Vol. 85, No. 2, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 29, 1978 Pages 632-640

FAST CATION FLUX FROM TORPEDO CALIFORNICA MEMBRANE PREPARATIONS: IMPLICATIONS FOR A FUNCTIONAL ROLE FOR ACETYLCHOLINE RECEPTOR DIMERS†‡

DONALD L. MILLER , HSIAO-PING H. MOORE, PAUL R. HARTIG AND MICHAEL A. RAFTERY

CHURCH LABORATORY OF CHEMICAL BIOLOGY
DIVISION OF CHEMISTRY AND CHEMICAL ENGINEERING
CALIFORNIA INSTITUTE OF TECHNOLOGY
PASADENA, CALIFORNIA 91125

Received September 14,1978

SUMMARY: Closed membrane vesicles derived from the innervated face of Torpedo californica electroplax respond to the cholinergic agonist carbamylcholine by a rapid efflux of cations. This response is detected by release of ^{22}Na enclosed within the vesicles and is considerably faster than previously reported for this in vitro system. It is considered likely that the rapid response is analogous to physiological phenomena since it has the appropriate pharmacological characteristics; it desensitizes upon prolonged contact with the agonist and it has a dose-response curve in a physiological range. It is further shown that a dimeric form of the acetylcholine receptor, stabilized by chemical modification methods, is fully active in terms of the carbamylcholine elicited response.

INTRODUCTION

Progress toward understanding neuromuscular transmission at a molecular level has been aided by studies of Torpedo and eel electroplax membrane preparations. Of the two major events leading to membrane depolarization, namely acetylcholine binding and cation translocation, the first has now been extensively studied in in vitro preparations (for a recent review see (2)). Cation translocation has been characterized to a much lesser extent. One approach has been to monitor the flux of vesicle entrapped cations in re-

† Supported by USPHS Grant NS-10294, by a grant from the Muscular Dystrophy Association of America and by a Postdoctoral Fellowship (to P.H.) from the Muscular Dystrophy Association.

‡ A preliminary account of this work has been presented (1).

ABBREVIATIONS

AcChR, acetylcholine receptor; Carb, carbamylcholine; α-BuTx, α-Bungarotoxin; H_{12}-HTX, perhydrohistrionicotoxin.

0006-291X/78/0852-0632$01.00/0

sponse to addition of cholinergic ligands (3). The usefulness of this approach has been limited by the large difference in the time-scale of the observed (3-6) cation efflux ($\tau_{1/2}$ ~ 7 min) for Electrophorus electricus compared with the millisecond response time of preparations studied in vivo. This slow response has nevertheless been extensively characterized with respect to various pharmacological agents (3,5,7-9).

We report here the results of studies carried out on membrane vesicle preparations from Torpedo californica electroplax for which we have observed rapid ^{22}Na efflux in response to the agonist Carb. The characteristics of this rapid efflux suggest that it has many properties that can be compared to the system in vivo, such as a similar apparent Carb dissociation constant, desensitization upon prolonged incubation with the agonist and blockage of the response by the toxins α-BuTx and H_{12}-HTX which are considered to be specific probes of the cholinergic ligand binding site and ion-translocation devise respectively. In addition we discuss the ionic composition necessary to elicit the response and the possible roles played by AcChR monomeric and dimeric forms in the flux mechanism.

MATERIALS AND METHODS

AcChR enriched membranes were prepared from electric organs of freshly killed Torpedo californica or from organs that had been frozen in liquid nitrogen immediately after dissection and stored at -80°C. The organs were first cut into pieces approximately 5 g each and suspended in 1 ml of ice-cold 400 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4 buffer per gram of organ. These were homogenized in a Waring Laboratory Blender at full speed for 2 × 1 min. The mixture was re-homogenized in a Brinkmann Polytron at full speed for 1 min. The homogenate was then centrifuged in a Sorvall GSA rotor at 5000 rpm for 10 min. The supernatant was collected, passed through two layers of cheesecloth, and centrifuged at 16000 rpm for 1 hour in a Beckman type 35 rotor. The supernatant was discarded and the pellets were resuspended in the same NaCl-sodium phosphate-EDTA buffer (0.2 ml per g of organ), using a Virtis-23 homogenizer at full speed for 2 × 20 sec. The final suspension of the membranes was made in a small volume (~0.03 ml per g of organ) of Ca^{+2} free Torpedo Ringers containing 400 mM NaCl (400 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 5 mM Tris, 0.02% NaN_3, pH 7.4) to maintain isotonicity. Resuspension was accomplished with a Virtis-23 homogenizer at medium speed for 2 × 20 sec. The entire isolation was done at 4°C, and the homogenization and suspension of membranes were carried out under argon. Iodoacetamide treated membranes were isolated in the same manner except for the inclusion of 5 mM iodoacetamide in the first homogenization step only.

To measure agonist-induced cation efflux, the membrane preparations (8-13 μM in α-BuTx sites) in Ca^{++} free Torpedo Ringers containing 400 mM NaCl

were incubated with 4.1 μM $^{22}NaCl$ (Amershan, 4.4 Ci/mmole) for 6-7 hours at 4°C to allow equilibration of the $^{22}Na^+$ inside and outside the vesicles. The flux assay was started by a 20 fold dilution of the radioactive membrane suspension into nonradioactive Dilution Buffer with or without a cholinergic effector(s). The Dilution Buffer consisted of 400 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 5 mM Tris, 4 mM $CaCl_2$, 0.02% NaN_3, pH 7.4 and was at room temperature. The mixture was immediately vortexed and at desired time intervals 200 μl aliquots were transferred with an Eppendorf pipet onto two layers of Millipore filters (plain, white, 0.8 μM pore size, 25 mm dia.) which had been presoaked in the Dilution Buffer and mounted on a Millipore manifold apparatus. The filters were immediately washed with 2 × 7.5 mls of nonradioactive Dilution Buffer (room temperature) and counted in a Beckman gamma counter with the counting windows optimized for ^{22}Na. The flux data were fit to single exponential curves.

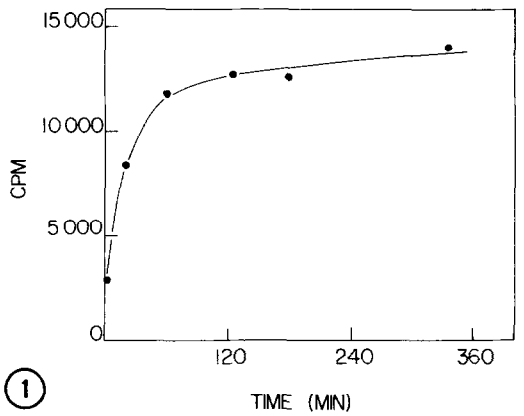
The concentration of α-BuTx sites in the membrane preparations was routinely assayed by the DEAE disc method of Schmidt and Raftery (10). Protein was assayed according to the procedure of Lowry et al (11) with bovine serum albumin as the standard. The membrane preparations used for the present study had a specific activity of 0.4 - 0.5 nmol toxin bound/mg protein.

Carb was obtained from Sigma Chemical Co. α-BuTx was purified from lyophilized venom of Bungarus milticinctus (Sigma Chemical Co.) according to the method of Clark et al (12). H_{12}-HTX was a generous gift of Dr.Y.Kishi.

RESULTS

Acetylcholine receptor (AcChR) enriched membrane preparations from Torpedo californica electroplax loaded with ^{22}Na exhibit a rapid component of sodium ion efflux upon addition of carbamylcholine (Carb). Figure 1 demonstrates the loading of membrane preparations by incubation in ^{22}Na containing buffer. The time to completion of loading varies from 2 to 6 hours from preparation to preparation. Figure 2 demonstrates that a rapid flux component which was essentially complete within 10 seconds was induced by Carb addition. The shaded area in Figure 2 indicates a zone of uncertainty in these measurements. The active cation efflux signal falls somewhere within this zone. With the present techniques we are unable to measure time points less than 10 seconds after mixing.

Agonist induced cation efflux in these preparations presumably arises from a subpopulation of sealed vesicles. In previous work (13) we have shown that intact Torpedo membrane vesicles are very sensitive to osmotic shock. The lower trace in Figure 2 shows the sodium retention by our membrane preparation following osmotic shock. 88% of the entrapped $^{22}Na^+$ was released by this treatment. Therefore, 12% or less of the total counts were non-vesicular in origin. Within the same experiment the level of ^{22}Na re-



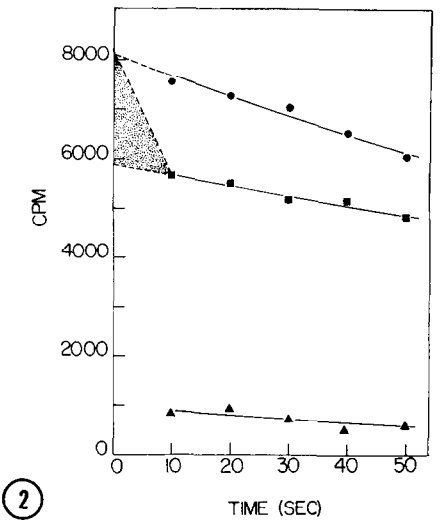


FIGURE 1: Time course of loading Torpedo membrane vesicles with $^{22}Na^+$. To a membrane preparation (8-13 μM in α-BuTx sites) in calcium free Torpedo Ringers containing 400 mM NaCl was added 4.1 μM $^{22}NaCl$. These were well mixed and incubated on ice. At the indicated times after the addition, 25 μl of the mixture was diluted into 500 μl of Dilution Buffer (see Materials and Methods) at room temperature. At 10 sec after the dilution, a 200 μl aliquot was pipetted onto Millipore filters and washed. The amount of entrapped $^{22}Na^+$ was determined by the radioactivity retained on the filters.

FIGURE 2: Release of loaded $^{22}Na^+$ from the membrane preparation by Carb and osmotic shock. A membrane preparation loaded with $^{22}Na^+$ was diluted 20 fold into Dilution Buffer (●), or Dilution Buffer plus 100 μM Carb (■) and the time course was followed by the Millipore filter method. The osmotic shock experiment (▲) was done by a 20 fold dilution into distilled water followed by immediate addition of 400 mM NaCl to the mixture. Solid lines indicate single exponential fits to the normalized data. The shaded area indicates a zone of uncertainty in these measurements.

tention following osmotic shock approximately equals the retention immediately after introduction of ^{22}Na in the loading solution. This baseline level of ^{22}Na retention presumably represents tightly bound and filter trapped sodium.

In contrast to recently published results on eel membranes (14), rapid, agonist induced efflux from Torpedo membrane vesicles occurs when buffers of the same ionic composition are used both inside and outside the vesicles. This active efflux is not sensitive to the divalent cation content of the buffer; buffers containing calcium and magnesium, magnesium alone, or EDTA with no divalent cations present all produce active efflux.

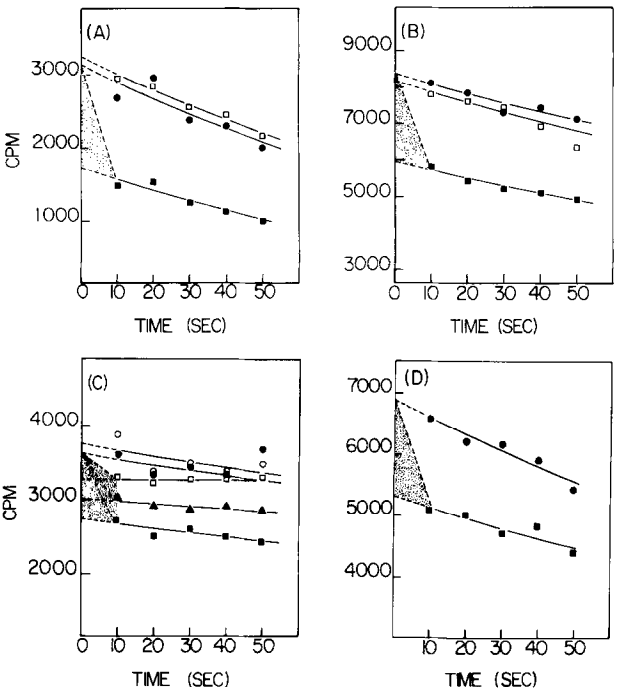


FIGURE 3: Effect of (A) α-BuTx (B) desensitization and (C) H_{12}-HTX on Carb-induced fast cation flux. (A) Membranes were first reacted with a six fold excess of α-BuTx at 0°C for 45 min before 20 fold isotonic dilution into Dilution Buffer (●) or buffer plus 100 μM Carb (□). The bottom curve (■) shows the control experiment done by isotonic dilution of non-toxinated membranes into 100 μM Carb. (B) Membranes were preincubated with 100 μM Carb at 0°C for 30 min. before isotonic dilution into Dilution Buffer (●) or buffer plus 100 μM Carb (□). (■) isotonic dilution of membranes into 100 μM Carb without preincubation with Carb. (C) Membranes were diluted 20 fold into Dilution Buffer with or without H_{12}-HTX and incubated at 0°C for 5 min. At time zero, buffer or Carb (100 μM) was added and efflux of $^{22}Na^+$ was followed. (●) no HTX, no Carb; (○) 5 μM HTX, no Carb; (■) no HTX, Carb; (▲) 5 μM HTX, Carb; (□) 20 μM HTX, Carb. (D) Membranes were isolated with 5 mM iodoacetamide added for the initial homogenization. After loading with ^{22}Na these membranes were diluted 20 fold into Dilution Buffer (●) or into buffer plus 100 μM Carb (■).

Active efflux from our Torpedo membrane preparations displays many of the properties of the in vivo response. Figure 3A demonstrates the blockage of efflux by α-BuTx. The toxin has no effect in the absence of Carb but completely blocks the Carb induced rapid efflux. Figure 3B illustrates the effect of desensitization on the efflux; pre-incubation of membranes with 100 μM Carb for 30 minutes blocks agonist induced rapid efflux without affecting

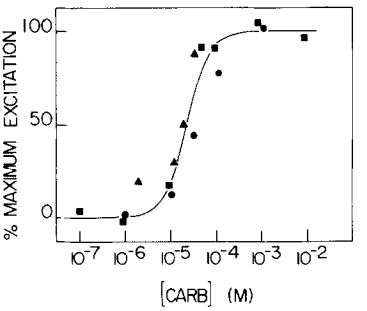


FIGURE 4: Dose-response curve of Carb-induced $^{22}Na^{+}$ efflux. The relative response as measured by the total amplitude of the fast efflux component is plotted as a function of Carb concentration in the dilution buffer (The data represents three independent measurements with three different membrane preparations). The data was analyzed by a nonlinear least squares program assuming multiple ligand binding equilibria. The solid line indicates the fitted curve with a titration midpoint at 25 μM Carb and a Hill coefficient of 1.8.

the background signal. Figure 3C shows the effect of H_{12}-HTX on the rapid efflux. Incubation of membranes with 5 μM H_{12}-HTX for 5 minutes did not affect the background signal, whereas incubation with 5 μM or 20 μM H_{12}-HTX blocked 30% and 57% respectively of the Carb induced efflux. Figure 3D shows that Torpedo membranes isolated in the presence of iodoacetamide exhibit agonist induced efflux to the same extent as untreated membranes. The acetylcholine receptor is largely or entirely in dimeric form in iodoacetamide treated membranes (15).

Figure 4 illustrates the dose-response curve for Carb induced rapid efflux. The midpoint of the titration is approximately 25 μM, in agreement with electrophysiological resuls on a related Torpedo species (16). A Hill plot of the dose-response data shows apparent cooperativity with a Hill coefficient of 1.8.

DISCUSSION

We observed a rapid cation efflux from Torpedo californica electroplax membrane vesicles in which the flux was complete before the first time point was taken at 10 seconds. This rapid flux signal represents a considerable increase in vesicle flux rates over those previously reported (3-9, 17-22)

for AcChR vesicles. In procedures that employ slow sampling techniques amplitude changes are the only data that can be used to investigate the mechanism of rapid ^{22}Na flux. The best diagnostic procedures available for verification of the significance of such amplitude changes are (i) the pharmacological specificity of the observed response, (ii) the dose-response curve for a known agonist and (iii) sensitivity of the response to the presence of the ligand for prolonged periods, i.e. desensitization.

The efficacy of α-BuTx, the most effective antagonist of cholinergic ligand binding, in blocking Carb induced ^{22}Na flux attests to the pharmacological specificity of the observed effects. In addition, the blockage produced by H_{12}-HTX, which is considered to operate by a different mechanism involving the ion-translocation moeity associated with the AcChR (23), further substantiates this notion.

At low ligand concentrations a slow flux component visible on the time scale of seconds might be expected. This was not observed. The change in amplitude of the flux signal which was observed at low agonist concentrations indicates that desensitization may have occurred before the decreased flux rate could empty the vesicle of its contents. The results obtained after preincubation with Carb (Figure 3B) support the notion that inactivation of flux occurred as a result of interaction with the ligand. Taken together, the data of Lee et al (24), Quast et al (25) and the results presented in Figure 3B support the idea that ligand induced inactivation of response and increased ligand affinity may both represent manifestations of desensitization.

Measurements of conductance changes at the neuromuscular junction (26) or of depolarization of single electroplax of Electrophorus electricus (27) have yielded Hill coefficients of approximately 1.7. Such cooperativity of response has also been claimed (3) for the slow efflux ($\tau_{1/2}$ ~ 7 min) of ^{22}Na induced by Carb in Electrophorus electricus vesicles but this has been disputed in a different study of the same slow response (9) in the identical

system. The apparent cooperativity we observe in our rapid ion flux measurements has a Hill coefficient of 1.8. The flux signal we observe is, however, an integrated flux amplitude which depends both on the flux rate and the rate of desensitization. Since both the flux and desensitization rates are agonist dependent, an apparent cooperativity that might not exist in flux could be introduced by desensitization. In addition, we measure an integrated flux signal that is not necessarily proportional to the maximum flux rate.

In a recent study of ^{22}Na efflux from Electrophorus electricus membrane vesicles Hess et al (14) observed a rapid response to the agonist Carb. This response was found to be dependent, however, on the presence of potassium chloride inside and sodium chloride outside of the membrane vesicles. For the fast response described here we do not find this to be the case.

A further feature of our results is of interest in terms of the molecular mechanism of synaptic transmission. We gave an early report (28) of the occurrence of 9S and 13S forms of Torpedo californica AcChR, one being a dimeric form of the other. It has now been shown (15,29-31) that the 13S form is covalently bonded by a disulfide bridge(s) through subunits of M.W. 65,000 daltons. We recently demonstrated by structural (32) and structure-function related (33) methods that the 65,000 dalton subunits were close to and in contact with the AcCh binding subunit in postsynaptic membranes of Torpedo californica. Reaction of the membranes with iodoacetamide to block all available sulfhydryl groups results in isolation of only the dimeric form of the AcChR following solubilization, indicating that this is the major naturally occurring form. The results presented here showing that such alkylation of membranes has no discernible effect on Carb mediated efflux lend strong support to the idea that the dimeric AcChR form is functional in synaptic transmission.

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