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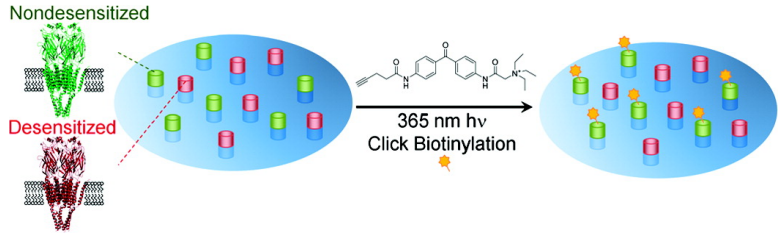

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An Activity-Based Protein Profiling Probe for the Nicotinic Acetylcholine Receptor

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Activity-based protein profiling (ABPP) is rapidly becoming one of the essential experimental approaches in understanding biological processes at the systems level.[1] ABPP reagents have been prepared for a number of important enzyme classes,[2−4] but ion channels are one important class of proteins for which ABPP probes have not been previously reported. Activation and deactivation of ion channels are central to some of the most important processes in neurobiology, such as neuronal excitability and synaptic plasticity.[5] A probe molecule that selectively labels a subset of the different activation states of a channel could be used as an activity-based probe.

The nicotinic acetylcholine receptor (nAChR) is an ion channel in the Cys-Loop superfamily that becomes cation-permeable upon binding the neurotransmitter acetylcholine. Like other neurotransmitter-gated channels, nAChRs typically undergo desensitization: a transition into a long-lived inactive state in response to prolonged exposure to acetylcholine.[6,7] In contrast to the closed states of nAChRs that predominate in the absence of a neurotransmitter, desensitized states typically have very high affinities for acetylcholine and nicotine[8,9] and predominate in the presence of a neurotransmitter. ABPP probes could be used to help characterize protein−protein interactions and posttranslational modifications associated with desensitization and reactivation of nAChRs. Such probes would thus be useful for investigating the neurobiology of desensitization in nicotine addiction[10,11] and neuromuscular disorders.[12]

We synthesized a candidate ABPP probe, named BPyneTEA (benzophenone-alkyne-triethylammonium), for state-dependent binding and photolabeling of nAChRs (Figure 1A). This candidate probe combines features of several "parent" structures that selectively bind to open or closed nAChRs.[13−15] We therefore characterized its action on nAChRs both electrophysiologically and biochemically to assess the effect of combining these features in a single structure.

To test the hypothesis that BPyneTEA can block both open and closed nAChRs, single-channel patch-clamp current recordings were obtained. To ensure that both open and closed states were observable, single-channel currents were recorded from a gain-of-function muscle-type nAChR mutant, αG153S,[16] activated using the weak agonist choline. Single-channel activity occurs as clusters of openings and closings that represent the conformational transitions of exactly one nAChR. Clusters are normally terminated by entry into long-lived desensitized states. In the presence of BPyneTEA, however, a cluster of activity may be terminated early by blockade of either closed or open states (Supporting Figure 3A).

Direct observation of individual BPyneTEA blockade events at the single-molecule level supports the hypothesis that this molecule binds both the open and closed states (Figure 1B and C). Blockade of the open state truncates open intervals within a cluster,[15,17] decreasing the mean open time with increasing BPyneTEA concentration (Figure 1D and Supporting Figure 3B).

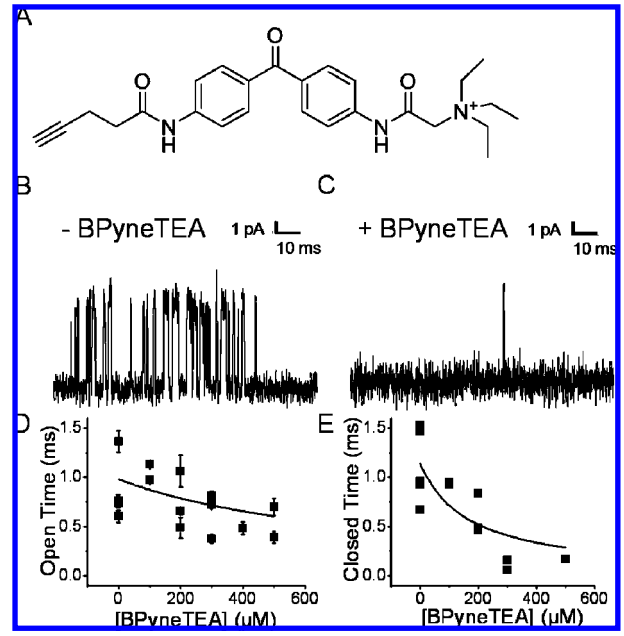

Figure 1. Single-channel electrophysiology demonstrates that BPyneTEA (A) binds and blocks the open and closed states of the nAChR. Single-channel activity occurs in clusters in the absence of BPyneTEA (B), but clusters are terminated early in the presence of 500 μM BPyneTEA (C). The decrease in the mean open time (D) and the lifetime of the fastest closed time component (E) with increasing BPyneTEA concentration indicate that BPyneTEA binding to the open and closed nAChR has an association rate constant of $\sim 10^6$ $M^{-1}\cdot s^{-1}$.

From the concentration dependence, the association rate constant for open-state blockade is $(1.3 \pm 0.7) \times 10^6$ $M^{-1}\cdot s^{-1}$ (best fit value ± standard error). This association rate constant depends on transmembrane voltage; an analysis of the voltage dependence suggests that BPyneTEA binds the nAChR 20 ± 10% into the transmembrane electrical field relative to the cell surface (Supporting Figure 4).

Blockade of the closed state truncates closed intervals within a cluster, decreasing the observed mean closed times with increasing BPyneTEA concentration (Figure 1E and Supporting Figure 3B). In this case, there are multiple kinetic components in the closed time distribution, but there is only one that decreases in a BPyneTEA-dependent fashion; this component represents the lifetime of the closed (and blocker-free) state. The association rate constant for binding to the closed state was determined from the BPyneTEA concentration-dependent decrease of the fastest closed time component and is $(5 \pm 2) \times 10^6$ $M^{-1}\cdot s^{-1}$ (best fit value ± standard error). Both open and closed states thus bind BPyneTEA with an association rate constant of $\sim 1 \times 10^6$ $M^{-1}\cdot s^{-1}$. The difference in blockade rate constants between the open and closed

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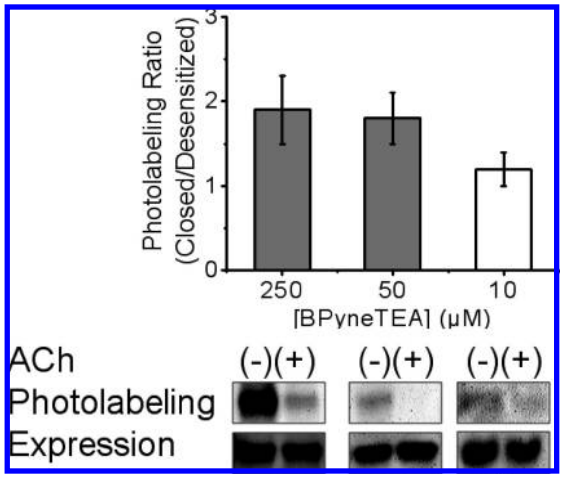

Figure 2. Photolabeling nAChRs expressed in live cells in the absence (closed) or presence (desensitized) of acetylcholine (ACh) demonstrates that BPyneTEA preferentially binds closed receptors. Closed receptors are labeled ~2-fold more efficiently than desensitized receptors at concentrations of 250 μM and 50 μM. At 10 μM BPyneTEA, differential labeling was not statistically significant.

states is not statistically significant (unpaired, two-sided t test, $p = 0.068$). The kinetic studies also allow estimation of upper limits for dissociation constants of BPyneTEA binding to closed and open states: $<\sim 20$ μM for the closed state and $<\sim 80$ μM for the open state (Supporting Figure 3B).

To test whether BPyneTEA selectively labels the closed (but activatable) state of the nAChR compared to the inactive desensitized state, we carried out photolabeling of nAChRs expressed in live HEK293 cells in the presence or absence of the desensitizing agonist acetylcholine. Closed or desensitized nAChRs were photolabeled with BPyneTEA, and copper(I)-catalyzed [3 + 2] cycloaddition (i.e., "click" chemistry, as adapted for bioconjugation[18]) of an azide-functionalized biotin was carried out to biotinylate the photolabeled receptors.[19] Biotinylated receptors were captured on streptavidin-coated beads, and nAChRs were visualized by Western blotting with an antibody against the nAChR α subunit. Quantification of the captured nAChRs (normalized for expression levels) shows that, at BPyneTEA concentrations ≥ 50 μM, the closed state is labeled more efficiently than the desensitized state by a factor of ~2 (Figure 2 and Supporting Table 1). At 10 μM BPyneTEA, weak labeling is observed, but its state selectivity is not statistically significant. Labeling was not observed in the absence of BPyneTEA or UV irradiation (Supporting Figure 1).

Selectivity for closed states compared to desensitized states is likely to be a crucial parameter in determining the utility of probes for investigation of nAChR desensitization *in vivo*. Because desensitization occurs primarily from the open state and is the thermodynamic minimum for the agonist-bound channel,[20] only channel populations that spend most of their time in the closed state will remain activatable. The selectivity of BPyneTEA for closed over desensitized conformations is modest (~2-fold) but high enough that comparison of subproteomes using mass spectrometry is expected to be feasible. The use of trypsin-catalyzed ^{18}O labeling of peptides for relative quantification of subproteomes by mass spectrometry[21] has allowed enrichments/depletions of <2-fold to be detected.[22] Optimization of the blocker and benzophenone moieties may allow improved selectivity for the closed state; the modular design of the molecule is expected to enable facile synthesis of second-generation probes.

The potential utility of a channel-targeted ABPP strategy depends on whether it will be generalizable to a large number of structurally distinct channels. Large changes in pore structure (as judged by accessibility to reactive probes in solution) have been observed for other Cys-Loop receptors such as the serotonin receptor,[23] as well as glutamate receptors[24] and potassium channels.[25] In addition, the many characterized state-selective channel blockers and inhibitors offer a rich set of potential pore-binding groups for ion channel targeted ABPP probes. Ion channels as a class thus share many of the advantages of enzyme active sites as ABPP targets and appear likely to be a generally useful target for ABPP techniques.

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Supporting Information Available: Synthetic, electrophysiology, and biochemical methods; discussion of blockade models and voltage-dependence of blockade; statistical analysis of live-cell labeling; labeling results in the absence of light or BPyneTEA. This material is available free of charge via the Internet at https://pubs.acs.org.

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