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CARBAMYLCHOLINE-INDUCED RAPID CATION EFFLUX FROM RECONSTITUTED MEMBRANE VESICLES CONTAINING PURIFIED ACETYLCHOLINE RECEPTOR[1]

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ABSTRACT

Membrane preparations containing essentially only the four polypeptides considered to constitute the acetylcholine receptor are purified from Torpedo californica electroplax. Treatment of these membranes with 2% (w/v) aqueous sodium cholate followed by removal of all insoluble matter results in a solubilized purified receptor preparation that can be reassociated with phospholipids during dialysis to remove the detergent. Such reconstituted receptor is shown to retain the capability of translocating $^{22}Na^{+}$ across the membrane in response to carbamylcholine binding in a highly reproducible manner. The dose response for this effect is similar to that observed for the original electroplax membrane preparation and the carbamylcholine induced signal is completely blocked by α-bungarotoxin.

INTRODUCTION

Following successful isolation and purification in large amounts of nicotinic AcChR[3] from electroplax tissue from sources such as Narcine (1-3), Torpedo californica (4-9), Torpedo marmorata (10-13, 3) and Electrophorus electricus (14-21) broadly based efforts to elucidate its molecular properties have been underway. The most extensive studies in vitro have thus far been carried out on membrane fractions highly enriched in AcChR derived from Torpedo (22-25) or Electrophorus (26, 27) which have been shown to retain

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[3] Abbreviations used: AcChR, acetylcholine receptor; α-BuTx, α-bungarotoxin; Carb, carbamylcholine; HTX, histrionicotoxin; Tris, tris(hydroxymethyl)-aminomethane.

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many properties that parallel those delineated by electrophysiological studies in vivo (28). Considerable knowledge has been accumulated concerning the interactions of AcChR enriched membranes with a wide variety of cholinergic ligands, local anesthetics and other agents such as histrionicotoxin (28).

The next level of biochemical characterization of the structure-function relationships of synaptic membrane proteins is the study of agonist induced cation flux across membranes reconstituted from purified protein components and phospholipids. In this manner it should be possible to define the membrane components necessary for both agonist binding and consequent cation translocation. In the past, reconstitution has been reported for membranes enriched in AcChR but also containing other polypeptide components (29-30). With AcChR purified by affinity chromatography from Torpedo we have reported (31, 32) that some preparations yielded reconstituted membrane vesicles that responded to the agonist Carb with an increased flux of $^{22}Na^{+}$ over background and that this effect could be blocked by α-BuTx; failure to detect this response has also been reported (33-35).

We present here a preliminary account of a highly reproducible rapid cation flux induced in membrane vesicles reconstituted from purified AcChR upon interaction of the system with the cholinergic agonist Carb. The system is shown to have a dose response similar to the electroplax membrane vesicles from which the purified and reconstituted AcChR was derived and to exhibit pharmacology expected for an in vitro model of a nicotinic cholinergic synapse.

MATERIALS AND METHODS

Preparation of Membrane Fragments:

Purified membranes enriched in AcChR (23, 24) were prepared using sucrose step gradients in a Beckman VTi 50 vertical rotor (36). Membranes fractions recovered from the middle band of the gradients were pooled and centrifuged for 1 hour at 30,000 rpm in a Beckman Type 35 rotor following a two-fold dilution into 10 mM Tris-Cl, pH 7.4. The membranes were resuspended with a Virtis-23 homogenizer in the same buffer and assayed for protein concentration and [^{125}I] α-BuTx binding sites (37). The specific activity of a typical membrane preparation was 1.0-1.25 nmol α-BuTx sites per mg protein.

Base Treatment:

Purified AcChR enriched membranes containing 10-15 mg of protein per ml were diluted ten-fold into ice-cold distilled water and the pH was adjusted to 11.0 with 0.2 N NaOH (38, 39). The membranes were stirred at 4°C for 1 hour and then centrifuged at 18,000 rpm in a Sorvall SS-34 rotor for 1 hour. The supernatants and a light particulate fraction ("soft pellet") sedimenting on top of the membrane pellets were removed and saved for gel analyses. The membrane pellets were resuspended in ice-cold distilled water and the pH treatment and centrifugation steps were repeated. The final membrane pellets were resuspended in 10 mM Tris-HCl, pH 7.4. The specific activity of a typical alkaline treated membrane preparation was 2.5-3.2 nmol α-BuTx sites per mg protein.

Sonication of Phospholipids:

Phospholipids were prepared for reconstitution by suspending asolectin (Associated Concentrates, Woodside, New York) in H_2O to 100 mg/ml. The suspension was sonicated under N_2 in a bath type sonicator at 4°C. The pH was adjusted to 7.4 with 0.2 N NaOH after 15 minutes and sonication was continued until the lipid suspension reached clarity.

Solubilization and Reconstitution of AcChR:

A mixture of appropriate concentrations of sonicated asolectin, cholate, NaCl and Tris-HCl, pH 7.4 was stirred at room temperature for 30 min. The alkaline treated membrane suspension containing 1.3 mg of protein was then added to this mixture to a final volume of 1.5 ml and final concentration of 20 mg/ml asolectin, 2% (w/v) cholate, 200 mM NaCl and 10 mM Tris-HCl, pH 7.4. After stirring at 4°C for 30 min., the sample was centrifuged in a Beckman type 65 rotor at 40,000 rpm (160,000 xg) for 1 hour. The supernatant was removed and dialyzed against 3 liters of 200 mM NaCl in 10 mM Tris-HCl, pH 7.4 for 20 hours.

^{22}Na Flux Assay:

For the measurement of agonist-induced cation efflux, the reconstituted preparations (10-17 μM in α-BuTx sites) were incubated with 0.87 μM $^{22}NaCl$ (Amersham, 21.1 Ci/m mole) for 12 hours at 4°C to allow equilibration of ^{22}Na inside and outside the vesicles. The flux assay was started at time zero by diluting the radioactive vesicle suspension 20-fold into nonradioactive Dilution Buffer with or without Carb at room temperature. (The Dilution Buffer consisted of 200 mM NaCl in 10 mM Tris-HCl, pH 7.4.) The mixture was immediately vortexed and at time intervals of 10 sec. 200 μl aliquots were transferred with an Eppendorf pipet onto three layers of DEAE-cellulose discs (Whatman DE81) which had been presoaked in the Dilution Buffer and mounted on a Millipore manifold apparatus. The filters were immediately washed with 2 x 7.5 mls of nonradioactive Dilution Buffer and counted in a Beckman gamma counter with the counting windows optimized for $^{22}Na^+$.

Gel Electrophoresis:

8.75% polyacrylamide gels (40) were run in 0.1% SDS and were stained for protein with 0.05% (w/v) Coomassie Brilliant Blue in 10% (v/v) acetic acid, 25% (v/v) methanol and destained in the same solution without the dye. Gel strips cut from slabs were scanned at 550 nm using a Gilford 240 spectrophotometer equipped with a linear-transport accessory.

RESULTS

Alkali extraction (41) of purified acetylcholine receptor membranes (38, 39) resulted in a preparation highly enriched in the four receptor subunits (Figure 1B) of M_r 40, 50, 60 and 65 x 10^3 daltons (6-8). The treated mem-

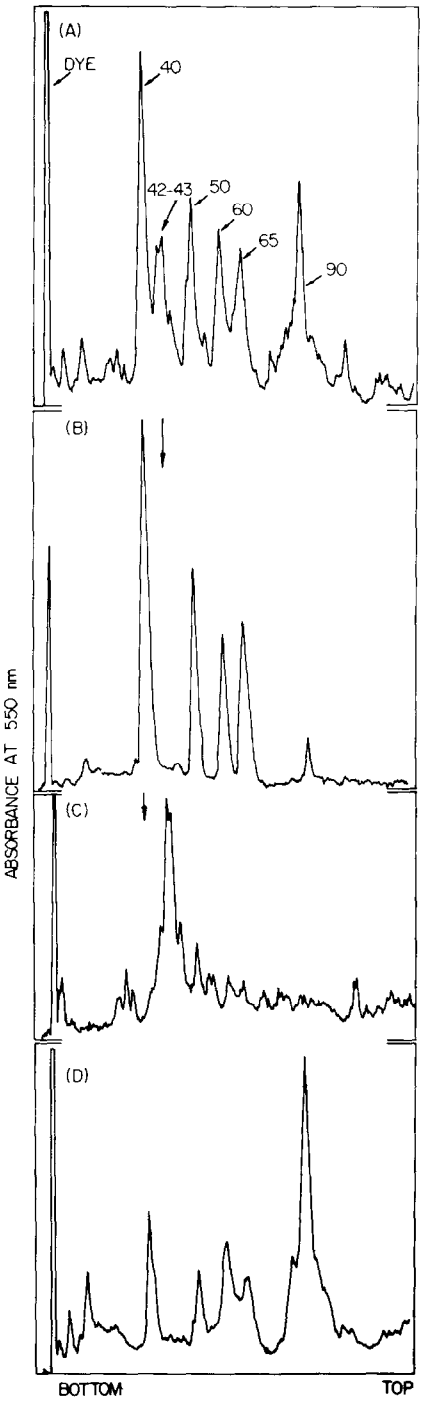

FIGURE 1: Densitometer scans of Coomassie Brilliant Blue-stained SDS gels of membranes before (A) and after (B) 2X treatment at pH 11. These alkaline treated membranes were used in the reconstitution studies. (C) and (D) are scans of gels of polypeptides recovered in the supernatant and "soft pellet," respectively.

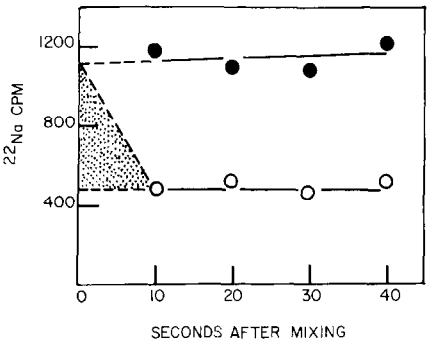

FIGURE 2: Carb-induced $^{22}Na^+$ efflux from membrane vesicles following treatment at pH 11. Membranes preequilibrated with $^{22}Na^+$ were diluted at time zero into buffer (10 mM Tris-HCl, pH 7.4) containing 100 μM (○) or 0 μM (●) Carb. Time points were taken as described in Methods for the flux assay of the reconstituted preparation.

branes were essentially devoid of the 43 x 10^3 dalton polypeptide (13) (Figure 1C) as well as most other minor polypeptide contaminants. The alkali treatment also removed more than 90% of the 90 x 10^3 dalton species, which was recovered in a light particulate fraction (Figure 1D) that sedimented on top of the dense membrane pellet following treatment at pH 11. Recent $^{22}Na^+$ flux studies have demonstrated that these treated membranes responded to Carb with a rapid efflux of vesicle entrapped $^{22}Na^+$ (38, 39) in a manner similar to untreated membranes (42, 43) and exhibited pharmacological properties expected for cholinergic membrane vesicles (43). An example of such Carb induced $^{22}Na^+$ efflux from pH 11 treated, AcChR-enriched membrane vesicles is shown in Figure 2.

In the present study the components (Figure 1B) of pH 11 treated membranes were solubilized in 2% (w/v) cholate and, following removal of undissolved material by centrifugation, were reassociated with exogenous phospholipids by extensive dialysis, resulting in the formation of reconstituted membrane vesicles. In duplicate experiments shown in Figures 3A and 3B, these reconstituted AcChR vesicles responded to carbamylcholine by releasing 30% and 36% of entrapped $^{22}Na^+$, respectively. This effect was eliminated by

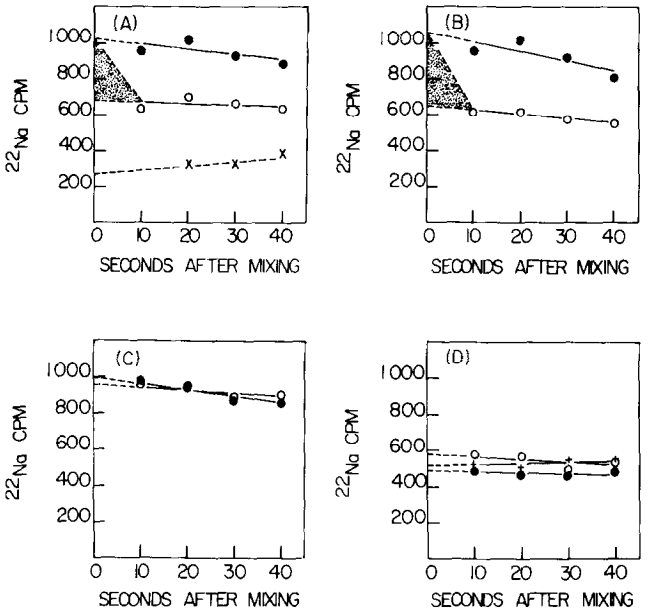

FIGURE 3: Carb-induced $^{22}Na^+$ efflux from reconstituted vesicles. (A) and (B) Reconstituted AcChR-containing vesicles preequilibrated with $^{22}Na^+$ were diluted 20-fold into Dilution Buffer (200 mM NaCl, 10 mM Tris-HCl, pH 7.4) containing 100 μM (○) or 0 μM (●) Carb or 0.1% Triton (X) and assayed for $^{22}Na^+$ retained as a function of time as described in "Methods." (C) Effect of α-BuTx on Carb-induced $^{22}Na^+$ efflux. Reconstituted AcChR-containing vesicles were incubated with a 3-fold excess of α-BuTx at 0°C for 30 minutes before isotonic dilution into Dilution Buffer containing 100 μM (○) or 0 μM (●) Carb and assayed for retained $^{22}Na^+$ as described in "Methods." (D) $^{22}Na^+$ efflux from liposomes. Liposomes were prepared in the same way as reconstituted AcChR vesicles except without addition of AcChR. These phospholipid vesicles were assayed for $^{22}Na^+$ efflux by diluting the preparation preequilibrated with the isotope into Dilution Buffer containing 100 μM (○) or 0 μM (●) Carb followed by assay for remaining $^{22}Na^+$ as described in "Methods." Liposomes were also diluted 20-fold into distilled water followed by addition of concentrated NaCl to 200 mM (+) prior to assay.

preincubation with the quasi-irreversible antagonist α-BuTx (Figure 3C). A similar flux experiment carried out with liposomes (Figure 3D) showed that the permeability of entrapped $^{22}Na^+$ in these nonreceptor-containing vesicles was not altered by Carb, demonstrating that the ligand was not acting non-specifically on lipids. The Carb-activated $^{22}Na^+$ efflux from pH 11 treated membranes (38, 43) and reconstituted membranes was rapid in onset and was complete prior to the first time point taken, as previously observed for untreated membrane preparations from T. californica electroplax (42).

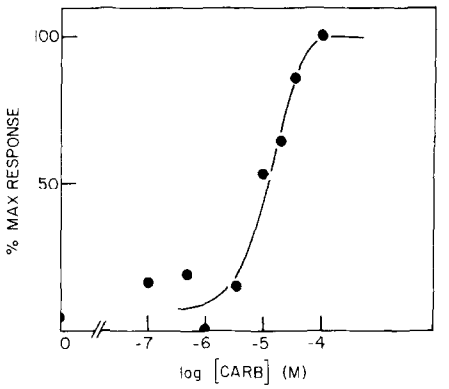

FIGURE 4: Plot of Carb dose response of $^{22}Na^{+}$ efflux from reconstituted AcChR vesicles. Vesicle preparations were diluted into "Dilution Buffer" containing various concentrations of Carb. The response, in each case determined from the average of the 4 time points from each $^{22}Na^{+}$ flux assay, was plotted against the log of the final Carb concentration.

Figure 4 shows a plot of the Carb dose-response curve for $^{22}Na^{+}$ efflux from vesicles reconstituted from purified AcChR; a midpoint of approximately 13 μM was observed for the response.

DISCUSSION

The electroplax membrane preparation described here has been shown to be composed of the same four polypeptides characteristic of electric ray AcChR purified by affinity chromatographic methods (6-8, 44, 45, 3). In essence, the sucrose density gradient procedure for preparation of AcChR enriched membranes from T. californica (23, 24) provides an excellent starting material (15-20% in receptor protein) that can be further purified by extraction (41) of non-receptor peptides for both T. nobiliana (38) and T. californica (39) electroplax. The extracted membranes containing only the AcChR protein in significant amounts have been shown to have unaltered properties when compared with the unextracted ones; the rate constant for [^{125}I] α-BuTx receptor complex formation is unaltered (39), local anesthetic analog (38) and [^{3}H] H_{12}-HTX binding (39) are unaffected and Carb induced translocation of $^{22}Na^{+}$ (38, 43) can be observed with unchanged pharmacology (43). Such membranes represent a preparation eminently suitable for reconstitution stu-

dies since, following dissolution in detergent solution, time-consuming chromatographic and analytical procedures are unnecessary prior to reconstitution.

A further modification has also been introduced in our preparation used for reconstitution. T. californica AcChR has been shown to occur as a 13.2 S protein (46) representing a dimeric form of the molecule in the membrane (47-50) linked through a disulfide bond(s) involving 65 x 10^3 dalton subunits. Studies of agonist induced $^{22}Na^+$ flux have demonstrated that this dimeric form is likely to be active in cation translocation (43). Accordingly, we have stabilized the dimeric form in the membranes used here by alkylation of all free sulfhydryl groups (50) during homogenization and membrane purification as described previously (37).

The $^{22}Na^+$ translocation studies we present display characteristics identical with those we reported earlier for unfractionated (42) and AcChR enriched, base extracted (43) membranes from T. californica electroplax: the Carb induced cation flux is rapid (the total amplitude of the effect being reached prior to the first time-point at 10 sec.), the effect can be completely blocked by pretreatment with the quasi-irreversible antagonist α-BuTx, and the dose-response curve for the agonist has a midpoint in the micromolar range, indicative of Carb association with a low affinity (nondesensitized) state of the receptor.

In previous studies in which purified AcChR was used in reconstitution experiments, varying degrees of success were reported (31-35). We utilized (31, 51) Triton-X-100 solubilized AcChR which, upon affinity chromatography (1) yielded a highly purified preparation. During the reconstitution process Triton was exchanged for sodium cholate since in the presence of phospholipids this detergent can be more readily removed by dialysis. As reported (31, 51) some preparations were successfully reconstituted to form membranes responsive to the agonist Carb and capable of being inhibited by α-BuTx. However, not all preparations of the purified, reconstituted receptor

responded to the agonist. The membrane preparation and reconstitution procedures we report here overcome these difficulties since complete reproducibility has been achieved; six separate reconstituted preparations derived from six AcChR enriched and base extracted membrane preparations all responded to Carb with enhanced $^{22}Na^{+}$ flux as shown in Figures 3A and 3B. The greater reproducibility of the studies reported here is possibly due to (i) stabilization of the AcChR in the dimeric form with prevention of disulfide-sulfhydryl interchange, (ii) dissolution in sodium cholate, rather than Triton-X-100, followed immediately by centrifugation to remove undissolved particles and dialysis to afford rapid reassociation with phospholipids.

In summary the results we report here show that agonist induced translocation of an alkali cation, $^{22}Na^{+}$, can be reproducibly achieved in membrane vesicles reconstituted from solubilized, highly purified postsynaptic acetylcholine receptor membrane preparations derived from Torpedo californica electroplax with pharmacological properties expected for a nicotinic acetylcholine receptor.

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