Receptor Agonists

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# Fluorescent Epibatidine Agonists for Neuronal and Muscle-Type Nicotinic Acetylcholine Receptors**

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Nicotinic acetylcholine receptors (nAChRs) are archetypical ligand-gated ion channels mediating synaptic transmission at the neuromuscular junction, autonomic ganglia, and in the central nervous system.[1,2] Selective binding of the natural neurotransmitter acetylcholine (ACh) is the key event for receptor activation which, after transmembrane conformational changes of the protein, leads to transient opening of an ion channel, thus triggering an action potential. Structural changes in ligand-gated ion channels upon activation have been observed, but the molecular mechanism of the transmembrane signaling reactions is far from being elucidated.[3,4]

Ligand binding to nAChR is typically investigated either by incubating the sample for some time with radioactive or fluorescent ligands, thereby driving the receptor to its desensitized state that can be probed,[5,6] or indirectly by measuring channel currents upon receptor activation.[6] Important new insights into how agonist binding to nAChR induces opening of its ion channel, and thus leads to transmembrane signaling, are expected to be gained from direct observation of the formation of single ligand–receptor complexes by simultaneous optical and electrophysiological measurements.[6] Although single receptors have been imaged on the surface of living cells in a few cases by fluorescence microscopy,[7,8] it was still impossible to resolve the activation of single AChRs because of the lack of ligands that are both brightly fluorescent and receptor agonists.

Fluorescent nAChR-activating molecules are thus required with minimal changes in receptor specificity and affinities.[9,10] Former attempts to label the natural, nonfluorescent agonist ACh, or analogues thereof, with fluorophores, such as dansyl,[9,11] pyrene,[10] or *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) (NBD),[12] yielded either fluorescent antagonists or poorly fluorescent agonists of moderate affinities,[11,12] which precludes their application to single-molecule/single-channel studies in living cells. However, these ACh derivatives allowed investigation of, for example, ligand binding and allosteric effects using purified nAChR from the electric organ of *Torpedo*.[13] Recently, fluorescent anabaseine agonists on the neuronal α7 subtype have been described to display increased fluorescence upon binding to ACh-binding proteins.[14] Additional important questions on the function of AChRs might be answered by investigating single agonist–receptor complexes: 1) How are different (activated/non-activated) AChRs distributed in neuronal cells and how is this distribution influenced by cellular and synaptic development?[15,16] 2) Does partitioning in microdomains or monomer–oligomer formation play a role in AChR-mediated signaling?[16,17] 3) Do AChRs interact with other cellular proteins and thereby influence other central cellular processes besides triggering action potentials?[18]

Herein, we report the synthesis and characterization of novel fluorescent agonists for nAChRs, based on labeling of the frog alkaloid epibatidine (EPB, **1**, Scheme 1), a potent agonist of various nAChRs.[19,20] These fluorescent EPBs bind to and activate neuronal and muscle-type nAChRs, and feature subtype selectivity. We demonstrate that the exceptional optical and pharmacological properties of the fluorescent agonists offer new possibilities to study AChRs on a single-cell and single-molecule level.

The synthetic ligands contain EPB to recognize nAChR, and a fluorescent moiety linked by a spacer to the 3′-position of the pyridine ring of EPB (Scheme 1). All synthesized compounds were purified by HPLC and characterized by mass spectrometry and NMR spectroscopy (see the Supporting Information). The optical properties of the fluorescent EPBs were identical to those reported for the original fluorescent groups (see Table 1 of the Supporting Information).

The receptor affinity and efficacy of the fluorescent EPBs were evaluated from radioligand binding and electrophysiology measurements to gain insight into subtype specificity for the major neuronal and muscle-type nAChRs. Compared to EPB, the affinity of its fluorescent derivatives was reduced by

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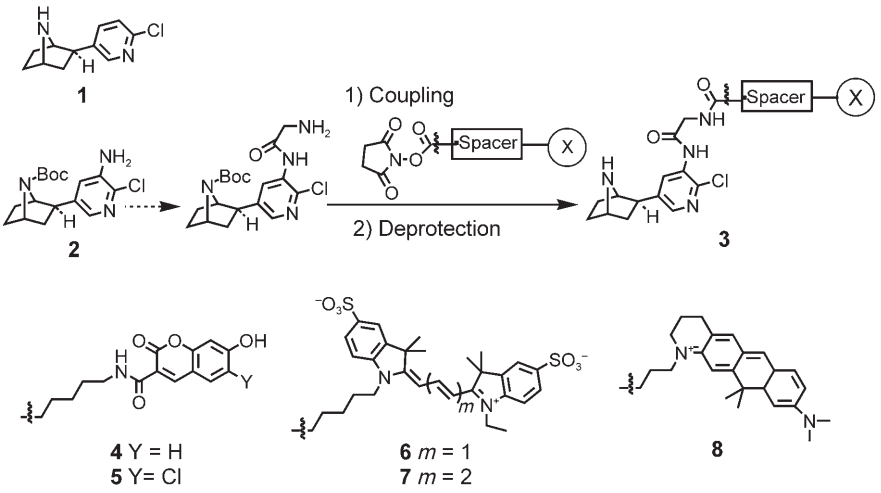


***Scheme 1.*** Fluorophore conjugation to EPB (**1**). 7-Boc-3′-aminoepibatidine (**2**; Boc: *tert*-butoxycarbonyl) was first modified with an aminoacetyl linker and then reacted with an *N*-hydroxysuccinimide-activated fluorophore X to yield after deprotection a fluorescent EPB (**3**); X: coumarin (EPB-Cou, **4**), 6-chlorocoumarin (EPB-Cl-Cou, **5**), Cy3 (EPB-Cy3, **6**), Cy5 (EPB-Cy5, **7**), Atto610 (EPB-Atto610, **8**). For details, see the Supporting Information.

one to two orders of magnitude for neuronal receptors and three orders of magnitude for muscle-type receptors (Table 1). As expected for EPB derivatives, the affinities for

***Table 1:*** Inhibition constants $K_i$ in nM of EPB ligands for nAChR subtypes evaluated from radioligand competition binding assays.

| Ligand | $\alpha4\beta2$[a] rat brain | $\alpha7$-5HT$_3$R[b] chicken/mouse | $\alpha_2\beta\gamma\delta$[b] *Torpedo* |
|---|---|---|---|
| EPB (**1**) | $0.37\pm0.19$[c] | $75\pm13$[c] | $51.6\pm9.5$[c] |
| EPB-Cou (**4**) | 8.5 | n.d. | $16\times10^3$ |
| EPB-Cl-Cou (**5**) | 4.75 | $25\times10^3$ | $(10.4\pm8.9)\times10^3$[c] |
| EPB-Cy3 (**6**) | 44 | $>1\times10^6$ | $(93\pm16)\times10^3$[c] |
| EPB-Cy5 (**7**) | $73\pm34$[c] | n.d. | n.d. |

[a] Inhibition of binding of [$^3$H]cytisine to rat-brain membranes by the indicated ligands; $\alpha4\beta2$ is the predominant nAChR in rat brain. [b] Protection by the ligands for [$^{125}$I]-α-bungarotoxin binding to $\alpha7$-5HT$_3$R in membranes of HEK293 cells or $\alpha_2\beta\gamma\delta$ nAChR in membranes of the electric organ of *Torpedo*. n.d.: not determined. [c] Data are mean ± standard deviation from two or three experiments.

$\alpha7$-5HT$_3$ chimeric receptors were very low,[19] but interestingly, the fluorescent molecules displayed an unprecedented selectivity for $\alpha4\beta2$ over $\alpha7$. EPB-Cou and EPB-Cl-Cou showed better affinities than EPB-Cy3 and EPB-Cy5, which might be a result of the larger size and/or presence of several charges on the cyanine dyes.

The ability of the fluorescent compounds to activate the nAChR was investigated by patch-clamp experiments. Compared to the currents evoked by 100 μM ACh, the neuronal-type receptors $\alpha4\beta2$ and $\alpha3\beta4$ showed 31 to 85% activation by 1 μM of the coumarin derivatives **4** and **5**, whereas 1 μM EPB-Cy5 evoked weak responses at $\alpha3\beta4$ and $\alpha4\beta2$ receptor subtypes (Table 2 and Supporting Information). Conjugation of the fluorescent groups to EPB decreased the efficacy for the neuronal nAChRs by two to three orders of magnitude, while, as expected, no activation of $\alpha7$ and $\alpha7$-5HT$_3$ receptors could be detected at concentrations up to 1–10 μM (Table 2). Interestingly, on the muscle nAChR gain of function mutant δS268K the fluorescent EPBs featured 50% effective concentration ($EC_{50}$) values comparable to that of EPB itself. Remarkably, the Cy5 and both coumarin derivatives were full agonists with a twofold lower $EC_{50}$ value compared to that of EPB, whereas EPB-Cy3 and EPB-Atto610 were partial agonists (Table 2 and Supporting Information). Although enantiomers of EPB itself are not discriminated,[21] substituted EPB derivatives might display, depending on their structure, stereoselective receptor recognition and activation that account for the partial agonist effect.

***Table 2:*** Activation of nAChR subtypes by EPB ligand conjugates evaluated from electrophysiology.[a]

| Ligand | $\alpha4\beta2$[b] rat | $\alpha3\beta4$[b] rat | $\alpha7$[c] | $\alpha_2\beta\gamma\delta$[d] human |
|---|---|---|---|---|
| EPB (**1**) | $23\pm7$ | $19\pm6$ | $(2.0\pm0.5)\times10^3$ | $53\pm4$ |
| EPB-Cou (**4**) | 59% | n.d. | 0% | $26\pm5$ |
| EPB-Cl-Cou (**5**) | 31% | 85% | 0% | $29\pm4$ |
| EPB-Atto610 (**8**) | n.d. | n.d. | 0% | $127\pm30$ (50%) |
| EPB-Cy3 (**6**) | n.d. | n.d. | 0% | $115\pm24$ (52%) |
| EPB-Cy5 (**7**) | ≤2% | 25% | 0% | $30\pm10$ |

[a] $EC_{50}$ values in nM stem from concentration–current curves. If no complete curve has been measured, the channel current evoked by 1 μM of ligand is listed as %-value of the current evoked by 100 μM ACh. Partial agonism is indicated in parentheses as percentage of maximal activation evoked by 100 μM ACh. n.d.: not determined. [b] Rat $\alpha4\beta2$ and $\alpha3\beta4$ receptors were expressed in oocytes. [c] EPB and EPB-Cou were investigated on rat $\alpha7$ receptor expressed in oocytes, the remaining ligands on $\alpha7$-5HT$_3$R chimeric receptors in HEK293 cells. [d] Human fetal muscle nAChR gain of function mutant δS268K was expressed in HEK293 cells.

Taken together, the successful design and synthesis of highly fluorescent, efficient agonists for both muscle and two major neuronal nAChR subtypes have been achieved. Fluorophore conjugation of EPB decreased similarly both the affinity and efficacy for neuronal receptors. However, for the muscle receptor efficacy was hardly affected. This might be because of structural and functional differences between subtypes of nAChR.

The results of the following two key experiments demonstrate the great potential of the fluorescent EPBs to investigate agonist binding and single-molecule imaging on individual living cells. First, HEK293 cells transiently expressing

  

the human wild-type adult muscle nAChR, imaged by fluorescence microscopy, showed an increasing fluorescence intensity upon perfusion with a solution containing 50 nM EPB-Cy5 until a steady level was reached; then, upon rinsing with buffer, the fluorescence intensity decreased gradually to the initial background level (Figure 1A; movie 1 of the

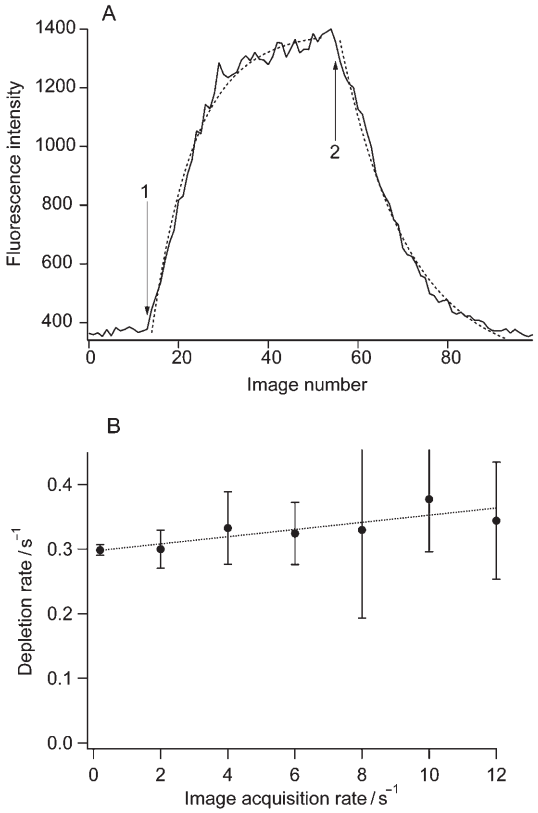


***Figure 1.*** Binding of fluorescent EPB to the surface of a living HEK293 cell expressing the adult wild-type muscle nAChR. A) Time course of the fluorescence intensity: addition of 50 nM EPB-Cy5 (arrow 1) leads to an increase of fluorescence; upon washing with buffer (arrow 2) the signal decreases to the initial value. Four images were acquired per second. The rate constants, evaluated from monoexponential fits (•••••) for three independent experiments, were for the fluorescence intensity accumulation $k_{acc} = (0.38 \pm 0.05)\ s^{-1}$ and depletion $k_{depl} = (0.256 \pm 0.032)\ s^{-1}$. B) Experiments at different image acquisition rates showed a weak linear dependence of $k_{depl}$ on the image acquisition rate. Evaluation yielded $k_{off} = (0.298 \pm 0.012)\ s^{-1}$; at 4 Hz $k_{bleach} = (0.022 \pm 0.006)\ s^{-1}$. The dissociation constant of EPB-Cy5 was $K_d = (103 \pm 53)$ nM. For details, see the Supporting Information.

Supporting Information). As fluorescence was only observed on transfected cells, identified by fluorescence of co-transfected green-fluorescent protein (GFP), it reflects the specific binding of the ligand to AChRs on the cell surface. Repetition of the experiments on the same cell with varying image acquisition rates showed only minor influence on the time course of fluorescence accumulation or depletion, which demonstrates that photobleaching did not significantly contribute to the decreasing fluorescence signal during washing (Figure 1B). The rate constants evaluated for binding of the fluorescent agonist to the receptor and its dissociation were $k_{on} = (2.48 \pm 0.06)\ \mu M^{-1} s^{-1}$ and $k_{off} = (0.26 \pm 0.03)\ s^{-1}$, respectively, which resulted in a dissociation constant $K_d = (103 \pm 53)$ nM for the complex of the ligand with the basal state of the adult wild-type muscle nAChR.

Next, we evaluated EPB-Cy5 for single-molecule fluorescence imaging of muscle nAChR in the plasma membrane of HEK293 cells (Figure 2A shows a micrograph of such a cell).

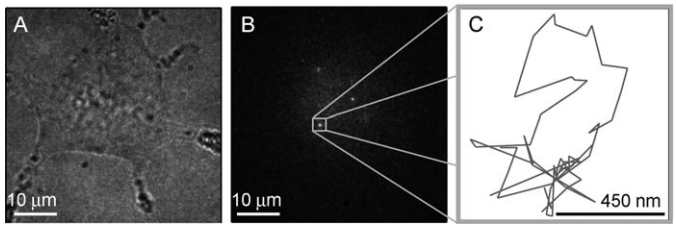


***Figure 2.*** Single-molecule microscopy. A) Transmission micrograph of an HEK293 cell expressing adult wild-type muscle nAChR. B) After adding 5 nM EPB-Cy5 to the cell shown in (A), the AChRs bind the fluorescent agonist and appear as single fluorescent spots, such as the one shown in the white box. C) Trajectory (red line) over 52 frames (≈4.3 s) of the single fluorescent receptor boxed in (B) diffusing in the cell membrane.

After perfusion with 5 nM Epi-Cy5, we observed on the upper surface of the cells bright fluorescent spots (Figure 2B) blinking and photobleaching in one step, which are typical properties of single molecules. By reason of their absence on nontransfected-cells, the spots represent nAChRs on which only one of the two binding sites is occupied by a fluorescent agonist. On average, trajectories of 28 frames could be acquired before the EPB-Cy5 molecules photobleached or dissociated from the receptor (movie 2 in the Supporting Information). Single-molecule trajectories, constructed by connecting the corresponding spots in consecutive frames using a custom-designed single-particle-tracking algorithm,[8] revealed the lateral diffusion of the nAChR within the cell membrane (Figure 2C).

In summary, we have shown the synthesis and characterization of EPBs comprising five structurally different fluorophores. These novel compounds are either partial or full agonists on muscle and on neuronal nAChR subtypes featuring (sub)micromolar receptor affinities and efficacies, and receptor-subtype specificity. The combination of exceptional optical properties—high quantum yield and photostability covering the visible spectrum—and high receptor affinities makes the fluorescent EPBs ideally suited for application to ligand-binding assays for screening purposes, and to elucidating the different facets of functioning of the nAChRs in vitro, in heterologous cells, or even native tissues, with single-molecule sensitivity.

## Experimental Section

Details of the synthesis (Scheme 1) of fluorescent EPB ligands and all other experiments are provided in the Supporting Information and also in references [22–24] for radioligand competition and protection binding experiments, in references [25–27] for expression and electro-

  

physiological characterization of the AChRs, and in reference [28] for recording fluorescence images and evaluating single-molecule traces on cell membranes.

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