Vol. 88, No. 2, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 28, 1979 Pages 735-743

RAPID CATION FLUX FROM TORPEDO CALIFORNICA MEMBRANE VESICLES: COMPARISON OF ACETYLCHOLINE RECEPTOR ENRICHED AND SELECTIVELY EXTRACTED PREPARATIONS

Hsiao-Ping Hsu Moore, Paul R. Hartig,
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

Received April 13,1979

SUMMARY: Rapid efflux of ^{22}Na from within closed vesicles derived from Torpedo californica electroplax membranes has been studied as an in vitro assay of acetylcholine receptor functionality. The most highly purified membrane preparations contained major polypeptides of M.W. 43 and 90 x 10^3 daltons in addition to the four peptides characteristic of the acetylcholine receptor (40, 50, 60, 65 x 10^3 daltons). Removal of these extra peptides by base extraction did not significantly alter the characteristics of carbamylcholine induced ^{22}Na efflux: the agonist dose response curve was similar, preequilibration with agonist caused desensitization, the irreversible antagonist α-Bungarotoxin blocked the efflux and the reversible blockade by the neurotoxin perhydrohistrionicotoxin was also retained. The dose response curve for perhydrohistrionicotoxin corresponded closely to its known binding characteristics for base extracted membranes.

INTRODUCTION

Characterization of synaptic transmission at the molecular level can be approached by study of isolated membrane preparations from Torpedo species highly enriched in AcChR. To date such in vitro studies have been conducted with respect to AcChR structure, composition, cholinergic ligand and neurotoxin binding properties (see 1) and to a lesser extent in terms of cation translocation (2-4) and reconstitution of such translocation from detergent solubilized membranes (5, 6) or purified AcChR (7, 8). Such assay of cation flux through membranes has been conducted using vesicles derived from Electrophorus electricus and Torpedo species electroplax and using myoblast systems (reviewed in 1). These studies have generally dealt with cation flux on a time scale (minutes) vastly different from the response time (milliseconds)

*Contribution #6024.
Abbreviations: AcChR, acetylcholine receptor; α-BuTx, α-Bungarotoxin; SDS, sodium dodecylsulfate; Carb, carbamylcholine; HTX, histrionicotoxin

0006-291X/79/100735-09$01.00/0

of preparations studied by electrophysiological methods. Recently, methodology has been reported whereby cation flux in vesicles from Electrophorus (9) and Torpedo californica (4, 6) can be studied on the time scale of seconds. These advances are important since they lend credence to the notion that the observed flux is truly related to physiological effects since the responses involved are at least as rapid as agonist induced desensitization (4, 10).

In continuing studies of characterization of the AcChR and its associated ion-channel it is important to attempt to relate molecular species with receptor functionality. In this manuscript we describe studies of agonist induced cation flux in two preparations. The first is a preparation of AcChR enriched membranes prepared by sucrose density centrifugation (11-13): this preparation contains the AcChR (with constituent polypeptides of M.W. 40, 50, 60 and 65 x 10^3 daltons (14-20)) in addition to two other major polypeptides of M.W. 43 and 90 x 10^3 daltons. The 43K polypeptide has been suggested (21) to represent a specific "ionophore". The second preparation is derived from the same AcChR enriched membranes by selective basic extraction under conditions of low ionic strength (22, 23) as previously described for erythrocyte membranes (24). This procedure results in membrane preparations essentially devoid of polypeptides other than AcChR subunits. It is shown that the percentage of vesicle entrapped ^{22}Na released by carbamylcholine and the effects of various pharmacological agents known to affect such flux are unaltered upon removal of non-receptor polypeptides from the membranes.

MATERIALS AND METHODS

Preparation of Membrane Fragments

Purified membranes enriched in AcChR were prepared using sucrose gradients in a Beckman VTi 50 vertical rotor (13). Membranes 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 (25). The specific activity of a typical membrane preparation was 1.0-1.25 nmol α-BuTx sites per mg protein.

pH Treatment

Purified AcChR membranes containing 10-15 mg of protein per ml were diluted ten-fold into ice-cold distilled water and the pH adjusted to the

desired values with 0.2 N NaOH. 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 pellets were resuspended in 10 mM Tris-Cl, pH 7.4. For AcChR enriched membranes, where removal of non-receptor proteins was not desired, the same treatments were followed except that the pH was kept at 7.4 throughout.

Flux Assay

Carb-induced ^{22}Na efflux from the purified and alkaline pH-treated membrane vesicles was assayed by the Millipore filtration technique previously described for crude membrane preparations (4), except that the Dilution buffer was 10 mM Tris-Cl, pH 7.4. The treated membrane vesicles in 10 mM Tris-Cl, pH 7.4 were 10-18 μM in α-BuTx binding sites during equilibration with $^{22}NaCl$. In those figures for which data points were averaged, time points of 10, 20, 30 and 40 sec. after dilution were taken.

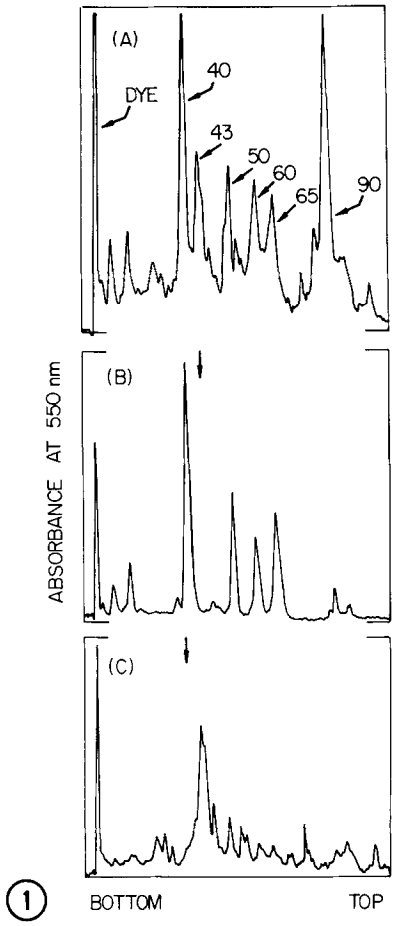
Gel Electrophoresis

8.75% polyacrylamide gels (26) were run in 0.1% SDS and were stained for protein with 0.05% (w/v) Coomassie Brilliant Blue in 10% acetic acid, 25% 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 AND DISCUSSION

We have previously demonstrated that a crude membrane vesicle preparation from Torpedo californica electroplaques catalyzes a rapid cation efflux upon addition of the agonist carbamylcholine (4). Purified membranes enriched in acetylcholine receptor can be obtained from high density regions of sucrose density gradients (11-13) and as shown by the following results, these purified membrane preparations retain and release ^{22}Na with the expected pharmacology of cholinergic vesicles. Osmotic shock with concomitant resealing leads to recovery of substantial interior volume in these vesicles (to be published).

Recently, the alkaline pH treatment originally used for selective extraction of erythrocyte membranes (24) has been applied to purified Torpedo membranes (22, 13, 23). Treatment at pH 11 removed nearly all of the 43K dalton polypeptide and many minor polypeptides from the membranes and produced a preparation greatly enriched in the four AcChR subunits (Figure 1). Treatment at lower pH values was less effective in removing these components. Most of the polypeptides of M.W. 90 x 10^3 daltons and M.W. less than 40 x 10^3 daltons (see Figure 1) occur in a particulate fraction that sediments on top



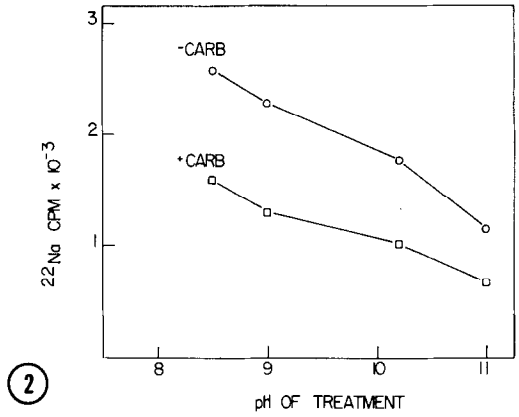


FIGURE 1: Densitometer scans of Coomassie Brilliant Blue-stained SDS gels of membranes before (A) and after (B) treatment at pH 11. (C) is a scan of a gel of the polypeptides recovered in the supernatant.

FIGURE 2: Effect of pH treatment on apparent vesicle volume. Equal aliquots of membranes were treated at the indicated pH values and loaded with ^{22}NaCl at pH 7.4. Counts retained in vesicles were determined from the average of 4 data points from the Millipore flux assay (see Methods) following dilution into 10 mM Tris-Cl pH 7.4 ± 100 µM Carbamylcholine.

of the dense membrane pellet following treatment at pH 11. This lighter particulate fraction can be discarded.

Following prolonged incubation with ^{22}Na at 4°C alkaline pH treated membranes retained the isotope when the external ^{22}Na was removed by the Millipore assay method (4). The apparent total vesicular volume (retained ^{22}Na) decreased as the pH value of the treatment was raised (Figure 2). All of

the treated membrane fractions responded to the agonist Carb by rapidly releasing entrapped ^{22}Na and the percentage of retained counts released remained relatively constant (or increased in some preparations) as the pH of the treatment was raised. Thus the removal of the 43K polypeptide and additional major and minor components by pH 11 treatment does not hinder agonist induced cation efflux. A similar preparation from Torpedo nobiliana has recently

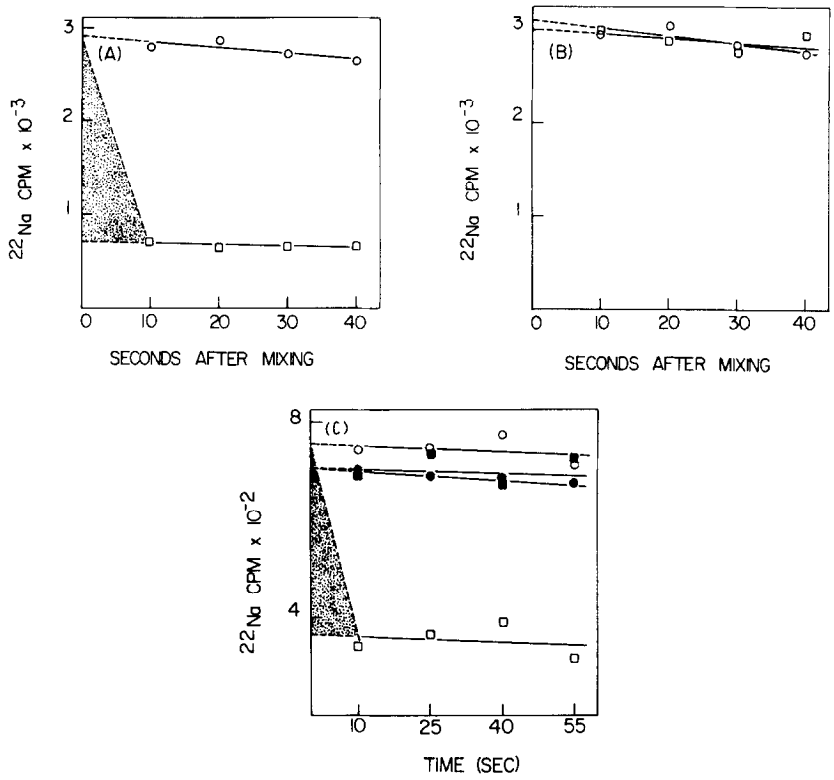


FIGURE 3: Carb induced $^{22}Na^+$ efflux from membrane vesicles treated at pH 11.
(A) Isotonic dilution into buffer (10 mM Tris-Cl, pH 7.4) containing 100 µM (□) or 0 µM (○) Carb.
(B) Effect of α-BuTx on carb-induced $^{22}Na^+$ efflux. Membranes were incubated with a 1.5 fold excess of α-BuTx at 0°C for 30 minutes before isotonic dilution into buffer containing 100 µM (□) or 0 µM (○) Carb.
(C) Effect of agonist induced desensitization on Carb induced $^{22}Na^+$ efflux. Membranes were preincubated with 100 µM Carb at 0°C for 30 minutes before a 20-fold isotonic dilution into buffer containing 100 µM (■) or 0 µM (●) Carb. Control experiments with membranes not preincubated with Carb are also indicated (dilution into buffer containing 100 µM (□) or 0 µM (○) Carb).

been described (22) that contained significant amounts of three of the AcChR subunits in addition to some peptides of lower M.W. Carb induced flux, blocked by α-BuTx, was described for this preparation.

Sodium efflux from pH 11 treated membranes exhibited the expected pharmacology for a nicotinic cholinergic receptor preparation, as also demonstrated for unfractionated, untreated membranes (4). The data in Figure 3 demonstrates that the Carb induced Na^+ efflux from pH 11 treated membranes is completely blocked by α-BuTx and is also eliminated by prior desensitization of the AcChR by Carb. The rate constant for [^{125}I] α-BuTx binding is not altered by treatment with base (23). In addition, pH 11 treated membranes bind the toxin, HTX, with similar affinity, kinetics and stoichiometry to that of untreated membranes (23). The treated membranes also exhibit a dose dependent blockage of sodium efflux by HTX (Figure 4). After correcting for depletion due to bound HTX the midpoint for this effect (2 μM) is very close to the dissociation constant (1.4 μM) obtained from direct binding studies (23).

Figure 5 shows the Carb induced dose-response curves for ^{22}Na efflux from both pH 11 treated and pH 7.4 "treated" membranes. In both cases a similar dose response curve was obtained. The responses exhibited midpoints of ap-

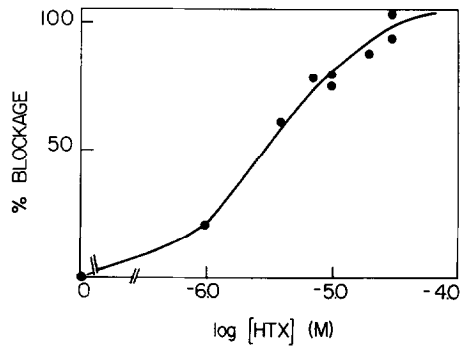


FIGURE 4: H_{12}-HTX inhibition of $^{22}Na^+$ efflux from pH 11-treated membrane vesicles. Membranes were incubated with various concentrations of H_{12}-HTX on ice for 30 minutes before isotonic dilution into buffers containing 100 μM Carb and the same H_{12}-HTX concentrations as the incubation medium. The percent blockage of ^{22}Na efflux was plotted against the logarithm of the concentration of added H_{12}-HTX. The response was determined from the average of 4 data points from the Millipore flux assay.

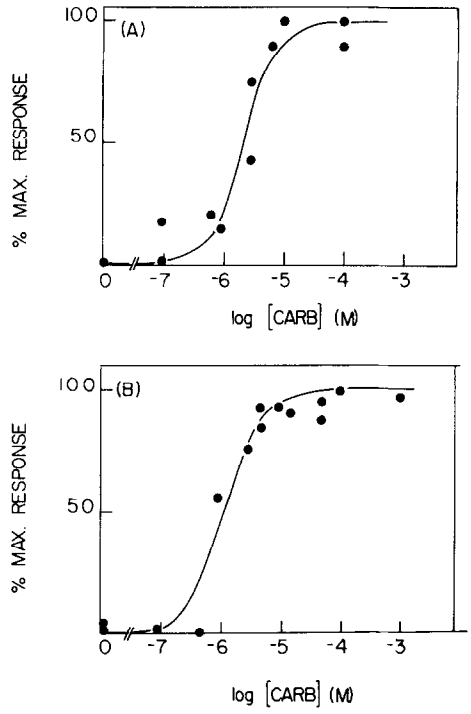


FIGURE 5: Plot of Carb dose response of ^{22}Na efflux from pH 7.4 (A) and pH 11 (B) treated membrane vesicles. Membranes were diluted into isotonic buffer containing various concentrations of Carb. The response, determined from the average of the 4 time points from the Millipore assay, was plotted against the concentration of added Carb in the solution.

proximately 1.5 μM with some apparent positive cooperativity. Previously a midpoint corresponding to a Carb concentration approximately 10 times larger was observed for ^{22}Na efflux from crude membranes (4). This difference can be ascribed to the differing salt concentrations in the various samples. Crude membranes (4) were assayed in 0.4 M NaCl while the pH 7.4 and 11 treated membranes in the present study were assayed in 10 mM Tris-Cl. Agonist binding to AcChR enriched membranes is inhibited by NaCl with an inhibition constant of 38 mM (27). The observed midpoint shift of the Carb induced response can be adequately explained by this inhibition of agonist binding.

Thus, the data presented in this communication demonstrate that removal of essentially all of the polypeptides other than those known to be part of the AcChR (from receptor purification studies (14-20)) does not greatly alter

the functional properties of the system as defined by various in vitro assays: (i) the kinetics of [^{125}I] α-BuTx binding remain the same (23), (ii) Carb induced cation flux is preserved with no alteration in its dose response characteristics and this effect is blocked by α-BuTx, (iii) pretreatment of the membranes with the agonist Carb abolishes cation flux in a manner reminiscent of desensitization and (iv) the neurotoxin HTX also inhibits Carb induced cation efflux with a dose response curve that agrees well with studies of [^{3}H]-HTX binding (23) to the same type of membrane preparation. Therefore, not only does HTX associate with the AcChR but this association is responsible for blockage of cation efflux induced by a cholinergic agonist.

Acknowledgments
This research was supported by USPHS Grant NS 10294, by a grant from the Muscular Dystrophy Association of America, by a grant from the American Heart Foundation and by a postdoctoral Fellowship from the Muscular Dystrophy Association of America (to Paul Hartig).
We thank Carolyn Sprague and Valerie Purvis for their expert typing and illustration production.

REFERENCES

1. Heidmann, T. and Changeux, J.-P. (1978) Ann. Rev. Biochem. 47, 317-357.
2. Popot, J.-L., Sugiyama, H. and Changeux, J.-P. (1976) J. Mol. Biol. 106, 469-483.
3. Sugiyama, H., Popot, J.-L. and Changeux, J.-P. (1976) J. Mol. Biol. 106, 485-496.
4. Miller, D. L., Moore, H.-P. H., Hartig, P. R. and Raftery, M. A. (1978) Biochem. Biophys. Res. Commun. 85, 632-640.
5. Hazelbauer, R. L. and Changeux, J.-P. (1974) Proc. Natl. Acad. Sci. 71, 1479-1483.
6. Epstein, M. and Racker, E. (1978) J. Biol. Chem. 253, 6660-6662.
7. Michaelson, D. and Raftery, M. A. (1974) Proc. Natl. Acad. Sci. 71, 4768-4772.
8. Michaelson, D., Duguid, J. R., Miller, D. and Raftery, M. A. (1976) J. Supramolecular Struc. 4, 419-425.
9. Hess, G. P., Lipkowtiz, S. and Struve, G. E. (1978) Proc. Natl. Acad. Sci. 75, 1703-1707.
10. Bernhardt, J. and Neumann, E. (1978) Proc. Natl. Acad. Sci. USA 75, 3756-3760.
11. Duguid, J. R. and Raftery, M. A. (1973) Biochemistry 12, 3593-3597.
12. Reed, K., Vandlen, R., Bode, J., Duguid, J. and Raftery, M. A. (1975) Arch. Biochem. Biophys. 167, 138-144.
13. Elliott, J., Blanchard, S., Wu, W. C.-S., Miller, J., Strader, C., Hartig, P., Moore, H.-P. H., Racs, J. and Raftery, M. A. (1979) Biochem. J., submitted.
14. Raftery, M. A., Vandlen, R., Michaelson, D., Bode, J., Moody, T., Chao, Y., Reed, K., Deutsch, J. and Duguid, J. (1974) J. Supramolecular Struct. 2, 582-592.

15. Weill, C. L., McNamee, M. G. and Karlin, A. (1974) Biochem. Biophys. Res. Commun. 61, 997-1003.
16. Raftery, M. A., Vandlen, R. L., Reed, K. L. and Lee, T. (1976) Cold Spring Harbor Symp. XL, 193-202.
17. Karlin, A., Weill, C. L., McNamee, M. G. and Valderrama, R. (1976) Cold Spring Harbor Symp. XL, 203-210.
18. Vandlen, R. L., Schmidt, J. and Raftery, M. A. (1976) J. Macromo. Sci. Chem. A10 (1 & 2), 73-109.
19. Hucho, F., Layer, P., Kiefer, H. R. and Bandini, G. (1976) Proc. Natl. Acad. Sci. 73, 2624-2628.
20. Vandlen, R. L., Wu, W. C.-S., Eisenach, J. C. and Raftery, M. A. (1979) Biochemistry, in press.
21. Sobel, A., Heidmann, T., Hofler, J. and Changeux, J.-P. (1978) Proc. Natl. Acad. Sci. 75, 510-514.
22. Neubig, R. R., Krodel, E. K., Boyd, N. D. and Cohen, J. B. (1979) Proc. Natl. Acad. Sci. 76, 690-694.
23. Elliott, J., Dunn, S. M. J., Blanchard, S. and Raftery, M. A. (1979) Proc. Natl. Acad. Sci., in press.
24. Steck, T. L. and Yu, J. (1973) J. Supramolecular Struct. 1, 220-232.
25. Blanchard, S., Quast, U., Reed, K., Lee, T., Schimerlik, M., Vandlen, R., Claudio, T., Strader, C., Moore, H.-P. H. and Raftery, M. A. (1979) Biochemistry, in press.
26. Laemmli, U. K. (1970) Nature (London) 227, 680-685.
27. Miller, J., Witzemann, V., Quast, U. and Raftery, M. A. (1979) Proc. Natl. Acad. Sci., submitted.