Agonist Binding to the *Torpedo* Acetylcholine Receptor. 1. Complexities Revealed by Dissociation Kinetics†

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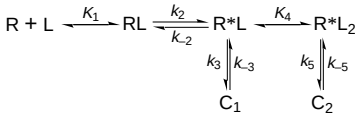
ABSTRACT: Examination of the kinetics of dissociation of [^{3}H]acetylcholine and [^{3}H]suberyldicholine from the membrane-bound acetylcholine receptor from *Torpedo californica* has revealed complexities in the high-affinity binding of nicotinic agonists. Each agonist binds to two high-affinity sites per receptor with an equilibrium dissociation constant of approximately 15 nM. When dissociation of [^{3}H]acetylcholine from the receptor complex was triggered by dilution, dissociation occurred as a monophasic process with an apparent rate of 0.023 ± 0.010 s^{-1}. However, when micromolar concentrations of unlabeled agonists (acetylcholine, carbamylcholine or suberyldicholine) were included in the dilution buffer this rate increased about 5-fold. This accelerating effect occurred even when the two high-affinity sites were initially saturated with the radioligand. This suggested the presence of an additional site (or subsite) for agonist with affinity in the micromolar range. However, at concentrations of 0−20 μM, no additional sites for [^{3}H]acetylcholine were detected at equilibrium. To explain these results, we propose that each high-affinity site is made up of two subsites, A and B, which are mutually exclusive at equilibrium. With [^{3}H]acetylcholine initially occupying site A, occupancy of site B by unlabeled ligand reduces the affinity for site A and accelerates the dissociation of the radioligand. Studies of dissociation of [^{3}H]suberyldicholine, a large bis-quaternary agonist, provide some clue as to the possible physical nature of these subsites. Whereas its dissociation rate was similar to that of [^{3}H]acetylcholine (0.028 ± 0.012 s^{-1}), this rate was only marginally, if at all, affected by the presence of unlabeled ligands. These results, in addition to those presented in the accompanying manuscript, lead to the proposal that [^{3}H]suberyldicholine is able to cross-link the two subsites or at least sterically occlude the second site.

In previous stopped-flow studies of the association of agonists, such as acetylcholine (AcCh)[1] or carbamylcholine (Carb), with the *Torpedo* nicotinic acetylcholine receptor (nAcChR), we observed complex kinetic behavior that was interpreted in terms of several ligand binding steps and multiple conformational changes (Quast et al., 1979; Dunn et al., 1980; Blanchard et al., 1982; Dunn & Raftery, 1993). Since all of the observed kinetic phases were too slow to account for the very fast opening of the receptor ion channel, they were considered to more likely reflect processes related to slow receptor desensitization (Katz & Thesleff, 1957). When agonist binding kinetics were monitored using either an extrinsic fluorescent probe, ethidium (Quast et al., 1979), or a fluorophore, 5-iodoacetamidosalicylic acid (IAS), covalently bound to the nAcChR [see Dunn and Raftery (1993)], the kinetics could be described by Scheme 1.

Scheme 1



According to this scheme, C_1 represents an equilibrium desensitized state of the receptor in which the agonist is bound with high-affinity ($K_d \approx 15$ nM for AcCh). Since each receptor carries two high-affinity sites for agonists (see below), the above scheme should be doubled to represent the intact receptor molecule. While the pathway to C_1 dominates the kinetics at low ligand concentration, at higher concentrations the binding of additional ligand to R*L drives the reaction toward C_2. A prediction of this mechanism is therefore that more than two binding sites will be occupied in the fully desensitized receptor at high agonist concentrations, i.e., in complex C_2. However, in direct binding studies carried out in many different laboratories, the stoichiometry of high-affinity binding sites for [^{3}H]AcCh or [^{3}H]Carb has been found to be two per nAcChR [reviewed by Stroud et al. (1990), Changeux et al. (1992), and McLane et al. (1996)].

In the present study, we have further investigated the mechanism of agonist binding using [^{3}H]AcCh and [^{3}H]-suberyldicholine (SdCh) to avoid potential complications that may arise using fluorescent reporter groups. The kinetics of agonist binding have previously been studied using radiolabeled AcCh (Boyd & Cohen, 1980a,b), fluorescent

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[1] Abbreviations: AcCh, acetylcholine chloride; α-BuTx, α-bungarotoxin; Carb, carbamylcholine chloride; DNPP, diethyl-*p*-nitrophenyl phosphate; IAS, 5-iodoacetamidosalicylic acid; nAcChR, nicotinic acetylcholine receptor; NBD, 7-nitro-2,1,3-benzoxadiazole; SdCh, suberyldicholine diiodide; *d*-TC, *d*-tubocurarine.

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agonists (Heidmann & Changeux, 1979; Prinz & Maelicke, 1983, 1992), and by changes in protein intrinsic fluorescence (Bonner et al., 1976; Barrantes, 1976, 1978). The study described here focuses on agonist dissociation kinetics. The results differ from previous reports since they indicate that, in addition to the two known high-affinity sites for agonists, at equilibrium there are additional sites to which agonists can bind. When these latter sites are occupied, the dissociation of bound agonist from the two primary sites is accelerated. The two types of binding sites (or domains) appear to be mutually exclusive since, at equilibrium, the stoichiometry of bound agonist is always 2 per receptor molecule. In the accompanying manuscript, these studies are extended to further analysis of agonist association kinetics, and a model is developed to accommodate the complexities observed in both the dissociation and association kinetics.

MATERIALS AND METHODS

Preparation of nAcChR-Enriched Membrane Fragments. Frozen electric organ from *Torpedo californica* was obtained from Pacific Biomarine, Venice, CA. nAcChR-enriched membrane fragments were prepared as previously described (Elliott et al., 1980) and were stored frozen at −86 °C. Before use, membrane aliquots were thawed and alkali-extracted to remove non-receptor proteins (Neubig et al., 1979; Elliott et al., 1979). Unless otherwise stated, the buffer used in each experiment was *Torpedo* Ringers (20 mM Hepes-Na^+, pH 7.4, 250 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, 0.02% NaN_3) and both equilibrium binding and kinetic experiments were carried out at room temperature (23 ± 2 °C). The concentration of α-BuTx sites was measured by the DEAE disc assay described by Schmidt and Raftery (1973) using [^{125}I]-α-BuTx from DuPont Canada, which was calibrated as previously described (Blanchard et al., 1979). Protein concentrations were determined by the Lowry method (Lowry et al., 1951). Specific activities of the alkali-extracted membrane preparations lay in the range 2−4 nmol of α-BuTx binding sites/mg of protein.

Radiolabeled Agonists. [^{3}H]Acetylcholine chloride was from DuPont Canada, and [^{14}C]acetylcholine chloride was from Amersham. Radiochemical purity was determined by thin-layer chromatography in 80% ethanol (Lewis & Eldefrawi, 1974) and specific activities (50−90 mCi/mmol) were determined by competition with unlabeled AcCh (Sigma Chemical Co.) for binding to nAcChR as described for [^{3}H]-AcCh by Neubig and Cohen (1979). Estimated specific activities of the different batches of radiolabeled AcCh used in this study were within 5% of the manufacturers' estimates. [^{3}H]Suberyldicholine diiodide (batches of 166 and 74 mCi/mmol) was synthesized as described previously (Mecklenborg & Orchin, 1958; Holmstedt & Whittaker, 1958; Sekull & Holland, 1961, 1963). In brief, suberyl dichloride (Aldrich) was reacted with 2 equiv of (dimethylamino)-ethanol in methylene chloride. The precipitated product was washed three times with methylene chloride, filtered, and dried *in vacuo* over phosphorus pentoxide and NaOH. The dried compound was then dissolved in water, titrated to pH 10 with NaOH, and extracted three times with methylene chloride. The combined organic extracts were dried over anhydrous magnesium sulfate and evaporated to yield an oil. This oil was used in a stoichiometric reaction with 2 equiv of [^{3}H]CH_3I (DuPont), followed by addition of an excess of unlabeled methyl iodide to yield [^{3}H]SdCh. Specific activities were determined as for radiolabeled AcCh above by competition with unlabeled suberyldicholine dichloride (Aldrich).

Equilibrium Binding of Radiolabeled Agonists. Acetylcholinesterase activity was inhibited by the addition of 0.005 vol of a 0.3 M solution of diethyl-*p*-nitrophenyl phosphate (DNPP, Sigma) in 2-propyl alcohol to a concentrated membrane suspension (approximately 10 μM in α-BuTx sites). After 3 min at room temperature, the DNPP-treated membranes were diluted about 5-fold in *Torpedo* Ringers and stored on ice until use. Acetylcholinesterase activity was shown to be negligible when assayed by the method of Ellman et al. (1961). Measurements of equilibrium binding by centrifugation assays were as described for [^{3}H]Carb binding by Dunn et al. (1980). Briefly, nAcChR-enriched membrane preparations were incubated with radiolabeled ligand for 30 min at room temperature. In many previous experiments, we have found that, at the ligand concentrations used, equilibrium is reached in <10 min under these conditions [see, e.g., Dunn et al. (1980)]. Duplicate 50 μL aliquots were taken and counted for radioactivity to allow estimation of the total ligand concentration. After centrifugation at 12 000g_{max} for 15 min, duplicate 50 μL aliquots of the supernatant were counted to allow estimation of the free ligand concentration. Total binding was calculated by subtracting free from total ligand concentrations. Nonspecific binding was measured in parallel samples in which an excess of unlabeled ligand was included in the incubation mixtures. In some experiments, equilibrium binding was measured by equilibrium dialysis using microdialysis chambers (Chemical Rubber Company) and 50K molecular weight cutoff dialysis tubing (SpectraPor). Aliquots (0.35 mL) of nAcChR-enriched membranes were dialyzed against 0.35 mL of radiolabeled ligand solution with rocking for 6 h at 4 °C. Total and free ligand concentrations were measured by taking duplicate 50 μL samples of each, adding 4 mL of hydrofluor (National Diagnostics) or CytoScint (ICN) and counting for [^{3}H] or [^{14}C] as appropriate. In dual labeling experiments with [^{14}C]AcCh and [^{3}H]SdCh, appropriate corrections were made for spillover of [^{14}C] into the [^{3}H] channel. Nonspecific binding was estimated in the presence of excess unlabeled ligand and in most cases was negligible.

Dissociation Measurements. To measure dissociation on subsecond time scales, a Biologic Rapid Filtration System (Biologic, Meylan, France) was used (DuPont, 1984; Agey & Dunn, 1989). DNPP-treated membranes were equilibrated with [^{3}H]AcCh or [^{3}H]SdCh by incubation for 30 min at room temperature. Aliquots of 0.25−0.4 mL were applied to a Whatman GF/C filter mounted in the filtration apparatus and excess buffer was removed under vacuum. Dissociation was induced by forced filtration for the desired time period with buffer alone or with buffer containing unlabeled ligand. Flow rates were varied from 5 to 0.5 mL/s to ensure adequate washing of the filter at each time period (Dupont, 1984). Nonspecific binding was measured in the presence of excess unlabeled ligand in the initial incubation mixture and was negligible at all time periods used. Similarly, there was no nonspecific binding of radiolabeled ligand to the filter measured in the absence of membranes. The Biologic filtration system was used to measure dissociation on time scales of 0.01−9 s. Manual techniques were used to measure dissociation kinetics over longer time periods. In these

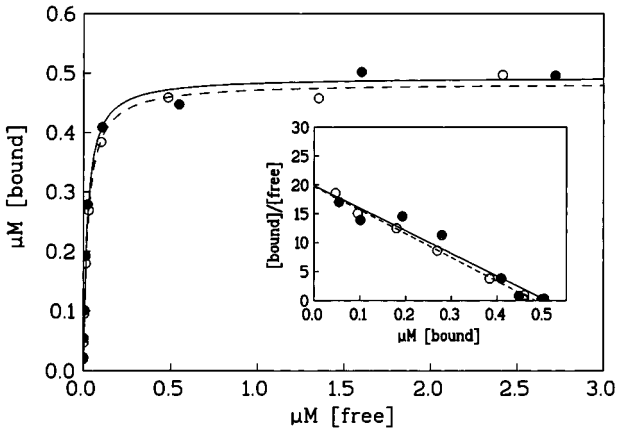

FIGURE 1: Equilibrium binding of [^{3}H]AcCh (●) and [^{3}H]SdCh (○) to nAcChR-enriched membrane preparations. Membranes (0.5 μM in α-BuTx sites) were incubated with varying concentrations of radiolabeled agonist for 30 min at room temperature, after which bound and free ligand was separated by centrifugation. Nonspecific binding, measured in the presence of excess unlabeled ligand, was negligible. Inset shows Scatchard plots of the data giving K_d = 23.8 nM and R_0 = 0.50 μM for [^{3}H]AcCh and K_d = 21.9 nM and R_0 = 0.48 μM for [^{3}H]SdCh. Data shown are representative of at least 12 experiments for each ligand.

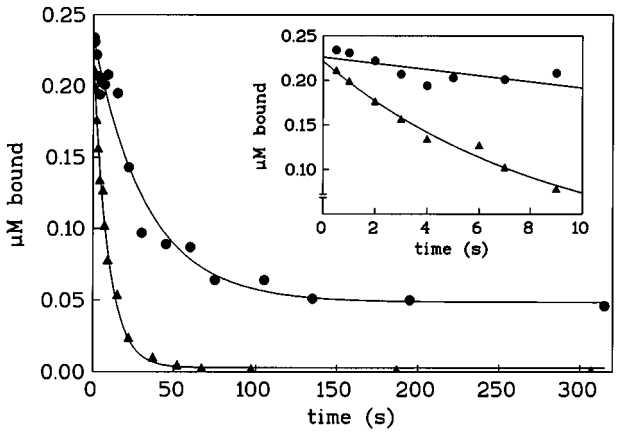

FIGURE 2: Dissociation of [^{3}H]AcCh measured after dilution of the receptor−ligand complex in *Torpedo* Ringers alone (●) or in Ringers containing 100 μM unlabeled AcCh (▲). Membranes (0.25 μM in α-BuTx sites) were first equilibrated with 0.5 μM [^{3}H]AcCh, sufficient to occupy at least 90% of the binding sites. Dissociation was initiated as described in the text and measured using the Biologic rapid filtration system (0.5−9 s) or by manual filtration techniques (times longer than 15 s). Inset shows expanded data on rapid time scales and illustrates the acceleration of dissociation in the presence of unlabeled AcCh. Solid lines are calculated using the best fit parameters obtained by nonlinear regression fitting to a simple exponential decay:

$$[RL]_t = [RL]_0 \exp(-k_{app}t) + [RL]_{eq}$$

where $[RL]_t$, $[RL]_0$, and $[RL]_{eq}$ are the concentrations of bound ligand at time = t, 0, and ∞, respectively, and k_{app} is the apparent rate constant. Values of k_{app} (obtained from the data in the composite curves, main panel) were 0.030 and 0.11 s^{-1} in the absence and presence of unlabeled AcCh, respectively.

experiments, complex formation between nAcChR-enriched membranes and [^{3}H]AcCh or [^{3}H]SdCh was allowed to come to equilibrium, and at the appropriate time, a 0.25 mL aliquot was diluted into 47.75 mL of *Torpedo* Ringers (i.e., a 200-fold dilution), in the absence or presence of unlabeled ligand. The mixture was immediately filtered through a Whatman GF/C filter under vacuum using a Hoefer filtration apparatus. Filters were rapidly washed with 5 mL of ice-cold Ringers, and, after drying and addition of 5 mL CytoScint, filters were counted for [^{3}H].

Data Analysis. Data were analyzed using the InPlot 4 program (GraphPad) and the equations described in the text.

RESULTS

Equilibrium Binding of Radiolabeled Agonists. The equilibrium binding of [^{3}H]SdCh to membrane-bound nAcChR is very similar to the binding of [^{3}H]AcCh (Figure 1). Binding of [^{3}H]SdCh is to an apparently homogeneous population of sites with a K_d of 15.9 ± 6.3 nM (n = 15). The binding of [^{3}H]AcCh is also characterised by K_ds in the range 10−30 nM, as has been found in many previous studies [e.g., Neubig and Cohen (1979) and Blanchard et al. (1982)]. For each agonist, the total number of high-affinity sites occupied is equal to the number of sites for [^{125}I]-α-BuTx, i.e., two sites per nAcChR molecule. As in many previous studies [e.g., Quast et al. (1979), Dunn et al. (1980), Blanchard et al. (1982), and Dunn and Raftery (1982a,b, 1993)], we have found no evidence for cooperativity between these binding sites.

Dissociation Kinetics of [^{3}H]AcCh. When dissociation of [^{3}H]AcCh was triggered by 200-fold dilution into *Torpedo* Ringers (Figure 2), dissociation occurred as a monophasic process with an apparent rate constant of 0.023 ± 0.010 s^{-1} ($t_{1/2} \approx 30$ s, n = 9). In this experiment, the initial complex was formed between 0.25 μM receptor (in [^{125}I]-α-BuTx sites) and 0.5 μM [^{3}H]AcCh, i.e., a ligand concentration sufficient to saturate greater than 90% of the high-affinity binding sites. In manual dilution experiments, it is not possible to reach conditions of "infinite dilution" due to technical limitations on the times required for filtering of large volumes. Thus some rebinding and lack of complete dissociation of bound ligand is inevitable. This is illustrated in Figure 2 where the residual binding seen at long times is due to lack of complete dissociation and not to nonspecific binding which was negligible in this experiment (see data in the presence of 100 μM AcCh in Figure 2). As a result of this rebinding, the dissociation rate constant is likely to be underestimated in these experiments. However, when 0.1 μM AcCh was included in the dilution buffer, i.e., a concentration sufficient to prevent rebinding but not high enough to markedly accelerate the dissociation (see below), the measured rate was not significantly different (0.025 ± 0.008 s^{-1}, n = 6). In the initial 9 s of the dissociation, the use of the Biologic automated filtration system obviates any problem of rebinding. During forced filtration with dilution buffer, free and dissociated ligand are continually removed. Thus no rebinding is likely and better estimates of the early dissociation kinetics can be obtained. As illustrated in the inset in Figure 2, little dissociation occurred on this time scale.

Acceleration of [^{3}H]Acetylcholine Dissociation in the Presence of Unlabeled Agonists. When 100 μM unlabeled AcCh was included in the dilution buffer, the rate of dissociation was increased to 0.106 ± 0.011 s^{-1} ($t_{1/2} \approx 6.5$ s, n = 6). The composite time course of the reaction using both automated and manual techniques show that, under these conditions, [^{3}H]AcCh was completely displaced and dissociation was again well described by a single exponential process (Figure 2). The lack of heterogeneity suggests that each of the two high-affinity sites act in a similar manner. The inset in Figure 2 shows the marked accelerating effect

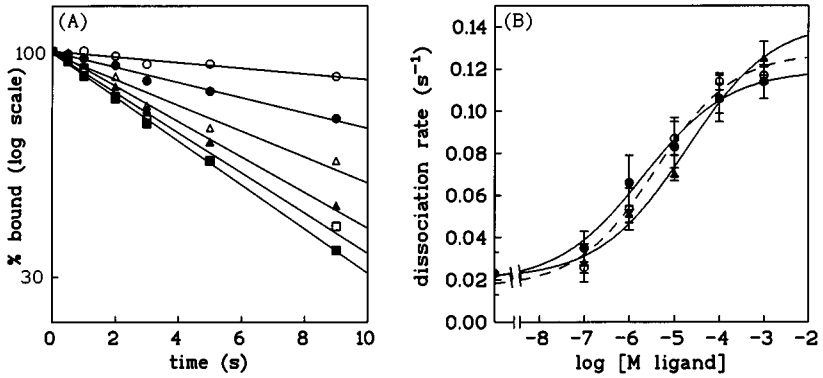

FIGURE 3: Effect of agonists on the rate of [^{3}H]AcCh dissociation. nAcChR-enriched membrane fragments were first equilibrated with [^{3}H]AcCh as described in the legend to Figure 1. Panel A shows representative semi-log plots of [^{3}H]AcCh dissociation occurring on rapid time scales during forced filtration with Ringers alone (○) or with 0.1 μM (●), 1.0 μM (△), 10 μM (▲), 100 μM (□) or 1 mM (■) mM AcCh. The agonist concentration dependence of the apparent dissociation rate constant, calculated from such semi-log plots are shown in panel B for AcCh (●), SdCh (○), and Carb (▲). Lines are calculated using the best fit parameters for a sigmoidal model:

$$\text{rate} = k_0 + \frac{k_{max} - k_0}{1 + [(10^{\log EC_{50}})^n/(10^{\log [L]})^n]}$$

where k_0 is the apparent rate in the absence of competing ligand, k_{max} is the maximum dissociation rate, [L] is the concentration of competing ligand, EC_{50} is the concentration of ligand that produces 50% of the maximal effect, and n is the apparent Hill coefficient. The fitted values for k_0, k_{max}, and EC_{50} are given in Table 1. In each case, the value of n was approximately 0.5. Data shown are pooled from six (AcCh), three (SdCh), and two (Carb) experiments.

Table 1: Effects of Agonists and Competitive Antagonists on the Dissociation of [^{3}H]AcCh and [^{3}H]SdCh

	[^{3}H]AcCh dissociation			[^{3}H]SdCh dissociation		
ligand	k_0 (s^{-1})	k_{max} (s^{-1})	EC_{50} (μM)	k_0 (s^{-1})	k_{max} (s^{-1})	EC_{50} (μM)
A. agonists						
AcCh	0.026 ± 0.012	0.117 ± 0.006	2.09 ± 0.12	0.029 ± 0.005	0.072 ± 0.006	15.3 ± 8.6
SdCh	0.020 ± 0.003	0.128 ± 0.014	2.88 ± 0.72	0.029 ± 0.005	0.053 ± 0.011	0.58 ± 0.13
carb	0.021 ± 0.003	0.140 ± 0.003	18.8 ± 4.7	0.026 ± 0.005	0.059 ± 0.006	54.3 ± 21.8
choline[a]	0.020 ± 0.007	0.017 ± 0.006	NE[b]	0.028 ± 0.009	0.026 ± 0.005	NE
B. antagonists						
d-TC	0.018 ± 0.002	0.203 ± 0.020	475 ± 184	0.029 ± 0.010	0.027 ± 0.008[c]	NE
β-erythroidine[a]	0.025 ± 0.015	0.025 ± 0.008	NE	0.028 ± 0.005	0.024 ± 0.009	NE

[a] Parameters listed represent mean ± sd from at least three independent experiments at 0 (k_0) and 1 mM (k_{max}) ligand. [b] NE, no effect. [c] Measured dissociation rate at 1 mM *d*-TC.

of unlabeled AcCh on rapid time scales. This effect was dependent on the concentration of unlabeled ligand (Figure 3, Table 1), giving an estimated EC_{50} for AcCh of 2.1 μM. Two other receptor agonists, SdCh and Carb, had similar accelerating effects on the rate of [^{3}H]AcCh dissociation with EC_{50} values of 2.9 μM and 18.8 μM respectively (Figure 3). The maximum rate of dissociation in the presence of any of these agonists was about 0.14 s^{-1} (Table 1). However, choline, which is a very poor agonist of the nAcChR (Quast et al., 1979; Dunn & Raftery, 1982b), did not affect the dissociation rate at a concentration of 1 mM (Table 1). As shown in Figure 3, the Hill coefficients for the dose-dependence of the effects of unlabeled ligands were approximately 0.5 in each case. A mechanistic interpretation of these low Hill coefficients is not, at this stage, practical since the apparent rates measured in these experiments are not true rate constants but are likely to be complex functions of several competing processes (see below and accompanying manuscript).

In studies of many receptor systems, competing ligands have been found to affect the dissociation of a previously bound radiolabeled ligand. While there are several possible explanations (see Discussion), this phenomenon has frequently been interpreted in terms of an additional binding site (or sites) which, when occupied by the competing ligand, induces a conformational change that alters the affinity of the first site. One explanation for the above observations is, therefore, that the nAcChR carries additional binding sites (K_ds in the micromolar range) for nicotinic agonists.

Lack of Evidence for Agonist Binding Sites with Micromolar Affinities. Since micromolar concentrations of the *Torpedo* nAcChR are readily available, agonist binding sites with micromolar affinities should be readily detectable. The results in Figure 4A illustrate that, at equilibrium, no additional sites for [^{3}H]AcCh were detected at ligand concentrations up to 20 μM. Furthermore, in a mixture of [^{14}C]AcCh and [^{3}H]SdCh, no evidence was obtained for ternary complex formation associated with each of the two binding sites (Figure 4B). The binding of [^{3}H]SdCh paralleled the displacement of [^{14}C]AcCh, and the total number of sites occupied remained constant and equal to the number of sites for [^{125}I]-α-BuTx, i.e., two per receptor molecule.

Since additional sites were not detected at equilibrium, any "ternary" complex in which the receptor−[^{3}H]AcCh complex can bind additional ligand molecules must be a transient phenomenon. On the basis of the results above and additional information provided both below and in the accompanying manuscript, it is proposed that such a transient state exists for AcCh and other mono-quaternary agonists. Although much of the evidence for this state is inferred

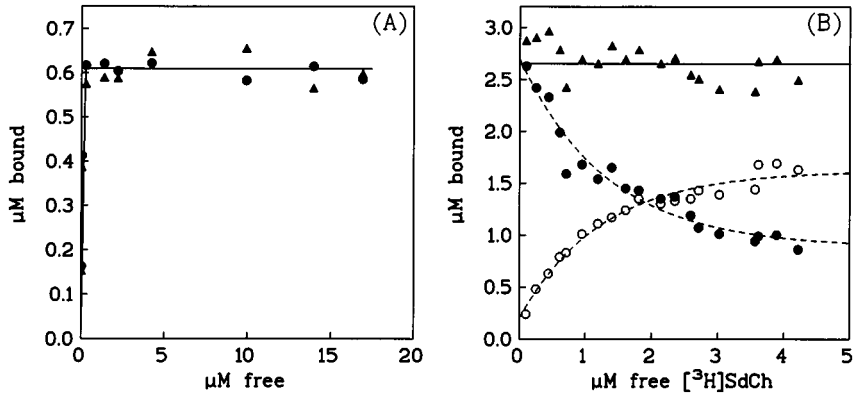

FIGURE 4: Lack of evidence for additional agonist binding sites with K_ds in the micromolar range. In A, nAcChR-enriched membranes (0.6 μM in α-BuTx sites) were incubated with increasing concentrations of [^{3}H]AcCh for 30 min at room temperature. Bound ligand was either estimated by centrifugation assay (●) or by filtration assay (▲) using the rapid filtration system and force filtering with Ringers at a flow rate of 5 mL/s for 0.3 s to minimize any ligand dissociation. Data are corrected for nonspecific binding (less that 0.5% of free) measured in the presence of 200 μM AcCh. The data in B show that in the presence of a mixture of [^{14}C]AcCh and [^{3}H]SdCH, the total number of sites occupied do not exceed the number of sites for α-BuTx. Membranes (2.5 μM in α-BuTx sites) were dialyzed against an equal volume of Ringers containing 10 μM [^{14}C]AcCh and varying concentrations of [^{3}H]SdCh (0−10 μM) and bound and free ligands were measured as described in the text. Data show the amount of bound [^{14}C]AcCh (●) and the amount of bound [^{3}H]SdCh (○) at each concentration of free [^{3}H]SdCh. Total binding (▲) did not change significantly (2.66 ± 0.17 μM).

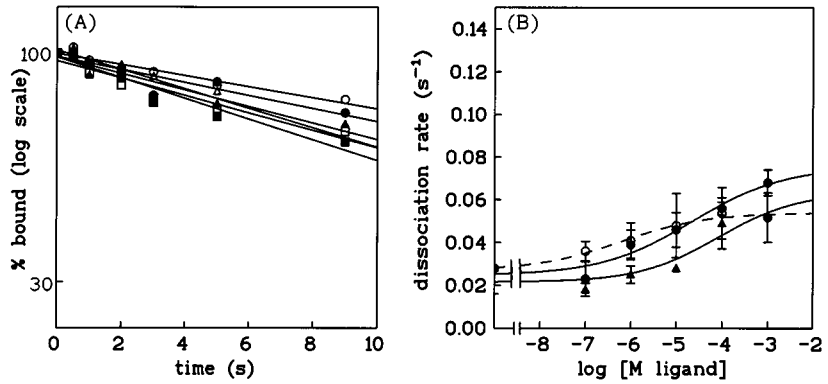

FIGURE 5: The presence of competing agonists has relatively little effect on the rate of dissociation of [^{3}]SdCh. Panel A shows the effects of increasing concentrations of unlabeled SdCh on the initial rate of dissociation of [^{3}H]SdCh. Experiments were identical to those shown in Figure 3 except for the nature of the labeled and competing ligand. Panel B shows the effects of AcCh (●), SdCh (○), and Carb (▲) on the rate of [^{3}H]SdCh dissociation. Data were analyzed as in Figure 3 and the results are presented in Table 1.

indirectly from analysis of ligand binding kinetics, studies of [^{3}H]SdCh binding has provided some insight into the possible physical nature of these multiple binding sites.

Dissociation Kinetics of [^{3}H]SdCh. The dissociation of [^{3}H]SdCh, triggered by 200-fold dilution into *Torpedo* Ringers, was also a monophasic process, occurring with an apparent dissociation rate of 0.028 ± 0.012 s^{-1}, i.e., quite similar to the rate observed for [^{3}H]AcCh. In contrast to [^{3}H]AcCh dissociation, however, the presence of unlabeled SdCh in the dilution buffer had very little effect on the dissociation rate (Figure 5A). While the agonists, AcCh, SdCh, and Carb, accelerated the dissociation rate slightly, this was much less pronounced than for [^{3}H]AcCh (Figure 5B and Table 1). SdCh is a large molecule in which two quaternary ammonium groups, which are presumed to be important for agonist recognition, are separated by a relatively long flexible backbone. Although the binding of bivalent ligands can be very complex [see De Lean et al. (1979)], a simple explanation is that the subsite for SdCh that stabilizes the second positive charge is the "additional" agonist binding site, whose occupancy results in accelerated [^{3}H]AcCh dissociation (see below). If, at equilibrium, [^{3}H]-SdCh is able to occupy both subsites to a significant extent, then any ternary complex formation and accelerating effects on the dissociation of this ligand will be considerably less than for monoquaternary agonists.

Pharmacology of Ligand Effects. The competitive antagonist, *d*-TC, also accelerated the rate of [^{3}H]AcCh dissociation, albeit at high concentrations ($EC_{50} \approx 0.56$ mM) but had no significant effect on the rate of [^{3}H]SdCh dissociation (Figure 6). β-Erythroidine, which is also a competitive antagonist and in structure represents about half of the *d*-TC molecule, had no effect on [^{3}H]SdCh dissociation and only a slight effect on [^{3}H]AcCh dissociation at very high concentration (>1 mM). The local anesthetics, lidocaine (10 mM) and prilocaine (5 mM), not only did not affect the dissociation rates but also, when included in both the initial incubation and filtration buffers, did not affect the accelerating influence of AcCh on [^{3}H]AcCh dissociation.

Two-Site (or Subsite) Model. The data above, together with the data provided in the accompanying manuscript, lead us to propose a model for ligand binding to the nAcChR which is illustrated in Figure 7. According to this model, in the nAcChR each high-affinity site for AcCh is made up by two subsites, sites A and B. Under equilibrium conditions, AcCh is bound predominantly to one of these sites (site A; see Discussion) with high-affinity ($K_d \approx 15$ nM). Dissociation by dilution alone occurs with an apparent rate

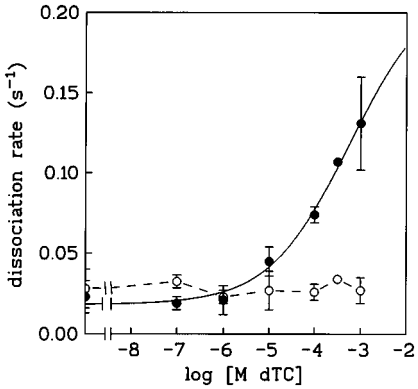

FIGURE 6: The effect of *d*-tubocurarine on the apparent rates of dissociation of [^{3}H]AcCh (●) and [^{3}H]SdCh (○). Data were obtained in experiments similar to those shown in Figures 3 and the parameters obtained from fitting to the [^{3}H]AcCh data are given in Table 1.

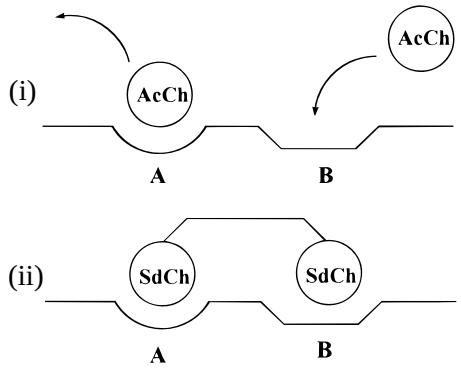

FIGURE 7: Two site (or subsite) model for AcCh (i) or SdCh (ii) binding. In i, AcCh is initially bound to site A at equilibrium. Dissociation from this site, induced by dilution, occurs with an apparent rate constant of 0.023 s^{-1}. However, when competing ligand, e.g., AcCh is included in the dilution buffer, this can occupy site B causing a conformational change that accelerates the dissociation of the first ligand from site A. The ternary complex is only transient and, at equilibrium, sites A and B are mutually exclusive. In ii, the bisquaternary ligand, SdCh, is depicted as occupying both sites A and B at equilibrium. The rate of dissociation of SdCh is, therefore, much less affected by the presence of competing ligand in the dilution buffer. This model represents only one of the two high-affinity binding sites in the nAcChR, i.e., in each receptor there would be two each of subsites A and B.

constant of 0.021 s^{-1}. However, when micromolar concentrations of competing ligand are included in the dilution buffer, it can occupy site B to accelerate the dissociation from site A. The ternary complex, in which both sites A and B are occupied, is transient, and at equilibrium, the sites are mutually exclusive. SdCh, being larger and bisquaternary, can occupy both sites A and B at equilibrium, and thus the presence of competing ligand has much less effect on its dissociation kinetics.

DISCUSSION

As noted in the introduction, we previously studied the kinetics of agonist binding to the membrane-bound nAcChR from *T. californica* (Quast et al., 1979; Dunn et al., 1980; Blanchard et al., 1982; Dunn & Raftery, 1993). From analysis of the multiple conformational transitions observed, we proposed a model involving multiple ligand binding steps (Scheme 1) in which, under some conditions i.e., high agonist concentration, more than two binding sites per receptor molecule could be occupied. However in numerous equilibrium ligand binding studies [reviewed in Stroud et al. (1990), Changeux et al. (1992), and McLane et al. (1996)], the stoichiometry of agonist binding sites has been demonstrated to be two sites per AcChR molecule. In an attempt to resolve this discrepancy, we have studied the dissociation kinetics of the radiolabeled agonists, [^{3}H]AcCh and [^{3}H]-SdCh, using rapid filtration methods following extensive dilution of the receptor−agonist complex. The initial conditions were such that, in the initial equilibrium complex, the AcChR was close to being saturated with radiolabeled ligand. We found that, in the absence of unlabeled ligand in the dilution buffer, the dissociation of [^{3}H]AcCh and [^{3}H]-SdCh at 23 °C was well described by a monophasic process with rates of 0.023 and 0.028 s^{-1}, respectively. However, when the dilution was conducted in the presence of unlabeled ligands (AcCh, Carb, SdCh, dTC), the rate of dissociation of [^{3}H]AcCh was accelerated in a ligand concentration dependent manner. The presence of local anesthetics in the dilution buffer did not affect either the dissociation rate or the ligand-induced acceleration suggesting that the binding site(s) for non-competitive inhibitors was not involved in the acceleration process. In addition, the concentration dependence of the AcCh effect ($EC_{50} \approx 2\ \mu M$) is about 50-fold lower than the K_d values of low-affinity sites that we have previously reported from fluorescence studies of agonist binding to NBD-labeled receptor (Dunn & Raftery, 1982a,b, 1993) and several orders of magnitude lower than the apparent dissociation constant for an inhibitory AcCh binding site described previously (Takeyasu et al., 1986; Forman et al., 1987). These two classes of sites do not, therefore, appear to be involved in the rate acceleration process.

In contrast to the complexities of the [^{3}H]AcCh dissociation kinetics, the dissociation rate of [^{3}H]SdCh was little affected by the presence of unlabeled ligands. We have interpreted these results in terms of a model depicted in Figure 7, in which each AcChR carries two high-affinity sites (sites A) for AcChR. However, in addition, each A site is associated with a secondary site or subsite (site B). Under equilibrium conditions, only one of the two subsites can be occupied by AcCh, i.e., only two AcCh molecules bind per AcChR but these are not necessarily bound in the same environment. Although a ternary complex in which AcCh occupies both sites A and B is observed in both the dissociation kinetics (above) and the association kinetics (accompanying manuscript), this is a transient phenomenon since sites A and B are mutually exclusive at equilibrium. Since the other ligand studied here, [^{3}H]SdCh, did not show the same dissociation characteristics as [^{3}H]AcCh, we propose that this large bischoline compound either bridges sites A and B or at least sterically blocks the access of competing ligands to site B.

In the above discussion, for convenience, we have suggested that site A is the "primary" site that is occupied at equilibrium although it is possible that sites A and B are equivalent but that occupancy of either one (A or B) reduces the affinity for the other by a negatively cooperative interaction. In order to investigate this further, we have studied the rate of [^{3}H]AcCh dissociation as a function of its concentration in the initial equilibration. If site B has lower affinity than site A, it might be expected that if this site becomes occupied to a significant extent at higher ligand concentrations, the observed rate of dissociation would increase. However, when [^{3}H]AcCh was varied between 0.1

and 10 μM the initial rate of dissociation, triggered by dilution, was not significantly affected (data not shown). These results favor ligand bound at site A as the predominant equilibrium species. This is discussed further in the accompanying manuscript.

In many other receptor systems [see Prinz and Striessnig (1993)], the dissociation rate of a radiolabeled ligand has been shown to be affected by the presence of unlabeled ligand. These results have frequently been interpreted in terms of negative cooperativity or the presence of multiple binding sites. The present results are not readily explained by negative cooperativity between the two "primary" agonist binding sites since, in the initial receptor−[^{3}H]AcCh complex, these two sites were effectively saturated prior to initiation of dissociation. Recently, Prinz and Striessnig (1993) proposed a model in which ligand-induced changes in dissociation rates could be explained if the ligands compete for multiple attachment points within a single binding site. This model also invokes a transient ternary complex and, as discussed further in the accompanying manuscript (Dunn & Raftery, 1997), is indistinguishable kinetically from the multisubsite model proposed here. In the present study, however, the differences in the dissociation behavior of [^{3}H]AcCh and [^{3}H]SdCh provide some information on the possible physical nature of the subsites.

There have been several previous reports of the kinetics of dissociation of agonists from the nAcChR complex and the consistency in the reported dissociation rates is striking, particularly in view of the structural differences in the agonists that have been studied. As reported in Results, the rate of [^{3}H]AcCh dissociation increased from 0.023 s^{-1} in the absence of competing ligand to a maximal value of about 0.12 s^{-1} in the presence of saturating concentrations of competing ligand. This maximal rate is close to that reported for ligand-induced dissociation of SdCh (0.06−0.10 s^{-1}; Barrantes, 1978), for dansyl-C_6-choline (0.3 s^{-1}; Heidmann & Changeux, 1979), and for NBD-5-acylcholine (0.2 s^{-1}; Prinz & Maelicke, 1992). In a previous report, Boyd and Cohen (1980b) studied the dissociation of [^{3}H]AcCh from *T. californica* AcChR by measurement of the rate of exchange of [^{3}H]AcCh for nonradioactive AcCh. Their observed dissociation rate of 0.15 s^{-1}, in the presence of competing ligand, is in good agreement with that reported here. Although these authors observed no concentration dependent effects when the nonradioactive ligand was varied between 3 and 60 μM, this is not a major discrepancy from the present results where the major effects occur at lower ligand concentration (see Figure 3).

In the present study, the estimated EC_{50} for the acceleration of [^{3}H]AcCh dissociation by AcCh was 2 μM. While this is not a true dissociation constant since the observed rate will be a complex function of several different liganded states of the AcChR, it is noteworthy that an equilibrium dissociation constant in the micromolar range was obtained from studies of the conformation of bound AcCh in the *Torpedo* AcChR complex by NOE-NMR fast exchange methods (Behling et al., 1988a,b). This suggests that these studies may have revealed some information about subsite B and the conformation of bound AcCh in this microenvironment. In these studies millimolar concentrations of AcCh were used and it is unlikely that AcCh bound to site A with a K_d of about 20 nM would have been in sufficiently rapid exchange to be detected.

The simple multi-subsite model we propose here implies that nicotinic agonists can bind to areas on the AcChR that are distinct from the primary binding site (site A) and suggests that these ligands may interact with the receptor in multiple ways. Such diversity in receptor binding properties has been observed by X-ray analysis of the interactions of human growth hormone (h-GH) with the extracellular domains of both the human growth hormone receptor and a prolactin receptor (Kossiakoff et al., 1993, 1994). These studies demonstrate extensive adaptability in the protein conformation that allows ligand binding to occur by induced fit mechanisms. It was shown that two receptor molecules could use the same molecular determinants to recognize different surfaces of h-GH. Thus the requirements for receptor−ligand recognition may be less well defined than for enzyme−substrate complex formation where catalysis has precise stereochemical requirements.

In the nAcChR, although the two primary sites appear to have identical equilibrium binding properties and similar dissociation kinetics, they cannot be truly identical. There is increasing evidence that these binding sites occur at subunit−subunit interfaces, specifically between $\alpha-\gamma$ and $\alpha-\delta$ interfaces [reviewed by Changeux et al. (1992); see also Dunn and Raftery (1997)]. In the nAcChR pentamer ($\alpha_2\beta\gamma\delta$) these interfaces can only be pseudosymmetric and not identical. The present finding of multiple subsites for AcCh binding suggests that there may be considerable flexibility in agonist recognition by the AcChR. In this respect, it is pertinent to note that there is considerable diversity in the structures of the agonists that are recognized, including acylcholines (e.g., AcCh, Carb, and SdCh) and compounds with varied ring structures (e.g., phenyltrimethylammonium, nicotine, and epibatidine). Although the charged nitrogen is certainly essential for binding, there appear to be few strict stereochemical requirements. This diversity of ligands that are recognized may be explained if, as suggested in a recent model study of the binding of charged cholinergic ligands, the quaternary ammonium group bonds via π-electron interactions to aromatic residues rather than to a fixed negative charge (Dougherty & Stauffer, 1990). If this is the dominant factor in the binding of agonists, then the directionality of binding of the residual parts of agonist molecules of differing structures will depend mainly on whether the protein provides amino acid side chains to dictate this. Determining whether or not the receptor provides such additional bonding interactions will surely depend on solving the structure at the atomic level.

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