BINDING OF [^{3}H] ACETYLCHOLINE TO ACETYLCHOLINE RECEPTOR FROM *TORPEDO CALIFORNICA* UNDER EQUILIBRIUM CONDITIONS: STOICHIOMETRY AND EVIDENCE FOR LONG-LIVED METASTABLE STATES.

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Introduction

The binding of acetylcholine (AcCh) by the acetylcholine receptor (AcChR) *in vivo* is the primary event that leads to the increased membrane permeability since it triggers the opening of transmembrane ion channels. A number of recent studies (see a recent review 1) suggest that this channel opening is associated with a conformational change of the AcChR as proposed by Nachmansohn as early as 1953(2). In *in vitro* experiments, however, prolonged exposure of the AcChR to AcCh or other agonists causes a gradual loss of the permeability response to agonists. This phenomenon is referred to as pharmacological desensitization. Recent biochemical studies with *Torpedo* membrane fragments suggest that while receptor activation is transient in nature and associated with lower-affinity for AcCh (K_d>1 μM) (3), desensitization is correlated with an increase in agonist affinity insofar as both of these latter phenomena follow qualitatively the same time course (4-9). Furthermore, evidence for a putative "pre-desensitized" state (10-11) is concurrent with more recent electrophysiological evidence that desensitization develops in two phases: an ini-

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tial fast phase (subsecond) followed by a slower phase which extends over tens of seconds to minutes (12-15). The recovery from desensitization was also found to display both a fast and a slow exponential phase with time constant of 12 sec and 4-5 min, regardless of the concentration or nature of the agonist. The fast and slow phases of desensitization onset and offset have been reported not to be due to independent causes but rather to be coupled (14). Thus the classical two-state cyclic scheme of desensitization scheme of Katz and Thesleff (15), would appear to require serious modifications. At present neither the mechanism of desensitization internally coupled to the complex receptor functional system nor its physiological significance in vivo is understood.

In binding studies under equilibrium conditions, where the receptor is exposed to the agonist for prolonged periods, the data represents the high-affinity binding properties of either a preexisting desensitized form or of AcChR "desensitized" through isomerization during the binding. In spite of recent advances (see recent reviews, 1, 17-20), there is still a great deal of disagreement about the equilibrium AcCh binding properties of isolated and even of membrane-bound AcChR. In direct binding studies of [^{3}H] AcCh with receptor-rich membrane fragments, overall equilibrium constants, $\bar{K}$ from $\sim$10 to 40 nM, either with positive cooperativity (20-24) or without cooperativity (25-27) have been reported. While some laboratories report one high-affinity AcCh binding site per α-toxin site (20-23, 26), others have found a ratio of 0.5 AcCh sites per α-toxin site (25, 27). In this paper we present evidence for the involvement of long-lived metastable states in the high-affinity binding of AcCh to the AcChR and we describe the effect of nonionic detergents on the apparent number of α-bungarotoxin (α-Bgtx) sites which may explain many of the aforementioned discrepancies (28).

Methods

AcChR-rich membrane fragments. The method for preparing membrane fragments from electric organ of Torpedo californica was essentially that of Sobel et al (29) and has been described elsewhere (28). The number of [^{125}I]α-Bgtx binding sites was determined by the DE-81 filter disk method as previously described (28, 30). Residual acetylcholinesterase activity of samples for [^{3}H]AcCh binding studies was inhibited as described in (28).

Modes of introducing [^{3}H]AcCh in binding studies. The following three modes of introducing [^{3}H]AcCh to receptor samples were used: Dialysis mode. [^{3}H]AcCh is introduced to the sample contained in a dialysis bag by slow diffusion through the membrane from a large pool of almost constant [^{3}H]AcCh concentration. From the point of view of the AcChR, the dialysis mode implies an open-system with respect to [^{3}H]AcCh. The true free concentration of [^{3}H]AcCh inside the dialysis bag at the end of the dialysis time was determined by the ultracentrifugation method (28). For binding studies with detergent solubilized receptor, the free concentration of [^{3}H]AcCh was determined from the counts in the filtrate derived from the dialysis bag content after 10 min centrifugation at 2000 g using the Centrifree (Amicon) micropartition system; Pulse -mode. Aliquots of [^{3}H]AcCh solution are directly added to aliquots of membrane fragments and rapidly mixed by vortexing. The pulse-mode implies a closed-system with respect to [^{3}H]-AcCh. The free ligand concentration was determined by the ultracentrifugation method with appropriate corrections for radioimpurities as described previously (28); Combination-mode. [^{3}H]AcCh is added to the membrane fragments by pulse-mode and this mixture is dialyzed against either a buffer containing the same free [^{3}H]AcCh concentration found in each sample or against plain buffer to yield a much lower final free [^{3}H]AcCh concentration.

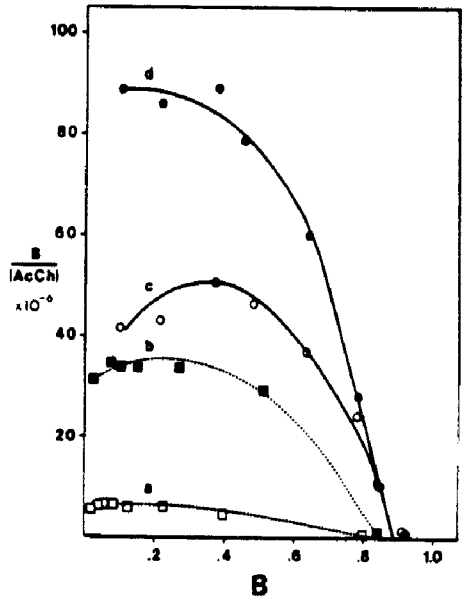

Figure 1. Scatchard representations demonstrating the time dependent increase in the affinity of membrane-bound receptor for AcCh and the lack of attainment of equilibrium in [^{3}H]AcCh equilibrium dialysis binding studies. Dialysis bags each containing receptor-rich membrane fragments (0.59 μM in α-Bgtx site) were subjected to equilibrium dialysis (two bags per flask) against varying [^{3}H]AcCh concentrations. One bag was removed after 3.5 hrs (---) and the other after 23 hrs (——) and AcCh , the free [^{3}H]AcCh concentration, and B, nmol of AcCh-bound per nmol of α-Bgtx binding sites, at these times was determined. a and c: the free ligand concentration was assumed to be equal to the concentration of [^{3}H]AcCh in the dialysis bath at the time of sampling. b and d: the free AcCh concentration was determined from the radioactivity in the supernatant after ultracentrifugation of the bag content.

Results and Discussion

Time dependence of [^{3}H]AcCh binding to AcChR in dialysis-mode.

When either plain buffer or α-Bgtx-blocked membrane bound or detergent solubilized AcChR solution is dialyzed against a [^{3}H] AcCh containing solution, >98% equilibration is achieved in less than 2 h, regardless of the initial [^{3}H]AcCh concentration. However, when [^{3}H]AcCh binding studies with the receptor-rich membrane fragments are carried out under the same equilibrium dialysis condition, the resulting binding curves vary with the dialysis time, as can be seen by comparing

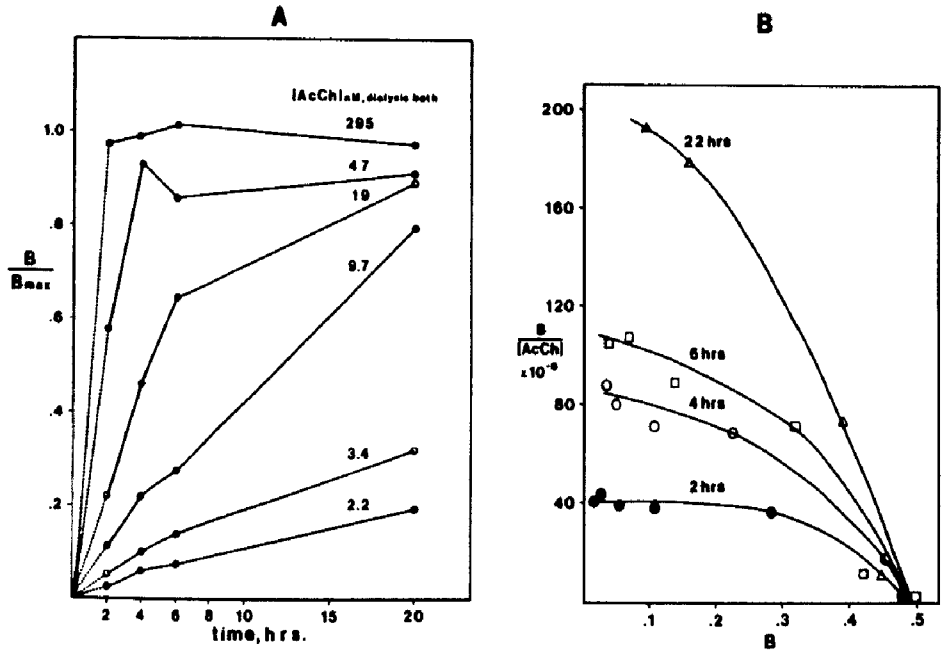

Figure 2. Time dependent AcCh affinity increase of detergent solubilized AcChR during equilibrium dialysis. Purified AcChR-rich membrane fragments (Band 3) were solubilized with 1% Lubrol WX and centrifuged. A. The fraction of AcCh binding sites occupied (B/B_{max}) at various AcCh concentrations (2.2 - 295 nM) in the dialysis bath, as a function of the dialysis time. The true free concentration of [^{3}H]AcCh inside the dialysis bag at the time of sampling was determined by centrifugation of the bag content at 2000 x g for 10 min using the Centrifree (Amicon) micropartition system. B. Scatchard representations generated from 2, 4,6 and 22 hr time points.

Figure 1b (3.5 h) and 1d (23 h). While the maximum value for AcCh bound, B, is the same for both dialysis times, there is a large increase in B values with time in the low [^{3}H]AcCh concentration range. It is apparent that in the low [^{3}H]AcCh concentration range equilibrium between the inside and outside of the dialysis bag is still not attained even after as long as 23 h. The two different Scatchard plots for both the 3.5 h (Figure 1a and b) and the 23 h (Figure 1c and d) time points differ because the actual free concentration of AcCh, determined by the ultracentrifugation method, is much lower than the concentration in the dialysis bath which is, in the equilibrium dialysis method, assumed to be equal to the free ligand concentration. Furthermore, the concentration of bound AcCh has been underestimated in the dialysis method since it

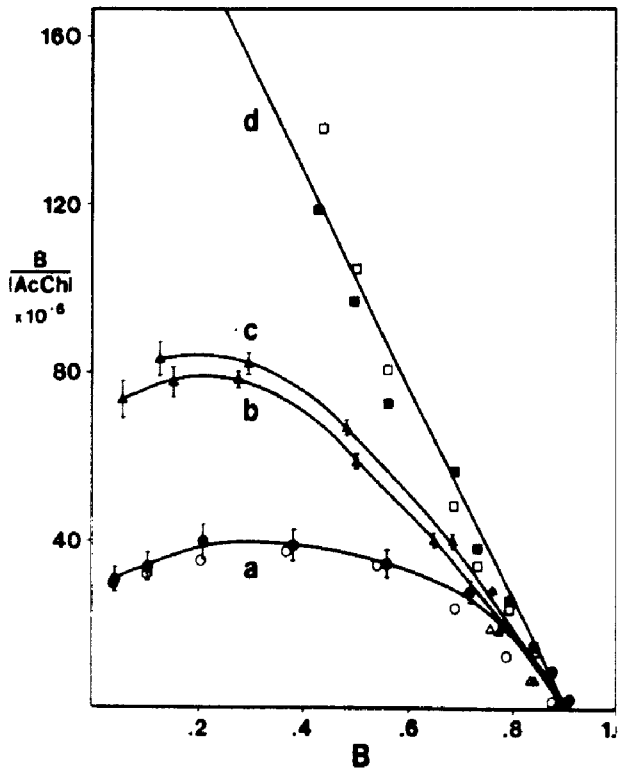

Figure 3. Scatchard representations of [^{3}H]AcCh binding to AcChR-rich membrane fragments at 4° C arising from different modes of AcCh addition. The same 0.2 mM DFP treated membrane preparation and the same final receptor concentration (0.48 μM in α-Bgtx binding sites assayed in the absence of detergent) was used in all of these experiments. The buffer composition was 10 mM PIPES (pH 7.0), 4 mM Ca^{++}, 0.5 mM EDTA, 100 mM NaCl, 0.8 mM Tetram. B, nmol of ligand bound per nmol of α-Bgtx binding sites. AcCh, molar concentration of free [^{3}H]AcCh at the time of sampling. a. ●——● A fixed amount of varying concentrations of [^{3}H]AcCh was added (pulse-mode) to aliquots of membrane fragment suspension preincubated with 0.1 mM Tetram, to give total [^{3}H]AcCh concentrations ranging from 15 nM - 1 μM. A portion of each sample was immediately centrifuged at 140,000 x g for 15 min and concentrations of bound and free [^{3}H]AcCh were determined from the radioactivity in 100 μL solution taken before and after ultracentrifugation with appropriate corrections for radiochemical impurities. Open circles (o), are data points from samples incubated for 17 hrs prior to ultracentrifugation. b. Δ——Δ An aliquot (550 μL) of each [^{3}H]AcCh-containing sample prepared in a was placed in a dialysis bag and dialyzed for 3 hrs against buffer containing the concentration of [^{3}H]AcCh determined in a to be the free [^{3}H]AcCh concentration. The content of each dialysis bag was then subjected to ultracentrifugation and the concentration of free and bound [^{3}H]AcCh was determined as in a. c. ▲——▲ Same as in b except that that the dialysis time was 17 hrs. d. ■——■ To aliquots of membrane suspension was added [^{3}H]AcCh to give concentrations ranging from 1 M to 20 μM. Each sample was dialyzed against plain buffer for 17 hrs to achieve final free concentrations in the 3 - 400 nM range. Free and bound [^{3}H]AcCh concentrations were determined from the counts before and after ultracentrifugation. This data yields $\bar{K}$ = 5 (± 1) x 10^{-9} M. □——□ Same as above except that the free [^{3}H]AcCh concentration was assumed to be equal to the [^{3}H]AcCh concentration of the dialysis buffer outside the bag after 17 hrs.

is inferred from the differences between the total and the free concentrations.

The time and concentration dependences observed in the binding of [^{3}H]AcCh with solubilized AcChR were qualitatively similar to that of membrane fragments and are presented in Figure 2. Although at high [^{3}H]AcCh concentrations maximum binding, ~0.5 AcCh per α-Bgtx site, has already been attained after 2 h, there is a large difference in binding between the 2 and 22 h points at lower concentrations. The fraction of bound AcCh at a given receptor concentration is a function of dialysis time and the rate of increase in $\underline{B}/\underline{B}_{max}$ to reach a time-independent value, presumably the equilibrium value, is faster at higher AcCh concentrations (Figure 2A). Below 10 nM AcCh concentration, the maximum value of $\underline{B}/\underline{B}_{max}$ has not been reached even after 22 h. Scatchard representations (Figure 2B) of the data clearly demonstrate that a slow progressive increase in the affinity of the solubilized AcChR for AcCh occurs with dialysis time and the curvature in the Scatchard plot suggests that true equilibrium binding data was not obtainable even after 22 h of dialysis.

Effects of different modes of introducing [^{3}H]AcCh on the binding of [^{3}H]AcCh to AcChR. Binding data of pulse-mode addition of [^{3}H]AcCh to freshly prepared receptor-rich Torpedo membrane fragments in the 2-200 nM free [^{3}H]AcCh concentration range, typically results in curved Scatchard plots, as shown in Figure 3a. Analysis of several similar experiments indicates that, if one assumes the binding to be in a state of equilibrium, the data may be characterized by $\bar{K}$ = 20 ± 10 nM and positive cooperativity (Hill coefficient, n_H = 1.7 ± 0.2), consistent with some of the earlier reports (see in 17, 24). Under these conditions no significant change in [^{3}H]AcCh binding was observed regardless of whether the ultracentrifugation of the incubation mixture was carried out within 30 min or after 24 h (Figure 3a, closed and open circles). The measured $\underline{B}$ values appear

to be time-independent. However, when the same incubation mixture was placed in a dialysis bag and subjected to the open-system condition afforded by dialysis, against buffer containing the same free [^{3}H]AcCh concentration found by the initial ultracentrifugation method, a continuous increase in binding as a function of time occurred, particularly in the low free [^{3}H]AcCh concentration range ([A] $<$ 0.1 μM), while the maximum value of B remained constant, as indicated by the abscissa intercept of Figures 3b and 3c. These results suggest that the population of AcChR in a higher affinity state is increasing during dialysis.

In another experiment equilibrium dialysis was carried out with all of the [^{3}H]AcCh usually introduced into the 50 mL of outside buffer solutions added instead to the 550 μL of membrane fragment samples, thus exposing the AcChR to a much higher concentration of [^{3}H]AcCh (1-20 μM). These samples were then individually dialyzed against 50-200 mL of plain buffer for 17 h to attain the final free [^{3}H]AcCh concentrations in the 3-400 nM range. The Scatchard plot generated by this experiment (Figure 3d) yielded a straight line with $\bar{K}$ = 5 ± 1 nM and no indication of positive cooperativity. Furthermore, the free [^{3}H]AcCh concentration determined by ultracentrifugation of the dialysis bag content and counting the radioactivity of the supernatant (solid triangles) was equal to the [^{3}H]AcCh concentration found in the outside dialysis solution (open triangles), indicating that a state of equilibrium had been attained under these conditions. These results show that, in addition to the time element, the transition to the higher-affinity state is facilitated by exposure to higher AcCh concentrations. This effect was found to be reversible since after exhaustive dialysis, membrane fragments preincubated with high concentrations of AcCh yielded essentially the same Scatchard curve as that derived from membrane fragments not previously exposed to AcCh. Therefore the intermediate curved Scatchard plots (Figure 3) obtained either by pulse-mode or

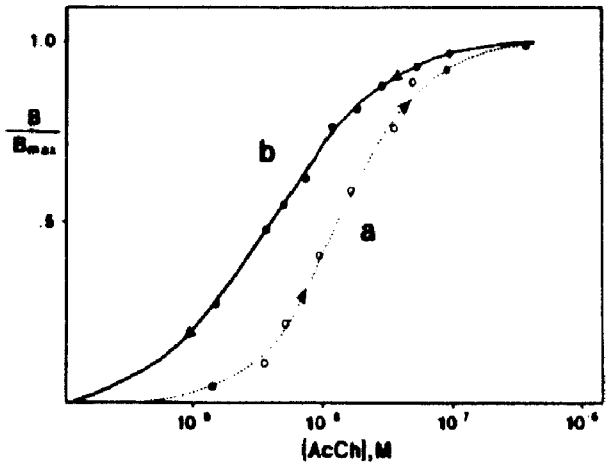

Figure 4. The degree of binding of [^{3}H]AcCh (B/B_{max}) at 4° C to Torpedo receptor-rich membrane fragments as a function of cyclic changes in the concentration of AcCh (logarithmic scale), showing hysteresis. a. o---o Binding curve obtained by "pulse mode addition" of [^{3}H]AcCh to the receptor, followed by ultracentrifugation to determine bound and free [^{3}H]AcCh concentrations (see Figure 3a); b. ●——● Binding data obtained by incubation of AcChR (0.48μM in α-Bgtx sites) with high AcCh concentrations (1 - 20 μM) followed by dialysis against initially AcCh free buffer to reach final free AcCh concentrations of 3 - 400 nM (see Figure 3d). The curve is a calculated line for $\bar{K} = 5 \times 10^{-9}$ M.

dialysis-mode AcCh addition cannot be analyzed in terms of overall equilibrium cooperativity since they appear to represent nonequilibrium distributions of rather long-lived, metastable high- and lower-affinity conformers and do not reflect equilibrium positive cooperativity, as previously believed (20-24).

Examination of our binding data indicates that when AcChR is exposed to cyclic changes of increasing and decreasing AcCh concentrations (see Figure 3a and 3d), a very pronounced hysteresis loop results, as shown in Figure 4. Since the binding of ligands acting as receptor antagonists, such as decamethonium, Bis-Q and dimethyltubocurarine, does not result in the unusual binding characteristics exhibited by AcCh (unpublished work), the hysteresis phenomenon appears to be related to a complex mechanism involving desensitization of AcChR by an

agonist. The hysteresis which results from the occurrence of long-lived metastable states (see a review 31) classifies the AcChR as a macromolecular memory system.

Stoichiometry of [^{125}I]α-Bgtx and [^{3}H]AcCh binding sites. During our investigation of the [^{3}H]AcCh and [^{125}I]α-Bgtx binding properties of acetylcholine-rich membrane fragments prepared from Torpedo electric organ in the presence of Ca^{++} and antiproteolytic agents, we made the following significant observations: preincubation of a given volume of these membrane fragments with 0.1% Triton X-100 or Lubrol WX results in a dramatic (up to 100%) increase in [^{125}I]α-Bgtx bound, relative to the same volume without detergent, and this increase is critically dependent on the particular density gradient fraction analyzed (Table I). In our recent experiments we employ a 42%, 37% and 30% sucrose step gradient for fractionating the crude membrane fragments. Toxin binding assays of these bands reveal that Bands 1 and 2 typically bind 50% to 70% more [^{125}I]α-Bgtx in the presence of detergent, and Band 3 consistently shows an even larger increase - often approaching 100% - under the same conditions, as shown in Table I.

There are at least two interpretations for this observation: contrary to other types of membrane preparation (32), a portion of these microsacs may be "outside-in" and therefore are not accessible to the toxin; or the detergent may loosen the membrane and expose a second sterically hindered α-Bgtx binding site or increase its affinity. Comparison of many [^{3}H]AcCh binding studies with membrane-bound versus detergent solubilized receptor and the fact that detergent treatment of membrane fragments does not increase the total number of AcCh binding sites, led us to favor the latter interpretation. The smaller detergent effect (∿50% increase) observed in membrane-bound receptor from the upper band (Band 1) taken from sucrose gradients may be attributable to loosening of the membrane through proteolysis, resulting in exposure of some of the second α-Bgtx bind-

Table I Characterization of a representative preparation of AcChR-rich membrane fragments isolated by discontinuous sucrose density gradient centrifugation.

Membrane fragments[a]	Band 1	Band 2	Band 3	Band 3 DFP treated
Location, % sucrose[a]	0/30	30/37	37/42	37/42
Volume, mL	20	16	16	10
α-Bgtx bound[b] no detergent, nmol/mL	1.36	1.02	1.52	2.30
α-Bgtx bound[b] in 0.1% Triton nmol/ml	2.08	1.70	2.96	4.60
% increase in α-Bgtx[b] bound with detergent	53	67	95	100
Specific activity[c] nmol/mg	0.8(1.2)	1.1(1.8)	1.3(2.3)	1.4(2.8)
AcChE activity[d] Units/nmol α-Bgtx site	33	14	5	0.03

a AcCh-rich membrane fragments were prepared from 90 g of electric organ of Torpedo californica. Three opaque bands were collected from the region of the interfaces of discontinuous sucrose density gradients.

b Nanomoles of [^{125}I]α-Bgtx bound, determined by DE-81 filter disc assays, in the absence and present of 0.1% Triton X-100.

c Specific activity: apparent moles of α-Bgtx binding sites determined in the absence or presence of 0.1% Triton X-100 (values in parentheses) per mg of protein.

d 1 enzyme activity unit is defined as 1 μmol of AcCh hydrolyzed per min. The activity in membrane fragments is expressed as units per mole of [^{125}I]α-Bgtx sites assayed in the presence of 0.1% Triton X-100.

ing sites. It should be recalled that while the kinetics of binding of α-Bgtx to Torpedo membrane-bound receptor is monophasic (20, 32-34), that for solubilized receptor is biphasic (34), suggesting that either the affinity of one of the two α-Bgtx sites on the receptor in membrane is strongly altered by solubilization (34), or that a sterically hindered second site becomes available to α-Bgtx upon detergent treatment.

Our [^{3}H]AcCh binding studies with membrane-bound receptor, using carefully standardized [^{3}H]AcCh (28), showed the stoichiometry of AcCh to α-Bgtx sites to be close to 1. This ratio becomes about 0.5 if the number of α-Bgtx sites determined in the presence of detergent is used, in agreement with the ratio obtained with our solubilized and purified AcChR. Therfore we conclude that the stoichiometry of the high-affinity AcCh binding site is 1 AcCh per receptor monomer (i.e., per M_r 290,000) or per two α-Bgtx sites assayed in the presence of detergent). It is pertinent to note that the investigators who have reported the stoichiometry of AcCh to α-Bgtx sites in membrane fragments to be 1 have assayed for toxin binding in the absence of detergent (20, 23, 26, 33), while those who have reported it to be 0.5 appear to have used 0.1% Triton X-100 (25, 35).

It may very well be that there are two widely different binding sites for AcCh and other cholinergic ligands per receptor monomer (i.e., per two α-Bgtx sites) (36, 37), and that only one of these can exhibit high-affinity for AcCh when binding studies are performed under the conditions described here. It should be recalled that the nonequivalent nature of the two α-toxin binding sites of the receptor has been demonstrated by showing that after disulfide reduction, only one of theα-toxin sites is affinity alkylatable by a low concentration of 4-(N-maleimido)benzyltrimethylammonium (MBTA) or bromoacetylcholine (36, 38-40).

Proposed reaction scheme. In Figure 5 we present a reaction scheme designed to rationalize the two main puzzling questions

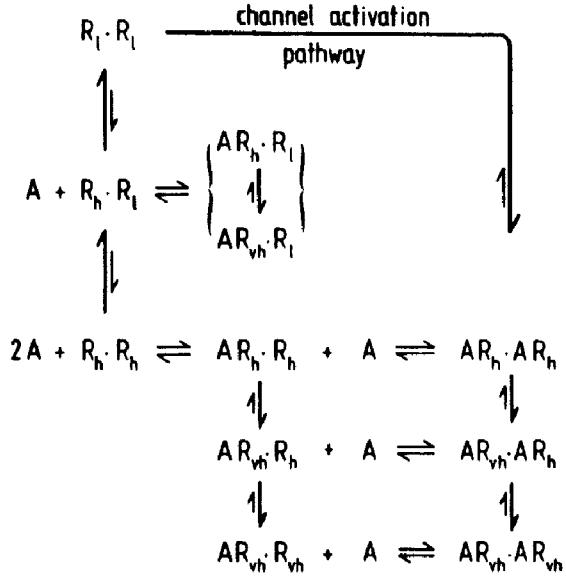

Figure 5. Proposed reaction scheme of AcCh binding to AcChR to account for the difference in AcCh-binding profile resulting from "single pulse mode addition" (closed-system with respect to AcCh) and "dialysis mode addition" (open-system). The simple bimolecular processes are represented by the horizontal sequences, whereas the vertical steps model the various slow structural isomerizations. R_l: low affinity conformer, (10^{-6} M<$\bar{K}$< 10^{-4} M); R_h: high affinity conformer, (10^{-8} M<$\bar{K}$<10^{-6} M); R_{vh}: very high affinity conformer, ($\bar{K} \sim 5$ (±1) x 10^{-9}M); $R_h \cdot R_l$: hybrid form of the two binding sites. The thick arrows indicate the preferential position of the isomerization equilibria. Large upper arrow indicates the channel activation pathway. A high AcCh concentrations (10^{-6} M), the low affinity conformer, R_l, is directly involved in the binding which results in channel opening and is subsequently transformed to its high affinity state. At low AcCh concentrations (10^{-6} M), the R_h and R_{vh} conformers are the dominant direct reaction partner for AcCh and thus constitute the direct route of the high affinity state pathway of the scheme. "Single pulse mode addition" of AcCh (closed system) favors the $AR_h \cdot R_l$ and $AR_{vh} \cdot R_l$ binding states, whereas "dialysis mode addition" (open system) ultimately leads to the $AR_{vh} \cdot AR_{vh}$ conformer.

raised by our AcCh binding experiments: 1) why AcChR is so slow in reaching an equilibrium state in AcCh binding during the dialysis-mode? 2) why pulse-mode AcCh binding results in less AcCh bound than the dialysis-mode and appears to be time-independent? In an attempt to describe the dialysis-mode binding data, a reaction model must account for the observed inequality of free [^{3}H]AcCh concentration, $[A_f]$, $[A_f]_{in} < [A_f]_{out}$, which develops between the inside and the outside of the dialysis bag (see Table I) even when the initial condi-

tions are $[A_f]_{in} = [A_f]_{out}$. The inequality can arise from the additional binding of AcCh to higher-affinity states of the AcChR, generated by conversion of unoccupied low affinity sites to high affinity sites, or unoccupied high affinity to still higher affinity sites. Such newly created high affinity sites would now bind a part of the remaining AcCh such that the free AcCh concentration inside the dialysis bag decreases below the initial value. In this situation the AcCh flux through the dialysis membrane is rate-limiting for the overall rate of net AcCh binding to AcChR. When the most stable state R_{Vh}, associated with the individual equilibrium constant $K_{Vh} \approx 10^{-9}$ M ($K_{Vh} < \bar{K} = 5$ nM), is the final very high affinity conformer, the conformers R_h must have K_h values which are larger than 10^{-9} M, perhaps $\sim 10^{-7}$ M (see 3). Hence at final equilibrium the concentrations of the intermediate AR_h state may be negligible compared to that of AR_{Vh}. Since our data suggest that cooperative interactions between at least two AcCh binding sites exist and given the stoichiometry of one AcCh binding site per monomer (9S), the receptor dimer (13S), R·R, is required for the smallest cooperative element in the scheme.

When the AcCh concentration is increased more rapidly (pulse-mode) than in the dialysis-mode of AcCh binding, rather long-lived nonequilibrium distribution of AcChR states with fewer occupied AcCh binding sites are maintained. Because these metastable nonequilibrium distributions depend on the rate of AcCh increase they must result from rate processes. In any case, the pulse-mode data suggest that the overall transitions from $R_l \cdot R_l$ to more high-affinity conformers can be "interrupted". On an elementary scale the overall equilibrium $R_lR_l \rightleftharpoons R_h \cdot R_h$ very likely involves the intermediate form $R_h \cdot R_l$. This hybrid $R_h \cdot R_l$, as a part of the sequence, $R_l \cdot R_l \rightleftharpoons R_h \cdot R_l \rightleftharpoons R_h \cdot R_l$ represents a suitable branching point where different rate processes can occur according to the scheme:

$$
\begin{array}{ccccccc}
 & & R_l \cdot R_l & & & & \\
 & & \text{slow} \uparrow\downarrow & & & & \\
A & + & R_h \cdot R_l & \overset{\text{rapid}}{\rightleftharpoons} & AR_h \cdot R_l & \rightleftharpoons & AR_{vh} \cdot R_l \\
 & & \text{slow} \uparrow\downarrow & & & & \\
 & & R_h \cdot R_h & & & &
\end{array}
$$

In the above reaction scheme the binding of AcCh to the conformer R_h of the hybrid $R_h \cdot R_l$ conserves the adjacent low affinity state. The longevity of this low affinity conservation suggests that, in addition to simple AcCh binding, structural transitions to a complex with higher life-times, $AR_{vh} \cdot R_l$, may also be involved. In the pulse-mode, the binding of AcCh to the hybrid $A + R_h \cdot R_l \rightleftharpoons AR_h \cdot R_l$, initiated by a bimolecular step, appears to be faster than the intramolecular transition $R_h \cdot R_l \rightleftharpoons R_h \cdot R_h$. Furthermore, under closed system conditions with respect to AcCh the conversion to the final R_{vh} conformation may be extremely slow. Thus under such conditions of fixed total AcCh concentration, nonequilibrium distributions with less AcCh bound states may be maintained for a very long time. The low affinity conservation pathway can therefore be considered as a frozen-in or deadend pathway.

Subsequent transfer of these metastable nonequilibrium distributions to the open system condition afforded by dialysis apparently enhances the coupling of the hybrid to the intramolecular step $R_h \cdot R_l \rightleftharpoons R_h \cdot R_h$ of the reaction scheme (see Figure 3b, c). The reaction steps $2A + R_h \cdot R_h \rightleftharpoons AR_{vh} \cdot R_{vh} + A \rightleftharpoons AR_{vh} \cdot AR_{vh}$, apparently give rise to larger driving forces to diverge the complexes from the low-affinity conservation pathway and induce a maximum shift to the side of the high-affinity complexes.

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