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FUNCTIONAL PROPERTIES OF ACETYLCHOLINE RECEPTOR MONOMERIC AND DIMERIC FORMS IN RECONSTITUTED MEMBRANES

Wilson C.-S. Wu† and Michael A. Raftery

Church Laboratory of Chemical Biology
Division of Chemistry and Chemical Engineering
California Institute of Technology
Pasadena, California 91125

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SUMMARY: The dimeric and monomeric forms of the acetylcholine receptor from Torpedo californica electroplax have been purified in the presence of lipids and reconstituted. A spectroscopic method was applied to study the rapid kinetics of cation transport mediated by each of the reconstituted AcChR oligomers. Both the AcChR dimer and monomer responded to carbamylcholine by mediating cation transport on the time scale of a few milliseconds. The responses to carbamylcholine were blocked by histrionicotoxin and by desensitization, demonstrating that both forms manifest pharmacological properties observed in vivo. Analysis of the fast ion transport produced by various agonist concentrations yielded estimated rates of transport through a single receptor channel. These were comparable for the monomer and dimer and in agreement with those obtained for a preparation containing a mixture of both oligomers.

INTRODUCTION

The acetylcholine receptor from Torpedo californica electroplax occurs as a 9S monomer and a 13.7S dimer following extraction into detergent solutions (1). The monomer is a pentamer (2,3) consisting of two 40,000 and one of each of 50,000, 60,000 and 65,000 dalton polypeptides while the dimer results from a disulfide linkage(s) between the 65,000 daltons subunits (4-7). A number of reports of reconstitution experiments have appeared recently (8-14) in which it was

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† PRESENT ADDRESS: Dept. of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06510

ABBREVIATIONS: AcChR, acetylcholine receptor; ANTS, 8-amino-1,3,6-naphthalenetrisulfonate; α-BuTx, α-bungarotoxin; Carb, carbamylcholine; HTX, histrionicotoxin.

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demonstrated that purified AcChR consisting of a mixture of monomers and dimers is functional in $^{22}Na^{+}$ transport on the time scale of seconds. In one reconstituted membrane system T. californica AcChR has been shown (15) to closely resemble the physiologically active receptor in terms of both the rapidity of cation translocation (millisecond time scale) and its quantitation. It is not known whether or not both of the AcChR oligomers are functional at this level. Preliminary reconstitution efforts (16) provided indications that both forms were active in $^{22}Na^{+}$ flux experiments, which studied transport on the time scale of seconds, providing the amplitude of the ion translocation while yielding no information concerning its rate. A final flux amplitude of 70 cations per channel was obtained from this study.

In the work reported here, we studied the rapid kinetics of ion transport mediated by purified AcChR dimer and monomer that had been reconstituted into separate preparations of membrane vesicles, utilizing a rapid spectroscopic method (19,15). We report that quantitative analysis of the kinetics yielded rates of ion translocation mediated by reconstituted AcChR dimer and monomer that are comparable to each other, as well as to those obtained previously for a preparation containing a mixture of both oligomers (15) and to the AcChR in native membranes (19).

MATERIALS AND METHODS

Purification and Reconstitution of AcChR Dimer and Monomer:

Highly purified AcChR-containing membranes were prepared as described earlier (17,18). Purified membranes at 2 mg protein/ml were solubilized by stirring at 4°C for 15 min in 10 mM Na Hepes, pH 7.4 and 200 mM $NaNO_3$ (termed Na Buffer) containing 2% Na cholate (w/v) and 4 mg/ml sonicated asolectin (8). Non-solubilized material was removed by centrifugation at 40,000 rpm in a Beckman 65 rotor for 1 hr. Approximately 8-12 mg of the solubilized AcChR (in 4 ml) were incubated with 50 ng of [^{125}I] α-BuTx (~40,000 cpm) at 0°C for 30 min. The sample was then layered on a 6.5 - 22% sucrose gradient prepared in Na Buffer containing 4 mg/ml sonicated asolectin. The gradient (~36 ml) was centrifuged in a Beckman VTi 50 vertical rotor at 46,000 rpm for 4 hr. Fractions of 35 drops (~1 ml) were collected and counted for [^{125}I] in a Beckman gamma counter. Peak fractions corresponding to AcChR dimer and monomer were pooled. Sonicated asolectin which had been solubilized in 2% Na cholate was added to each of the two purified AcChR oligomers to reach a final asolectin concentration of 14 mg/ml. Detergent was removed by dialysis against 1000 volumes of Na Buffer at room temperature for 20 hr.

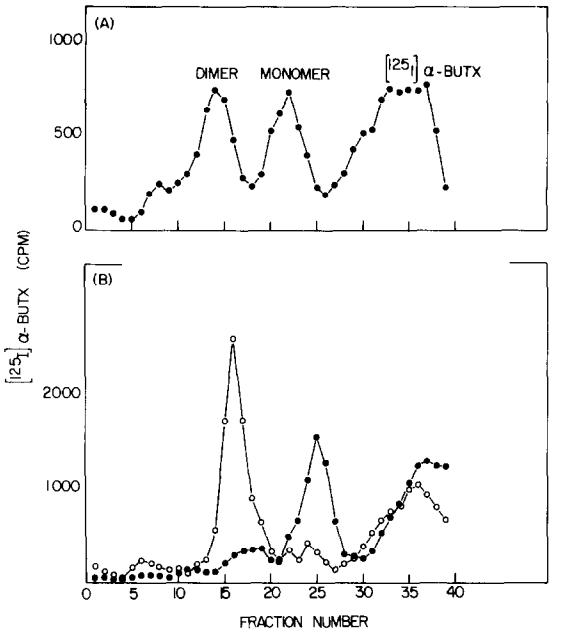


FIGURE 1: [^{125}I] α-BuTx Binding Profiles of Cholate-solubilized AcChR Membranes Centrifuged Through Sucrose Gradients:
(A) Purified native membranes and (B) purified dimer (○) and monomer (●) that had been reconstituted into membrane vesicles were solubilized and centrifuged through 6.5 - 22% sucrose gradients in a Beckman VTi 50 vertical rotor as described in Methods.

Loading of ANTS within Reconstituted Vesicles:

The fluorophore (ANTS) molecule was loaded into vesicles by the freeze-thaw procedure previously described (15).

Stopped-Flow Study of Tl^+ Flux into Reconstituted Vesicles:

Measurement of Tl^+ transport was performed in a stopped-flow instrument as described (19). Tl^+ flux was initiated by rapid mixing (machine dead time of ~3 msec) at 25°C of a reconstituted vesicle suspension with an equal volume of 28 mM $TlNO_3$ in 10 mM Na Hepes, pH 7.4 and 172 mM $NaNO_3$ (termed $TlNO_3$ Buffer) either lacking or containing Carb. Fluorescence emission was monitored, recorded and analyzed as reported (19).

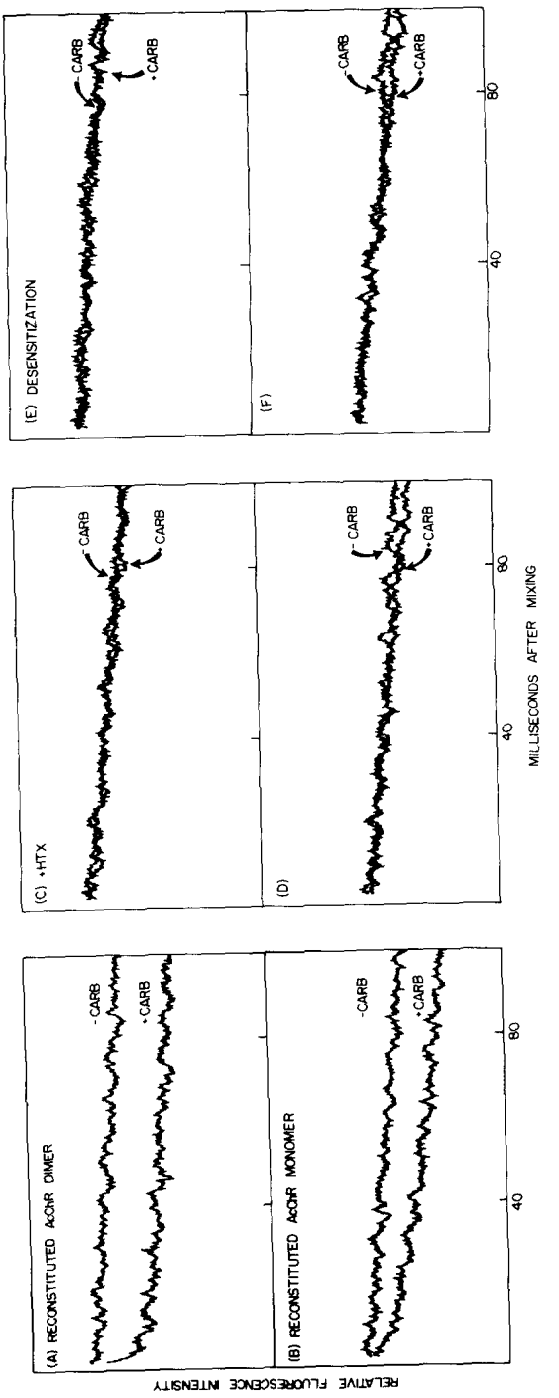
RESULTS AND DISCUSSION

Using the purification conditions described here solubilized AcChR dimers and monomers are obtained in approximately equal amounts (Figure 1A) and in quantities sufficient for reconstitution into membrane vesicles. Each receptor oligomer appeared to be reasonably stable in reconstituted membranes in terms of spontaneous conversion to the other oligomeric form since the α-BuTx binding pro-

files in Figure 1B reveal the predominant occurrence of the expected AcChR oligomer in the reconstituted membranes derived from each of the two purified oligomeric forms. In contrast to membrane-bound preparations, AcChR dimer in detergent solution was found to be significantly less stable; more than 50% of such purified dimers spontaneously converted to monomers over a similar period of time (2-3 days).

Transport of Tl^+ into reconstituted vesicles containing purified AcChR dimer or monomer was studied by measuring Tl^+-induced quenching of fluorescence of ANTS entrapped in the vesicle interior (19,15). As observed previously for reconstituted AcChR membrane vesicles (15), a spontaneous fluoroscence decay, representing leakage of Tl^+, was recorded when vesicles entrapping ANTS were mixed with Tl^+ in a stopped-flow instrument. The carbamylcholine-activated influx of Tl^+ into membrane vesicles yielded a marked acceleration of fluorescence decay. The fluorescence traces in Figures 2A and 2B (lower traces in each) show the response to 1 mM Carb by ANTS-reconstituted vesicles containing purified AcChR dimer and monomer, respectively. For the preparation containing dimer, the rate of fluorescence decay was accelerated from a half time of 600 msec recorded in the absence of Carb to 6 msec when 1 mM Carb was introduced. The same concentration of Carb accelerated the fluorescence decay mediated by AcChR monomer from a 500 msec half time to 19 msec. The Carb-induced response by both reconstituted preparations was blocked by 10 μM HTX (Figures 2C and 2D), a toxin reported to possibly interact with AcChR-associated ion channels (20,21). Desensitization of each reconstituted AcChR oligomer induced by preincubation with 100 μM Carb was also shown to abolish the rapid fluorescence decay (Figures 2E and 2F) produced by 1 mM Carb. These results demonstrate that both the AcChR dimer and monomer reconstituted into membranes are each capable of binding HTX and undergoing agonist-induced desensitization to manifest the specific pharmacological blockage of cation transport shown previously for native preparations (22,23).

To achieve a quantitative comparison of the capability of both AcChR forms to translocate cations, the rates of ion transport mediated by each reconstituted


(A) RECONSTITUTED AcChR DIMER
-CARB
+CARB
(B) RECONSTITUTED AcChR MONOMER
-CARB
+CARB
(C) +HTX
-CARB
+CARB
(D)
-CARB
+CARB
(E) DESENSITIZATION
-CARB
+CARB
(F)
-CARB
+CARB
40
80
MILLISECONDS AFTER MIXING
RELATIVE FLUORESCENCE INTENSITY

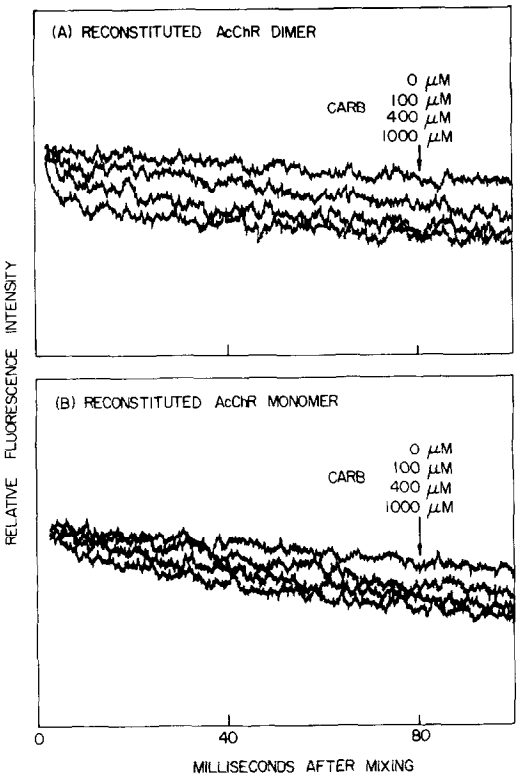


FIGURE 3: Dependence of the Rate of Tl^+ Transport on Carb Concentration: Fluorescence traces showing the kinetics of Tl^+ influx across reconstituted membranes containing purified AcChR dimers (A) and monomers (B) with various agonist concentrations. Fluorescence traces shown in descending order were recorded for Tl^+ flux activated by 0, 100, 400 and 1000 μM Carb.

preparation were determined for various concentrations of the agonist. Figures 3A and 3B show that increasing the concentration of Carb produced fluorescence

FIGURE 2: Carbamylcholine-Induced Influx of Tl^+ Across Reconstituted Membranes Containing Purified AcChR Dimer or Monomer: Reconstituted membrane vesicles containing (A) purified dimer or (B) purified monomer and entrapping ANTS (Syringe 1) were rapidly mixed at time = 0 in a stopped-flow instrument with $TlNO_3$-Buffer (see Materials and Methods) containing no (upper trace in each) or 2 mM (lower trace in each) Carb (Syringe 2) and the fluorescence was recorded over a period of 100 msec. Since equal volumes of membranes and Carb solutions were mixed, the final concentration of agonist was half of that initially present in Syringe 2. (C) Purified dimers and (D) purified monomers in reconstituted membrane vesicles loaded with ANTS were preincubated with 10 μM HTX for 5 min at 25°C (Syringe 1) and were rapidly mixed with 10 μM HTX in $TlNO_3$-Buffer (Syringe 2) containing no or 2 mM Carb.
(E) Purified dimers and (F) purified monomers in reconstituted membrane vesicles entrapping ANTS were preincubated with 100 μM Carb at 25°C for 30 min (Syringe 1) and were rapidly mixed with $TlNO_3$-Buffer (Syringe 2) containing no or 2 mM Carb.

TABLE I

COMPARISON OF ION FLUX RATES AT VARIOUS CARBAMYLCHOLINE CONCENTRATIONS

	k_{app} (SEC^{-1})[1]		CATIONS/SEC/CHANNEL[2]	
[CARB]	DIMER	MONOMER	DIMER	MONOMER
100 μM	4.1	3.1	1.69×10^4	2.22×10^4
250 μM	15.5	8.6	6.40×10^4	6.15×10^4
400 μM	47.9	9.6	1.97×10^5	6.86×10^4
750 μM	77.0	26.7	3.17×10^5	1.91×10^5
1000 μM	112.0	35.7	4.61×10^5	2.57×10^5

1. The apparent flux rates (k_{app}) were determined by computer averaging 5-6 fluorescence traces for each Carb concentration and fitting each averaged trace to Equation 1 (19), which described fluorescence decay as a function of time.

2. The number of cations transported per second by each activated receptor channel was estimated by obtaining the apparent cation transport rate across a single vesicle for each Carb concentration and dividing it by the average number of AcChR molecules per vesicle as described previously (15). For reconstituted AcChR dimer there were on the average 2.05 channels per receptor-containing vesicle and for reconstituted AcChR monomer there were 1.18 channels per vesicle (assuming 1 channel per 2 α-BuTx sites). The final concentrations of α-BuTx sites in the ANTS vesicle preparations were 8×10^{-8}M for dimer and 5.1×10^{-8}M for monomer. The phospholipid concentrations were 2.6×10^{-3}M and 2.15×10^{-3}M for dimer and monomer, respectively. Assays of α-BuTx sites and phospholipids were determined as in earlier experiments (15).

traces with higher decay rates. The left-hand column of Table 1 shows the apparent flux rate constants (k_{app}) obtained for activation up to 1 mM Carb. It is clear that the concentrations used are below saturating levels for both preparations, in agreement with the previous observation for reconstituted (15, containing a mixture of oligomers) and native (19) AcChR membranes. Using the k_{app}, the number of cations that were transported per sec by a single activated AcChR channel was estimated for each agonist concentration. The transport numbers for both the dimer and monomer are within a factor of 2 of each other, as shown on the right-hand column of Table 1. The transport numbers for the individual oligomers are approximately within a factor of 2 of those obtained for reconstituted AcChR consisting of a mixture of both oligomers. The numbers calculated here are substantially higher (by 1-2 orders of magnitude) than that estimated from $^{22}Na^+$ flux assay (16) Assuming a dose response behavior similar to the preparation of

mixed oligomers (15), the maximal transport rate for the purified AcChR dimer would be slightly more than 10^6 when extrapolated to saturating Carb concentrations. These results thus provide the quantitative evidence necessary to demonstrate that both the dimeric and the monomeric forms of AcChR are fully functional in terms of mediating the very fast ion transport known to occur for an AcChR in vivo.

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