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Agonist binding and affinity state transitions in reconstituted nicotinic acetylcholine receptors revealed by single and sequential mixing stopped-flow fluorescence spectroscopies

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Abstract

The affinity state of nicotinic acetylcholine receptors (nAcChoRs) reconstituted into either dioleoylphosphatidylcholine (DOPC) or a mixture of dioleoylphosphatidylcholine, dioleoylphosphatidic acid, and cholesterol (DOPC/DOPA/cholesterol) has been determined using single and sequential mixing stopped-flow fluorescence spectroscopies. These techniques have millisecond temporal resolution, permitting low- and high-affinity conformational states of the nAcChoR to be resolved following mixing with the fluorescent partial agonist Dns-C_6-Cho from their characteristic Dns-C_6-Cho dissociation rates. Our studies reveal that prior to agonist-induced affinity state conversion, nAcChoRs reconstituted into either DOPC or DOPC/DOPA/cholesterol are predominantly in a conformational state that has a low affinity for agonist. Prolonged exposure to Dns-C_6-Cho converts nearly all DOPC/DOPA/cholesterol-reconstituted nAcChoRs to the high-affinity state. In contrast, Dns-C_6-Cho converts only half of all DOPC-reconstituted nAcChoRs to the high-affinity state. The other half persists in a low-affinity state characterized by a K_d for Dns-C_6-Cho of 0.61 ± 0.07 μM. This K_d is similar to that previously reported for Dns-C_6-Cho binding to low-affinity, resting-state nAcChoRs in native membranes. However, affinity state conversion of DOPC-reconstituted nAcChoRs may be facilitated by re-reconstituting them into bilayers composed of DOPC/DOPA/cholesterol. These results indicate that the lipid bilayer composition modulates nAcChoR agonist-induced affinity state transitions. 0005-2736/98/$ – see front matter © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Acetylcholine receptor; Cholesterol; Lipid–protein interaction; Reconstitution

1. Introduction

The nicotinic acetylcholine receptor (nAcChoR) is the prototypical member of a superfamily of structurally and functionally similar intrinsic membrane proteins that also includes the $GABA_A$, glycine, and 5-HT_3 receptors [1–3]. Detailed biophysical and biochemical studies have shown that in the absence of agonist, native nAcChoRs exist in an equilibrium between two conformational states: a resting state that is characterized by a low affinity for agonist and a desensitized one that has a high affinity for agonist [4,5]. In the absence of agonist, most nAcChoRs in native membranes are in the low-affinity

Abbreviations: nAcChoR, nicotinic acetylcholine receptor; DOPA, dioleoylphosphatidic acid; DOPC, dioleoylphosphatidylcholine; Dns-C_6-Cho, [1-[5-(dimethylamino) naphthalene]sulfonamido]-*n*-hexanoic acid β-(*N*-trimethylammonium bromide) ethyl ester; TPS, *Torpedo* physiological saline

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resting state. The binding of two agonist molecules to receptors in the resting state leads to a conformational isomerization first to an open channel state and ultimately to a set of closed desensitized states [6–8]. Agonists may also bind to receptors in the desensitized state, but this does not lead to channel opening.

Previous studies using reconstituted nAcChoRs have demonstrated that specific lipids are needed to maintain normal receptor function [9–12]. In particular, cholesterol or cholesterol analogs and negatively charged lipids appear to be necessary to preserve agonist-induced channel opening in reconstituted nAcChoRs [13–15]. However, the role that lipids play in preserving the ability of reconstituted nAcChoRs to bind agonist and undergo agonist-induced transitions to higher affinity states remains unresolved. Toxin binding assays suggest that even in the absence of agonist, receptors reconstituted into bilayers devoid of cholesterol (or cholesterol analogs) and negatively charged phospholipids are predominantly in the desensitized state and, therefore, fail to undergo an affinity state transition upon agonist exposure [14,16]. This conclusion was supported by the results of photolabeling experiments showing that even in the absence of agonist, nAcChoRs reconstituted into phosphatidylcholine are labeled by the photoactivatable probe [^{125}I]TID in a pattern that is indicative of receptors in the desensitized state [16]. However, Criado et al. concluded from their own studies of toxin binding kinetics that nAcChoRs reconstituted into phosphatidylcholine are stabilized in a receptor state that has a low affinity for agonist [17]. Finally, in contrast to all of the above mentioned studies, Dalziel et al. [18] and Ochoa et al. [13] have reported that nAcChoRs reconstituted into DOPC retain their ligand binding properties and their ability to undergo agonist-induced affinity state transitions.

We have recently developed a new approach for detecting and characterizing agonist binding and affinity state transitions in nAcChoR-rich membranes using stopped-flow fluorescence spectroscopy [19]. This approach resolves agonist binding to high- and low-affinity receptor states from the dissociation rate of the fluorescent partial agonist, Dns-C_6-Cho, following chemical dilution into acetylcholine. Because the binding of Dns-C_6-Cho to nAcChoRs can be resolved within milliseconds of mixing, Dns-C_6-Cho-induced desensitization may be detected as a time-dependent increase in the number of high-affinity binding sites upon exposure to the agonist. The present work uses this approach to define the conformational state of nAcChoRs reconstituted into DOPC and to determine whether such receptors undergo a change in affinity state upon equilibration with agonist. We have focused on nAcChoRs that have been reconstituted into pure phosphatidylcholine (DOPC) because previous studies have failed to arrive at a consensus regarding the affinity state of such receptors or their ability to undergo agonist-induced affinity state transitions. For comparison, we also assessed the affinity state of nAcChoRs reconstituted into DOPC/DOPA/cholesterol (molar ratio 63:12:25), a lipid mixture that simulates the steroid and charge density of native membranes.

2. Materials and methods

2.1. Membrane preparation, reconstitution, and characterization

Electric organs were obtained from *Torpedo nobiliana* (Biofish Associates Georgetown, MA) as previously described and approved by the Massachusetts General Hospital Animal Care and Use Committee [20]. The nAcChoR was reconstituted using a modification of the procedure of Ellena et al. in which Affi-Gel 201 was derivatized with DL-*N*-acetylhomocysteine thiolactone and used in place of Affi-Gel 401 [21]. The affinity column was washed with either DOPC alone or a mixture of DOPC, DOPA, and cholesterol to obtain the desired membrane lipid composition. The protein content of reconstituted nAcChoR membranes was determined as described by Markwell et al. using bovine serum albumin as the standard [22], and the number of agonist binding sites was estimated by assuming a molecular mass of 290 000 and two binding sites/receptor. The cholesterol and phospholipid content of reconstituted membranes were determined by previously reported methods [23,24]. Lipid/protein ratios typically ranged from 200 to 400. In some cases, receptors reconstituted into DOPC were re-reconstituted into DOPC/DOPA/cholesterol using the method of Ochoa et al.

[13]. For experiments using native membranes, acetylcholinesterase activity was inhibited with diisopropylfluorophosphate. The number of agonist binding sites in native membrane preparations was determined as previously described [19]. Dns-C_6-Cho was synthesized according to the procedure of Waksman et al. [25].

2.2. Stopped-flow fluorescence spectroscopy

Single and sequential mixing stopped-flow fluorescence spectroscopies were performed with an Applied Photophysics SX.17MV stopped-slow spectrofluorimeter (Leatherhead, UK). An excitation wavelength of 290 nm was provided by a 150-W xenon arc lamp. The bandpass on the monochrometer was 2.5 nm. Fluorescence was detected through a 530-nm high-pass filter on a logarithmic timebase typically for either 10 or 100 s. The temperature was kept at 10.0 ± 0.2°C with a circulator bath. In a typical experiment, 5 to 10 individual shots were signal averaged to reduce noise. Signal averaged fluorescence traces were transferred to a Macintosh computer and analyzed using the commercially available analysis program Igor 3.0 (Wavemetrics, Lake Oswego, OR).

2.3. Single mixing stopped-flow fluorescence spectroscopy

Reconstituted nAcChoRs were first preequilibrated with the fluorescent partial agonist Dns-C_6-Cho for 1–2 h and then loaded into one of the spectrofluorimeter's premix syringes. Acetylcholine (2 mM in TPS) and Dns-C_6-Cho were loaded into the other to maintain a nearly constant aqueous concentration of the fluorescent partial agonist upon mixing. The two solutions were rapidly mixed (1:1, v/v) into an optical cell where the fluorescence intensity was recorded.

2.4. Sequential mixing sopped-flow fluorescence spectroscopy

Sequential mixing stopped-flow fluorescence spectroscopy was performed as previously described [19]. In brief, reconstituted receptor membranes were loaded into one of the spectrofluorimeter's premix syringes and a solution of Dns-C_6-Cho was loaded into the other. Receptor membranes and Dns-C_6-Cho were then mixed (1:1 v/v). After the desired preincubation period, the receptor/Dns-C_6-Cho solution was mixed (1:1 v/v) with a solution containing 2 mM acetylcholine plus Dns-C_6-Cho at half the concentration present in the premix syringe to maintain a nearly constant free Dns-C_6-Cho concentration with the second mixing step. This second mixing step occurred within the optical cell where changes in fluorescence emission were recorded. The time axis on all stopped-flow fluorescence traces refers to the time after the second mixing step. The reported nAcChoR and Dns-C_6-Cho concentrations are those during the preincubation period.

2.5. Interpretation of stopped-flow fluorescence traces [26]

Under conditions of energy transfer from receptor tryptophan to the dansyl moiety of Dns-C_6-Cho, the binding of Dns-C_6-Cho to the nAcChoR produces an increase in fluorescence emission above 530 nm [4,26,27]. Subsequent chemical dilution into the nonfluorescent agonist acetylcholine reduces this fluorescence as acetylcholine exchanges with Dns-C_6-Cho for agonist binding sites on the receptor [4]. Because the different agonist binding affinities exhibited by nAcChoR conformational states largely reflect their different agonist dissociation rates [28,29], Dns-C_6-Cho binding to high- and low-affinity receptors may be distinguished from the time scale of the fluorescence decay upon dilution into a nonfluorescent agonist; Dns-C_6-Cho dissociates slowly from high-affinity receptors but rapidly from low-affinity ones. Sequential mixing stopped-flow fluorescence spectroscopy allows membranes to be preequilibrated with Dns-C_6-Cho for periods of time ranging from milliseconds to minutes before dilution into acetylcholine. With very short preincubation times, two fluorescent decays are observed in native nAcChoR membranes. There is a fast decay on the time scale of milliseconds, reflecting Dns-C_6-Cho dissociation from one of the two agonist binding sites on resting-state nAcChoRs. This site has a K_d of 1.1 μM. Although Dns-C_6-Cho also binds to the other resting-state site, it does so with a much lower affinity and dissociates from this site within the 1 ms mixing time of the

spectrofluorimeter. Therefore, this site is not detected. There is also a slow decay on the time scale of seconds, reflecting Dns-C_6-Cho dissociation from the two high-affinity sites on desensitized nAcChoRs ($K_d \sim 3$ nM). With longer preincubation times that permit low-affinity nAcChoRs to undergo significant Dns-C_6-Cho-induced affinity state conversion to the high-affinity state prior to mixing with acetylcholine, the amplitude of the slow decay increases while that of the fast decay decreases. With very long preincubation times, no fast decay is detectable in native membranes because essentially all receptors have been converted to the high-affinity state prior to mixing with acetylcholine.

3. Results

3.1. Single mixing stopped-flow fluorescence spectroscopic studies of nAcChoRs reconstituted into DOPC

Fig. 1A shows typical fluorescence decays obtained when nAcChoR-rich native membranes or nAcChoRs reconstituted into DOPC (0.7 μM agonist binding sites) were equilibrated for 1–2 h with 8 μM Dns-C_6-Cho and then rapidly mixed with 1 mM acetylcholine. To facilitate comparison between these two preparations, the initial fluorescence intensity of each trace has been normalized to 1.0. Native and DOPC-reconstituted membranes each exhibited a slow fluorescence decay on the slow time scale previously reported for Dns-C_6-Cho dissociation from high-affinity, desensitized nAcChoRs [4,19]. We noted that these slow decays deviated from a simple first-order process, but could be well fit to a double exponential equation. Fig. 1B shows the same traces as in Fig. 1A except that the first second has been expanded to reveal an additional (intermediate) fluorescence decay in the trace obtained with nAcChoRs reconstituted into DOPC. This intermediate component occurred on the time scale of tens of milliseconds and was not observed in traces obtained using native membranes. The amplitude and rate of this additional decay, determined by fitting the fluorescence trace to a triple exponential equation, were 0.379 ± 0.003 and 48 ± 1 s^{-1}. The rapid rate of the intermediate decay is indicative of Dns-C_6-Cho dissociation from a class of sites that has a low affinity for agonist. Preequilibrating membranes with the competitive antagonist α-bungarotoxin prior to performing these stopped-flow studies eliminated the intermediate and slow decays (data not shown).

Fig. 2A shows the slow fluorescent decays ob-

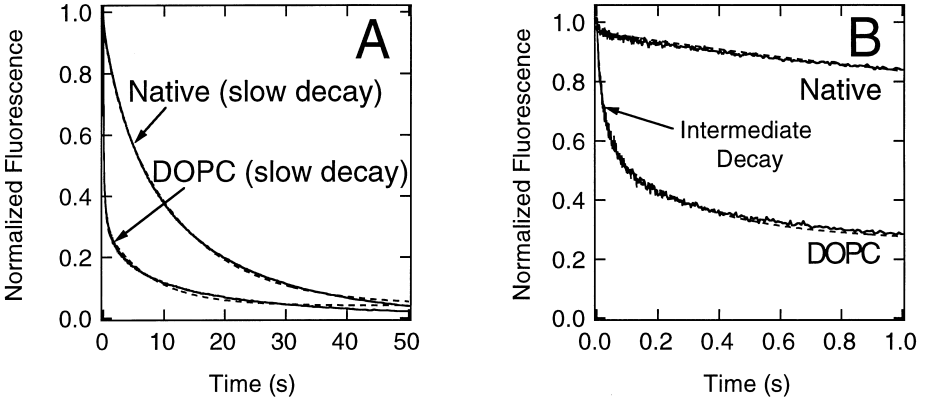

Fig. 1. Single mixing stopped-flow fluorescence traces of native and DOPC-reconstituted nAcChoRs. Panel A shows the change in fluorescence intensity recorded when nAcChoRs reconstituted into DOPC or nAcChoR-rich native membranes (0.7 μM agonist binding sites) are first preequilibrated with Dns-C_6-Cho for 1–2 h and then rapidly mixed with 1 mM acetylcholine. On this time scale a slow fluorescent decay is observed reflecting competitive displacement of the fluorescent agonist Dns-C_6-Cho by acetylcholine from high-affinity agonist binding sites on nAcChoRs. Panel B shows the same traces as panel A with the first second expanded to reveal an additional (intermediate) decay in the trace recorded using nAcChoRs reconstituted into DOPC. No significant intermediate decay is observed in nAcChoR-rich native membranes. The dotted lines are derived by fitting the fluorescence traces to either a double exponential (native) or triple exponential (DOPC-reconstituted) equation.

served when receptors reconstituted into DOPC (final concentration 0.13 μM agonist sites) were preequilibrated with either 0.25 μM or 8 μM Dns-C_6-Cho and then rapidly mixed with 1 mM acetylcholine. This low concentration of receptor was used to minimize depletion of Dns-C_6-Cho from the aqueous phase. On the slow time scale shown in this figure, the two traces nearly overlap. Fig. 2A is expanded in Fig. 2B to show that the amplitude of the intermediate decay is significantly larger in the trace obtained using the higher concentration of Dns-C_6-Cho. Because the amplitude of the intermediate and slow decay reflect the number of low- and high-affinity sites, respectively, that had bound Dns-C_6-Cho during equilibration, we evaluated the affinity of Dns-

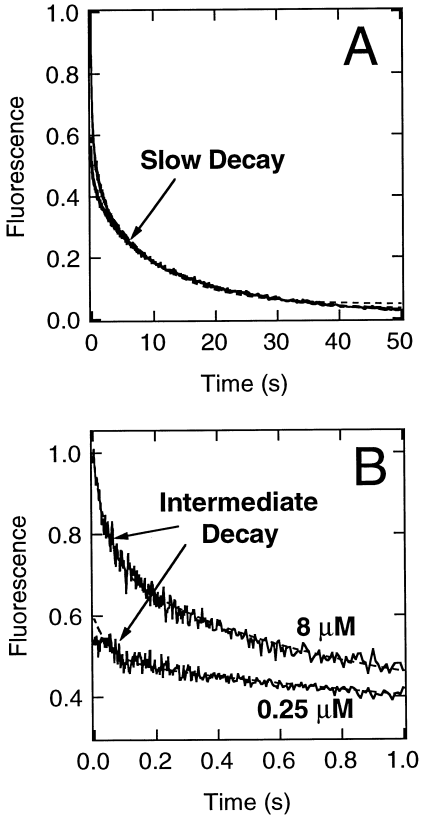

Fig. 2. Single mixing stopped-flow fluorescence traces of DOPC-reconstituted nAcChoRs. Panel A shows the change in fluorescence intensity recorded when nAcChoRs reconstituted into DOPC (0.13 μM agonist binding sites) that have been preequilibrated with either 0.25 or 8 μM Dns-C_6-Cho for 1–2 h are rapidly mixed with 1 mM acetylcholine. Panel B shows the first second of panel A. The dotted lines are fits of fluorescence traces to a triple exponential equation.

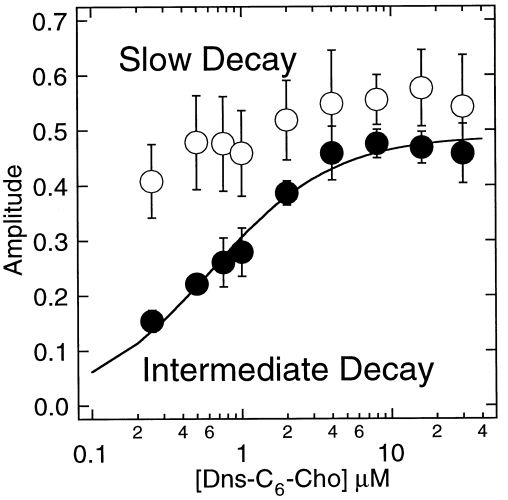

Fig. 3. Plot of the amplitudes of the intermediate (●) and slow (○) decays following preequilibration with Dns-C_6-Cho at the indicated concentrations. In each Dns-C_6-Cho titration, the component amplitudes were normalized to the total fluorescence change observed using 30 μM Dns-C_6-Cho, the highest concentration studied. The line is a fit of the data to Eq. 1 yielding a K_d of 0.61 ± 0.07 μM, a Hill coefficient was 1.1 ± 0.2, and an Amp_{max} of 0.49 ± 0.02.

C_6-Cho for these sites by measuring the amplitudes these decays as a function of Dns-C_6-Cho concentration (Fig. 3). There was little increase in the amplitude of the slow decay with increasing Dns-C_6-Cho concentration, indicating that these sites were nearly saturated even at concentrations of Dns-C_6-Cho as low as 0.25 μM. In contrast, the amplitude of the intermediate decay increased significantly with Dns-C_6-Cho before reaching a plateau by approximately 4 μM, consistent with the titration of low-affinity Dns-C_6-Cho binding sites. The concentration of Dns-C_6-Cho producing a half-maximal intermediate decay amplitude was determined by fitting the amplitude data to the logistic equation:

$$\mathrm{Amp} = \mathrm{Amp}_{max} \times \left(\frac{[\mathrm{Dns{-}C_6{-}Cho}]^n}{[\mathrm{Dns{-}C_6{-}Cho}]^n + K_d^n} \right) \qquad (1)$$

where Amp is the amplitude of the intermediate decay, Amp_{max} is the maximal amplitude reached at high Dns-C_6-Cho concentrations, n is the Hill coefficient, and K_d is the Dns-C_6-Cho concentration producing a half-maximal amplitude. The value of K_d was determined to be 0.61 ± 0.07 μM and the Hill coefficient was 1.1 ± 0.2. In three separate preparations, the amplitudes of the intermediate and slow

decays represented 46 ± 9% and 54 ± 9%, respectively, of the total reduction in fluorescence observed when receptors that have been preincubated with saturating concentrations of Dns-C_6-Cho (> 8 μM) are diluted into 1 mM acetylcholine.

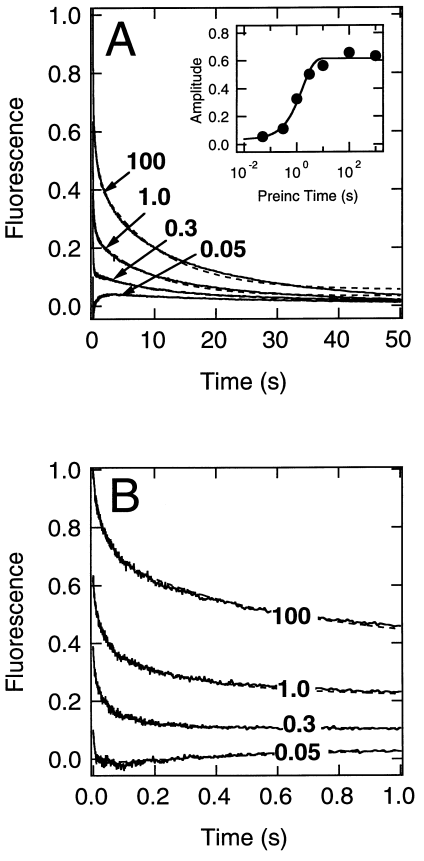

Fig. 4. Sequential mixing stopped-flow fluorescence traces of DOPC-reconstituted nAcChoRs. Panel A shows the change in fluorescence intensity recorded when nAcChoRs reconstituted into DOPC are preincubated with 10 μM Dns-C_6-Cho for the indicated times (in seconds) and then chemically diluted into 1 mM acetylcholine. The dotted lines are fits of fluorescence traces to a triple exponential equation. The inset is a plot of the amplitude of the slow decay as a function of preincubation time. The slow decay amplitude was taken as the sum of the amplitudes of the two slower components in a triple exponential fit of the trace. The line is a fit of the amplitude data to an exponential equation yielding an apparent rate of 0.6 ± 0.1 s^{-1}. Panel B shows the first second of panel A to reveal, depending on the preincubation time, the fast or intermediate decays. A small fluorescence enhancement on the time scale of several hundred ms is also noted in the 0.05-s preincubation trace which reflects Dns-C_6-Cho interactions with the agonist-inhibitory site [19].

3.2. *Sequential mixing stopped-flow fluorescence spectroscopic studies of nAcChoRs reconstituted into DOPC*

Fig. 4A shows typical fluorescence traces obtained when nAcChoRs reconstituted into DOPC (0.4 μM) were preincubated with 10 μM Dns-C_6-Cho for times ranging from 50 ms to 100 s and then rapidly mixed with acetylcholine. With Dns-C_6-Cho–nAcChoR preincubation times sufficiently short to minimize Dns-C_6-Cho-induced affinity state conversion of nAcChoRs (i.e. 50 ms), the amplitude of the slow decay was 0.06. This indicated that few of the nAcChoRs were in the high-affinity state at the time of dilution into acetylcholine. However, with longer preincubation times, the amplitude of the slow decay increased reflecting an increase in the number of the high-affinity nAcChoRs. The apparent rate with which Dns-C_6-Cho induced this affinity state conversion, determined by fitting the slow decay amplitude data in Fig. 4A to an exponential equation, yielded a value of 0.6 ± 0.1 s^{-1} (inset). Fig. 4B reveals the first second of the traces in Fig. 4A. With long preincubation times (i.e. 100 s), the intermediate decay was similar to that observed in single mixing studies using receptors that had been preequilibrated with Dns-C_6-Cho prior to diluting into acetylcholine (compare with Fig. 1B). However, with short preincubation times (i.e. 50 ms), an exponential decay was observed that was significantly faster than the intermediate decay observed in the single mixing studies (Fig. 4B). This suggested that when nAcChoRs were exposed only briefly to Dns-C_6-Cho, they bound the fluorescent agonist with an affinity that was even lower than that observed at equilibrium. In 24 experiments using Dns-C_6-Cho concentrations ranging from 0.5 to 20 μM, the fast decay rate averaged 160 ± 60 s^{-1}. This in the range previously reported for Dns-C_6-Cho dissociation from one of the agonist binding sites on the nAcChoR resting state. The affinity of this site for Dns-C_6-Cho was determined from a plot of the amplitude of the fast decay as a function of Dns-C_6-Cho (Fig. 5). This amplitude increased with Dns-C_6-Cho concentration and then plateaued. The concentration of Dns-C_6-Cho producing a half-maximal increase in the amplitude of the fast decay was 1.4 ± 0.2 μM and the Hill coefficient was 1.1 ± 0.2.

3.3. *Stopped-flow fluorescence spectroscopic studies of nAcChoRs reconstituted into DOPC/DOPA/cholesterol*

To determine whether reconstituting nAcChoRs into a more native-like lipid membrane enhanced the ability of receptors to undergo an affinity state transition upon agonist exposure, we examined the kinetics of Dns-C_6-Cho dissociation from nAcChoRs reconstituted into a mixture of DOPC, DOPA, and cholesterol (molar ratio 63:12:25). In previous studies, this lipid mixture has been shown to support nAcChoR channel activation. Fig. 6A shows typical fluorescence traces obtained when nAcChoRs reconstituted into DOPC/DOPA/cholesterol are preincubated with 10 μM Dns-C_6-Cho for various times and then chemically diluted into 1 mM acetylcholine. As observed in fluorescence traces obtained using nAcChoRs reconstituted into DOPC, the slow decay is relatively small with short preincubation times. However, with longer preincubation times, the amplitude of the slow decay increased to 0.87 with an apparent rate of 0.7 ± 0.2 s^{-1} (see inset) With long preincubation times, the amplitude of the fast decay was $13 \pm 2\%$ of the total reduction in fluorescence upon diluting into acetylcholine ($n = 3$ preparations), a value that is significantly less than the $46 \pm 9\%$ observed when nAcChoRs are reconstituted into DOPC alone (Fig. 6B). Similarly, re-reconstituting DOPC-reconstituted nAcChoRs into DOPC/DOPA/cholesterol reduced the amplitude of the intermediate decay to $24 \pm 2\%$ ($n = 3$ preparations).

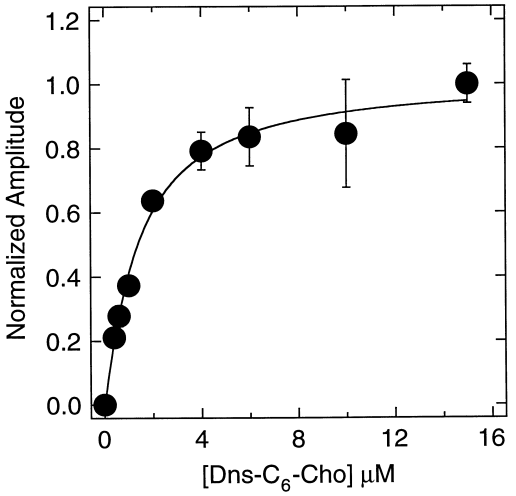

Fig. 5. Dns-C_6-Cho concentration dependence of the fast decay amplitude using a 50-ms preincubation time. The amplitudes have been normalized to that obtained using 15 μM Dns-C_6-Cho. The concentration of DOPC-reconstituted nAcChoRs was 0.13 μM. The data points are an average (±S.D.) of three separate determinations using the same preparation. The data is fit to Eq. 1 yielding a K_d of 1.4 ± 0.2 μM and a Hill coefficient of 1.1 ± 0.2.

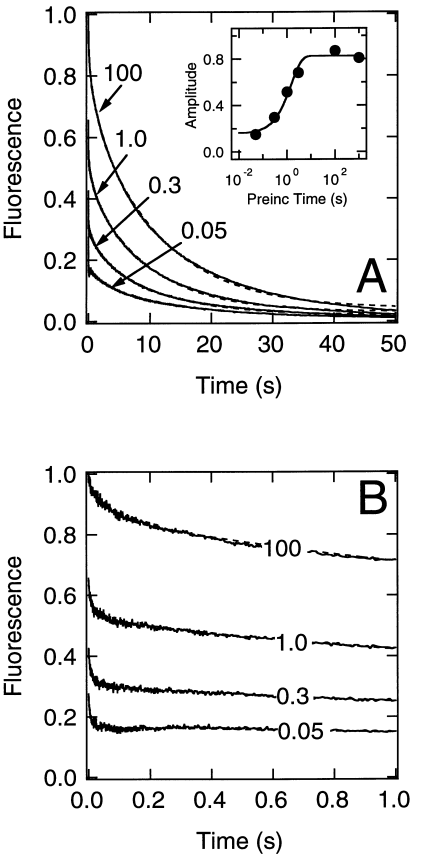

Fig. 6. Sequential mixing stopped-flow fluorescence traces of DOPC/DOPC/cholesterol-reconstituted nAcChoRs. Panel A shows the change in fluorescence intensity recorded when nAcChoRs reconstituted into DOPC/DOPC/cholesterol are preincubated with 10 μM Dns-C_6-Cho for the indicated times and then chemically diluted into 1 mM acetylcholine. The dotted lines are fits of fluorescence traces to a triple exponential equation. The inset is a plot of the amplitude of the slow decay as a function of preincubation time. The line is a fit of the amplitude data to an exponential equation yielding an apparent rate of 0.7 ± 0.2 s^{-1}. Panel B shows the first second of panel A to reveal, depending on the preincubation time, the fast or intermediate decays. A small fluorescence enhancement on the time scale of several hundred ms is also noted in the 0.05-s preincubation trace which reflects Dns-C_6-Cho interactions with the agonist-inhibitory site [19].

4. Discussion

In single mixing studies, native and DOPC-reconstituted nAcChoRs were preequilibrated with the fluorescent partial agonist Dns-C_6-Cho and then rapidly mixed with acetylcholine. In experiments using either membrane preparation, a slow fluorescence decay was observed reflecting Dns-C_6-Cho dissociation from high-affinity binding sites on desensitized nAcChoRs [4,19]. However, in experiments using DOPC-reconstituted nAcChoRs, an additional fluorescence decay was observed. This decay was essentially complete by 200 ms and could be eliminated by preincubating membranes with α-bungarotoxin. This is indicative of Dns-C_6-Cho interactions with low-affinity agonist binding sites on DOPC-reconstituted nAcChoRs. An estimate of the K_d of these sites was made by titrating them with Dns-C_6-Cho and then using the amplitude of the intermediate decay as a measure of the fraction of sites that had bound Dns-C_6-Cho prior to its being displaced by acetylcholine. This experiment is shown in Fig. 3 and yielded a K_d of 0.61 ± 0.07 μM.

It is possible to estimate the fraction of all low-affinity sites that have bound Dns-C_6-Cho from the fractional amplitude of the fast decay provided that the relative fluorescence intensity of Dns-C_6-Cho bound to low- and high-affinity states is known. We have previously computer-modeled the fluorescence intensity (amplitude) of Dns-C_6-Cho bound to low- and high-affinity sites in native membranes and determined that Dns-C_6-Cho fluorescence varies little with the affinity of the site to which it is bound [19]. Thus, the fractional amplitudes of the intermediate and slow decays may be taken directly as approximate measures of the fractions of Dns-C_6-Cho-bound sites having low and high affinity, respectively. The absence of an intermediate decay in traces obtained with nAcChoR-rich native membranes equilibrated with saturating concentrations of Dns-C_6-Cho indicates that Dns-C_6-Cho converts essentially all preexisting low-affinity receptors to a state that has a high affinity for agonist. Similarly, nearly all DOPC/DOPA/cholesterol-reconstituted nAcChoRs are converted by Dns-C_6-Cho to a high-affinity state as indicated by the small fractional amplitude of the intermediate decay in these membranes ($13 \pm 2\%$). In contrast, the relatively large fractional amplitude of the intermediate decay observed when nAcChoRs are reconstituted into DOPC alone ($47 \pm 9\%$) implies that many nAcChoRs persist in a low-affinity state even after exposing them to excess Dns-C_6-Cho for 1–2 h. The amplitude of this intermediate decay may be reduced by re-reconstituting them from DOPC into DOPC/DOPA/cholesterol, suggesting that a significant fraction of nAcChoRs can recover their ability to undergo affinity state conversion to the high-affinity state if they are returned to a lipid environment that more closely resembles that found in nAcChoR-rich native membranes.

To define the affinity state of reconstituted nAcChoRs prior to agonist-induced affinity state transitions, we used Dns-C_6-Cho preincubation times as brief as 50 ms before dilution into acetylcholine. With a preincubation time this short, agonist affinity state conversion during Dns-C_6-Cho preincubation is minimized. In all experiments, a Dns-C_6-Cho concentration of 10 μM was used to saturate the low- and high-affinity sites detected with single mixing experiments. We also expect that at this Dns-C_6-Cho concentration, the process of Dns-C_6-Cho binding to preexisting receptor conformational states will be in pseudoequilibrium even when using preincubation times as short as 50 ms [19]; assuming an association rate constant as slow as 10^7 M^{-1} s^{-1}, the $t_{1/2}$ for binding is less than 7 ms. This allowed us to monitor the conformational transitions that follow Dns-C_6-Cho binding. Fig. 4A and Fig. 6A show that with a 50-ms preincubation time, the amplitude of slow decay was relatively small, indicating that few nAcChoRs were in the high-affinity state at the time of dilution into acetylcholine. However, the amplitude increased with longer preincubation times as Dns-C_6-Cho shifted approximately half (DOPC) or nearly all (DOPC/DOPA/cholesterol) agonist binding sites on reconstituted nAcChoRs to the high-affinity state.

In addition to the low-affinity sites detected following equilibration of reconstituted receptors with Dns-C_6-Cho, we also detected α-bungarotoxin displaceable, low-affinity sites by using a preincubation times of 50 ms. These low-affinity sites appear to be conformationally distinct from those observed following equilibration with Dns-C_6-Cho because they are characterized by a faster dissociation rate constant and a higher K_d for the fluorescent agonist. However, the kinetic constants defining this low-af-

finity site are essentially identical to those of the transient low-affinity resting-state sites detected under identical conditions in nAcChoR-rich native membranes [19]. It seems reasonable to assume, therefore, that the low-affinity site detected in reconstituted nAcChoR membranes following a 50-ms preincubation time also reflects Dns-C_6-Cho interactions with the resting state.

In previous studies, the ability of reconstituted nAcChoRs to undergo the transition from low- to high-affinity has been most commonly evaluated indirectly with a α-bungarotoxin competition assay [9,11,13,14,16]. This assay compares the rates of α-bungarotoxin binding when (1) added to membranes simultaneously with agonist or (2) added to membranes that have been preequilibrated with agonist. The nAcChoR is typically judged to be in the low-affinity state and capable of undergoing an affinity state transition with agonist exposure if the simultaneous addition of α-bungarotoxin and agonist results in an initial rate of α-bungarotoxin binding that is greater than that determined following preequilibration with agonist [14,16]. Conversely, if these binding rates are not different, then the nAcChoR is assumed to preexist in the high-affinity state. Unfortunately, the initial rates of α-bungarotoxin binding under these two experimental conditions typically differ by less than a factor of 2 even when using nAcChoRs that have been reconstituted into lipids that are believed to maintain the receptor's ability to undergo agonist-induced affinity state transitions [14]. The small difference may make data interpretation difficult even under the best of circumstances. Because preequilibrating DOPC-reconstituted nAcChoRs with agonist leaves a significant fraction of the nAcChoRs in a relatively low-affinity state, the difference in the α-bungarotoxin binding rates measured in this assay is predicted to be even smaller; the rate of α-bungarotoxin binding to nAcChoRs preincubated with agonist will be relatively high because of the persistence of nAcChoRs that bind agonist with low affinity. Furthermore, the α-bungarotoxin competition assay assumes that the apparent rate of desensitization induced by agonist upon simultaneous addition is slow on the time scale of α-bungarotoxin binding. Our studies show that even at a concentration of 10 μM, Dns-C_6-Cho significantly alters the preexisting equilibrium in less than 1 s.

A smaller number of studies have suggested that nAcChoRs reconstituted into DOPC are fully capable of undergoing a transition from the low-affinity to the high-affinity state [13,18]. However, these studies used a lipid dilution technique that does not remove endogenous lipids. These endogenous lipids may support function even when present in the membrane at relatively low mole fractions. For example, adding just 6 mol% cholesterol to DOPC-reconstituted nAcChoRs will significantly promote channel activation [30]. Furthermore, because a substantial pool of cholesterol is strongly associated with the nAcChoR, dilution may leave a significant amount of cholesterol at the receptor's annular and nonannular cholesterol binding sites [31]. In contrast, our studies used affinity column purification, a process that removes endogenous lipid.

McCarthy and Moore have used a structural assay to define the conformational state of reconstituted nAcChoRs [16,32]. They characterized the labeling of native and reconstituted nAcChoRs by the photoreactive, hydrophobic probe [^{125}I]TID in the absence of agonist and following agonist preequilibration, then used the labeling pattern as an indicator of the nAcChoR's conformational state. They found that receptors reconstituted into DOPC exhibited a pattern of labeling that closely resembled that observed in agonist desensitized native membranes. This data was interpreted as evidence that nAcChoRs reconstituted into DOPC were in the desensitized state. These results are not necessarily in conflict with ours. White and Cohen have shown that agonist-modulated [^{125}I]TID incorporation reflects interactions with the M2 transmembrane domain, a site that is quite distinct from the extramembraneous agonist binding domain characterized in the present study [33,34]. Therefore, it is conceivable that the difference between our results and those of White and Cohen reflects the probing of different regions of the receptor.

In a recent study, Rankin et al. used the fluorescent channel blocker ethidium to detect nAcChoR channel activation and concluded that nAcChoRs reconstituted into DOPC alone fail to open in response to agonist [30]. Similarly, radiotracer studies have shown that agonists do not induce ion flux through nAcChoRs reconstituted into DOPC [35,36]. Our studies prove that the inability of

DOPC-reconstituted nAcChoRs to open and allow ion flux does not result from their being locked in the high-affinity, desensitized state as previously suggested. Rather, such receptors largely preexist in a conformational state that exhibits a low affinity for agonist, and a substantial fraction remain that way even following prolonged agonist exposure. However unlike the resting-state conformation, these low-affinity receptors remain in a closed state even upon exposure to high concentrations of agonist.

In summary, we have characterized the affinity state of nAcChoRs reconstituted into bilayers composed of DOPC alone or a mixture of DOPC, DOPA, and cholesterol prior to and following agonist-induced affinity state transitions. In contrast to several previous studies using α-bungarotoxin binding as a measure of nAcChoR affinity state, our results indicate that prior to agonist-induced affinity state conversion, nAcChoRs reconstituted into either DOPC or DOPC/DOPA/cholesterol are predominantly in a conformational state that has a low affinity for agonist. Equilibration with the fluorescent agonist Dns-C_6-Cho converts nearly all DOPC/DOPA/cholesterol-reconstituted nAcChoRs to the high-affinity state. However, equilibration with Dns-C_6-Cho converts only half of all DOPC-reconstituted nAcChoRs to the high-affinity state. The other half persist in a low-affinity inactivatable conformational state, characterized by a K_d for Dns-C_6-Cho that is similar, but not identical, to that previously reported for its binding to low-affinity, resting-state nAcChoRs in native membranes.

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