane potential was kept at −60 mV. Inward currents are displayed downwards. Ligands and buffer were applied with a software controlled RSC-200 perfusion system (Bio-Logic, Claix, France). Pulses (2 or 3 s) of ACh (100 μM) were repetitively applied every 60 s while α-CnTx or its fluorescent variant were added at various concentrations.

The change in peak-current response was measured as a function of time and α-CnTx concentration so that the rate constants of the inhibition kinetics ($k_{el,on}$ and $k_{el,off}$) could be evaluated from exponential fits that yield ($k_{el,on} \times [\alpha\text{-CnTx}] + k_{el,off}$) and $k_{el,off}$, respectively. Antagonist-binding curves were computed from the equilibrium-peak responses for each concentration and fitted to Equation (1):

$$I = \frac{I_0}{1 + (IC_{50}/[\text{antagonist}])^{-n}} \quad (1)$$

by using the Levenberg–Marquardt algorithm of Igor Pro (Wavemetrics Inc., Lake Oswego, OR, USA), where I is the peak current at a particular antagonist concentration, I_0 is the peak current in the absence of antagonist, IC_{50} is the half-maximal inhibitory concentration, and n is the Hill coefficient.

Fluorescence experiments: Cells grown and transfected on glass coverslips (0.17 mm thick) were mounted on a modified epifluorescence wide-field microscope (Axiovert 200, Zeiss, Jena, Germany). To investigate Cy5 and Alexa 647 fluorescence, circularly polarized light from the 632.8 nm line of a HeNe laser (Coherent, Auburn, CA, USA) was directed by a dichroic mirror (Q645LP, Chroma Corp., Rockingham, VT, USA) into a microscope objective (C-Apochromat 63x W Korr, 1.2 NA, Zeiss) to illuminate a 22 μm diameter region of the sample illuminated. Fluorescence was collected by the same objective, passed through a filter (HQ710/100, Chroma), and imaged on an intensified CCD camera (I-Pentamax 512 EFT, Roper Scientific, USA). GFP fluorescence was achieved by excitation with the 488 nm line of an Ar^+ laser (Innova Sabre, Coherent, USA). To minimize photobleaching cells were illuminated for only 50 ms per image by using a shutter (LS3T2, Vincent Associates, Rochester, USA). Illuminated cells were continuously perfused by using a VC-77SP fast step perfusion system (Warner Instruments Corp, Hamden, CT, USA).

Binding kinetics of α-CnTx–Cy5: The association of α-CnTx–Cy5 to nAChR was measured repetitively with the same cell. The cell was incubated with α-CnTx–Cy5 (30 nM) for 1–25 min, washed rapidly (10 s) to remove free-fluorescent ligand from the solution, and fluorescence images were recorded. The time dependency of average fluorescence intensity of the whole cell was fitted by single-exponentials to yield ($k_{on} \times [\alpha\text{-CnTx–Cy5}] + k_{off}$). After complete removal of the ligand (total wash time ≥ 20 min) the measurement was repeated.

In order to determine the dissociation-rate constant, cells were first incubated for 2 min (unless stated otherwise) with α-CnTx–Cy5 (30 nM) followed by continuous washing with buffer. A series of fluorescence images was taken with a delay of 15 s after the start of the wash to ensure that no free fluorescent ligand remained in solution. After complete removal of the ligand (total wash time ≥ 20 min) the measurement was repeated. All experiments were performed with at least three different cells from independent cultures. The dissociation-rate constant of α-CnTx–Cy5 from nAChRs was evaluated from the decay of fluorescence intensity during washing. This decrease in fluorescence intensity is a superposition of ligand dissociation and photobleaching of the fluorophore and can be described by:

$$F = F_0 \exp(-(k_{off} + k_{bl} \times t_{exp} \times \nu) \times t) = F_0 \exp(-k_{app} \times t) \quad (2)$$

where F is the fluorescence intensity at time t, F_0 is the fluorescence intensity of the first image, k_{bl} is the photobleaching rate, t_{exp} is the exposure time per image, and ν is the frequency at which images were recorded. The intercept of a single linear fit of the apparent fluorescence decay rate $k_{app} = (k_{off} + k_{bl} \times t_{exp} \times \nu)$ as a

function of ν was used to obtain k_{off}. The k_{off} was evaluated from image series that were taken at frequencies ranging from 0.2 to 8 Hz with at least three different cells from independent preparations. To quantify the binding kinetics, the use of an imaging system with high quantum efficiency (>50%) and high signal-to-noise ratio is required.

Fluorescent single-cell binding assay for unlabeled ligands: The affinities of unlabeled α-CnTx and dTC were measured by fluorescence competition-binding assays. Cells were repetitively incubated with increasing concentrations of competitor for 8 min; control experiments at longer incubation times showed this was sufficient to reach equilibrium. They were then incubated with a mixture of competitor and α-CnTx–Cy5 (30 nM). The initial fluorescence intensities that were evaluated by averaging the second image over the whole cell were fitted to:

$$F = \frac{F_0}{1+(IC_{50}/[\text{competitor}])^{-n}} \quad (3)$$

Here F and F_0 are the fluorescence intensities measured in the presence and absence of competitor, IC_{50} is the half-maximal inhibitory (competitor) concentration, and n the Hill coefficient.

Single-molecule imaging: Single-molecule measurements were performed with the same epifluorescence microscope as was used for bulk fluorescence imaging. Incubation times with α-CnTx–Cy5 (3–30 nM) were reduced to some seconds so that only about 1–5% of all nAChRs on the cell surface bound a fluorescent ligand. Single-molecules images were recorded at a frequency of 4 Hz. Cells were illuminated for 50 ms with excitation intensities of about 0.5 kW cm^{-2}. Labeling was found to be specific as no single molecules were observed on nontransfected cells (i.e., cells not expressing nAChRs). Single molecules were identified by using the standard criteria of fluorescence intensity, single-step photobleaching, and spot size.[21]

Discrimination between nAChR subtypes: Separate cell cultures were cotransfected, as described above, either with DNA encoding nAChR and cytosolic GFP or α7/5-HT_3 receptor chimera[23] and cytosolic CFP (Clontech). One day after transfection, the two cell types were cocultured on glass coverslips (25 mm) for 24 h. nAChRs were probed sequentially first by incubation with α-CnTx–Cy5 (100 nM) for 20 min, and then with α-BgTx–A647 (10 nM; Molecular Probes) for 90 min. After incubation with ligand, the cells were quickly washed with buffer to remove free fluorescent ligand and then toxin binding was imaged. Fluorescence images were acquired on a laser scanning-confocal microscope (LSM 510, Zeiss) by using appropriate laser and filter settings.

Single-cell fluorescent binding assay in microfluidic structures: Binding experiments inside a glass microstructure were carried out by using a home-built optical trapping system. A 1064 nm light beam from a Nd^{3+}:YVO_4 laser (J-20; BL10-106Q, Spectra Physics, CA, USA) was expanded to slightly overfill the back aperture of a high NA (numerical aperture) objective (Plan-Apochromat, 63x Oil, 1.4 NA; Zeiss) on an inverted microscope (Axiovert 100TV; Zeiss). Thereby, a diffraction-limited, 50 mW optical trap was formed in the sample plane. The optical trap was positioned in the center of the microfluidic channel inside the microstructure by using steering mirrors (Figure 4).

For fluorescence experiments, a 30 μm region of the sample was illuminated with the 632.8 nm light of a HeNe laser (05-HLP-171, Melles Griot, Carlsbad, CA, USA) at an average power of 4 mW. The emitted fluorescence was passed through a set of filters (Z488/633/1064RPC and Z488/633M, Chroma) to reject back-scattered excitation and trapping light, and was imaged on a CCD camera (Pixelfly, PCO, Kelheim, Germany) at a frequency of 0.5 Hz, with 50 ms acquisition time.

The microfluidic circuit consisted of two syringe pumps (Versa6, Kloehn, Bonaduz, Switzerland), two multiposition valves (EMHMA-CE and E60-CE, VICI, USA) for handling liquids, and a microfabricated glass Pyrex 7740 microchip with 50 μm (high)×110 μm (wide) channels (kind gift from L. Ceriotti and E. Verpoorte, IMT, Neuchâtel, Switzerland). The microfluidic system comprised two inlet channels, one for ligand and one washing buffer, which merged into a central channel. Medium around the cells flowed from only one inlet channel at a time and was changed by switching the flow from one channel to the other. Fluorescence measurements were performed by trapping a cell and then repetitively alternating between 1 min incubations with α-CnTx–Cy5 (300 nM) and washing buffer.

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