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Cy3-3-acylcholine: A fluorescent analogue of acetylcholine for single molecule detection

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Abstract—We synthesized a novel fluorescent analogue of acetylcholine, Cy3-3-acylcholine. The molecular weight of the products agreed with structural predictions. Discrete intensity changes of fluorescent spots due to a single ligand binding/unbinding to nAChR were visualized by TIRF microscopy. The agonist effect of the Cy3-3-acylcholine on nicotinic acetylcholine receptor (nAChR) was confirmed electrophysiologically. This newly synthesized fluorescent analogue will enable us to conduct more elaborate studies on single channel interaction processes between nAChR and ligands.

Ion channels are important molecular machines that govern various cell functions such as membrane excitation or expression of genetic information. One example is the nicotinic acetylcholine receptor (nAChR), a well-characterized ligand-gated ion channel.[1] nAChR possesses important roles not only in neurotransmission but also in Alzheimer's disease[2] and anti-inflammatory effects.[3] The relationship between ligand binding and single channel gating events has been inferred only indirectly until now because the simultaneous measurement of single ligand binding and gating was impossible. Recently, however, we reported an experimental apparatus that has the potential to make simultaneously electrical and optical measurements of a channel within an artificial lipid bilayer[4] (see Supporting information). This is instrumental to our final aim in developing an experimental system that investigates signal transduction processes (beginning at ligand binding to gate opening and closing) of channel molecules at the single molecule level.

In this study, we report the synthesis of a fluorescently labeled analogue of acetylcholine (ACh), Cy3-3-acylcholine, to visualize interactions between the ACh receptor (AChR) and agonists at the single molecule level. Several fluorescent agonists for acetylcholine receptor channels, *N*-7(4-nitrobenzo-2-oxa-1,3-diazole) (NBD)-acylcholines for example, have been reported so far.[5–8] They are, however, not suited for our present purpose for reasons such as short fluorescent life time or inappropriate emission wavelength. Therefore, it is necessary to develop suitable, alternative fluorescent analogues for single molecule observation of ligand–channel interactions. The binding of a newly synthesized ligand to nAChR was visualized by total internal reflection fluorescence (TIRF) microscopy as spots on the channel-reconstituted vesicles. The action of this newly synthesized ligand on nAChRs was investigated electrophysiologically. In this study we utilized chicken neuronal nicotinic acetylcholine receptor α4β2 which can form heteromeric functional receptors when expressed in *Xenopus laevis* oocytes.[9] This newly synthesized fluorescent analogue of ACh is potentially a powerful tool to investigate single channel–ligand interactions. The use-

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fulness of this newly synthesized fluorescent analogue of ACh is discussed below.

The synthesis of Cy3-3-acylcholine is outlined in Scheme 1. After the Cy3 moiety was attached to 4-aminobutyric acid **1**, the acid **2** was esterified with *N*,*N*-dimethylethanolamine followed by quaternization with methyliodide.[10–13] Cy3-3-acylcholine **4** was characterized by UV measurement and MALDI–TOF–MS (calcd 801.3556 for M^+; found 801.3595). nAChR was purified from electric tissues of *Torpedo californica*[14] and reconstructed into diphytanoylphosphatidylcholine (DPhPC)/cholesterol vesicles.[15] The ratio of DPhPC/cholesterol was 4:1 and the ratio of lipid/protein was 200:1. A few μL of reconstructed vesicle solution was put onto the surface of a glass coverslip that was cleaned with KOH and ethanol. Vesicles that did not adsorb to the glass surface were rinsed with a buffer containing 0.5 M NaCl, 10 mM MOPS (pH 7.4), and 5 mM $CaCl_2$ several times and rinsed one last time with the same buffer but with added photobleaching reducing reagents.[16] A final concentration of 20 nM Cy3-3-acylcholine was added and observed under TIRF with a fluorescent microscope that was equipped with a YAG laser (λ = 532 nm) and II-CCD camera (Fig. 1a). Figure 1b shows a fluorescence image of DPhPC/cholesterol vesicle adsorbed on the surface of a glass coverslip.

Binding of Cy3-3-acylcholine to nAChR makes the vesicles fluorescent allowing for visualization under laser illumination. In the absence of Cy3-3-acylcholine (vesicles only) or in the absence of vesicles (Cy3-3-acylcholine only), no fluorescent spots were observed. Therefore, synthesized Cy3-3-acylcholine has the activity to bind to nAChR in reconstructed DPhPC/cholesterol vesicles. We also confirmed that 10 μM nonfluorescent acetylcholine competitively blocked Cy3-3-acylcholine binding to the channel.

Fluorescence intensities from spots on a glass vary over a wide range since the number of channels reconstituted into a liposome varies by liposome. We only analyzed spots from which we could see single molecule binding events. Figure 2 shows representative fluorescence intensity changes in the presence of 20 nM Cy3-3-acylcholine. The intensities fluctuated and approached zero in a single step, indicating we could directly see single agonist binding/unbinding to nAChR incorporated vesicles. The results shown here indicate three levels of fluorescence during the time course of intensity changes. We analyzed more than a hundred spots and found that there were at least three levels of intensity in each case. Because there were only two binding sites on a channel, these three levels may correspond to the number of ligands bound to a single channel protein. Assuming that intensity steps as shown in Figure 2 correspond to binding events of single ligand molecules to a single channel protein, the dissociation constant of Cy3-3-acylcholine to the receptor channel is of the order of 10 nM, showing this compound is suitable for the simultaneous electrical and optical measurements of single molecule bindings in which the fluorophore concentration in the solution should be of the order of 10 nM or less to reduce the background noise. If the dwell time for two bound ligands corresponded to a spike-like current in the ligand-stimulated nAChR that is usually observed in electrophysiological studies (~10 ms), then the temporal resolution of this experiment (video rate, 33 ms) is too short to completely detect the event. For simultaneous measurements, better temporal resolution is needed by using a high speed detector such as an Avalanche Photo Diode.

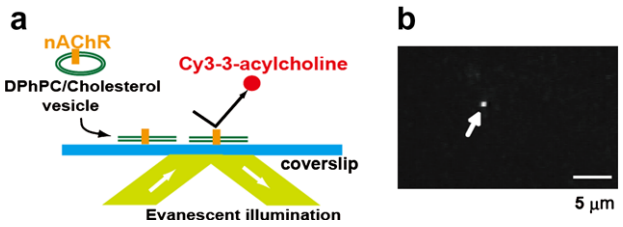
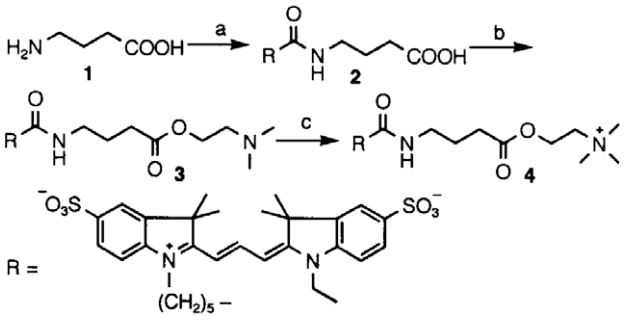


Figure 1. (a) Schematic drawing of ligand binding observation. (b) Images of a nAChR containing vesicle adsorbed on the glass surface (arrow). Binding of Cy3-3-acylcholine to nAChR makes the vesicles fluorescent allowing for visualization under a fluorescent microscope.



Scheme 1. Reagents and conditions: (a) Cy3 monofunctional dye, 0.1 M sodium borate buffer, pH 8.5, room temperature, 2 h; (b) oxalyl chloride, *N*,*N*-dimethylethanolamine, DMF, room temperature, 4 h; (c) CH_3I, DMF, room temperature, 2 days.

Xenopus oocytes were placed in a Perspex recording chamber which was perfused with standard oocyte saline (SOS) (7–10 mL/min) by a gravity-fed system.[17] Atropine (0.5 μM) was added in SOS to suppress the endogenous muscarinic acetylcholine receptor responses.[18] Membrane currents were recorded by the two-electrode voltage clamp method using a GENECLAMP 500B amplifier. Figure 3 shows the agonist actions of the fluorescent probe on α4β2 nicotinic AChRs, which were expressed in *Xenopus* oocyte. Upon application of 100 nM acetylcholine, the current rapidly activated and soon desensitized. Saline itself caused an insignificant response except for a short pulse caused by changing the medium. Cy3-3-acylcholine also opened the channel rapidly, while the desensitization was slower than that due to acetylcholine. Since the desensitization is modified by Cy3-3-acylcholine, it is conceivable that the single channel open state will last longer when activated by Cy3-3-acylcholine than by acetylcholine. The longer open dwell time is expected to overcome the challenges in making simultaneous measurements of Cy3-3-

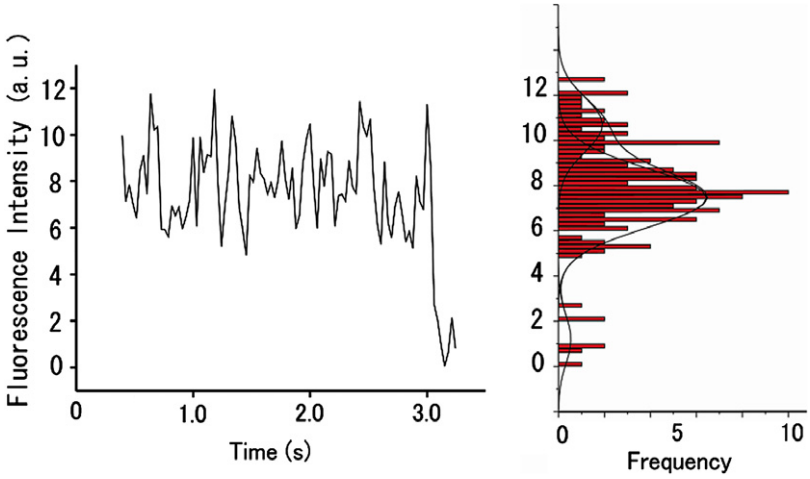


Figure 2. A representative example of discrete changes in fluorescence intensity for immobilized proteins. The intensity histogram on the right shows that there were three levels in fluorescence. The histogram was fitted with the sum of three Gaussian distributions.

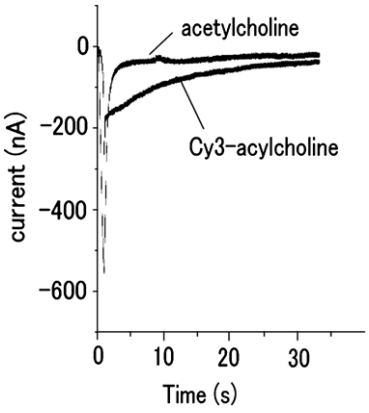


Figure 3. Acetylcholine and newly synthesized analogue activity on nAChR. Electrical response of nAChRs expressed in *Xenopus* oocyte by an agonist. *Xenopus* oocytes injected with nAChR cDNA (chicken α4 and β2) had two intracellular electrodes inserted. Current was measured under a voltage clamp at −100 mV. Fifty microliters of 100 nM acetylcholine and 100 nM Cy3-3-acylcholine was applied at $t = 0$. The time constants for desensitization (the time taken for current to decay from its peak to 50%) were 0.36 ± 0.14 s for acetylcholine and 25 ± 17 s for Cy3-3-acylcholine (mean ± SD, $n = 3$).

acylcholine binding to nAChR and opening of the channel.

In summary, we synthesized a novel fluorescent analogue of ACh, Cy3-3-acylcholine, to enable simultaneous optical and electrical measurements of single nAChRs. The molecular weight of the products agreed with structural predictions. Discrete intensity changes of fluorescent spots due to a single ligand binding/unbinding to nAChR reconstructed and bound to lipid vesicles were visualized by TIRF microscopy. The agonist effect of the Cy3-3-acylcholine on nAChR was confirmed by voltage clamp electrophysiology using a *Xenopus* oocyte expression system. This newly synthesized fluorescent analogue of ACh will enable us to conduct more elaborate studies on single channel interaction processes between nAChR and ligands.

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Supplementary data

Synthesis and characterization of Cy3-3-acylcholine, and details for imaging of single fluorescent analogues, expression of nAChR, and electrophysiology are provided in the supplementary files. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.12.002.

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