Proc. Natl. Acad. Sci. USA
Vol. 76, No. 12, pp. 6265–6269, December 1979
Biochemistry

Correlation of polypeptide composition with functional events in acetylcholine receptor-enriched membranes from *Torpedo californica*

(cation flux/partial inactivation/functional polypeptides)

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

Communicated by H. A. Barker, September 27, 1979

ABSTRACT **Membrane vesicles containing partially inactivated acetylcholine receptor (AcChoR) channels may produce a full $^{22}Na^+$ flux response because an excess of channels may exist above the level needed to completely empty the vesicles of ions. Therefore, attempts to use ion flux amplitudes as indicators of AcChoR function may fail due to the presence of these excess AcChoR channels. Random inactivation of variable fractions of AcChoR channels in vesicles by the irreversible binding of the neurotoxin α-bungarotoxin provides a tool for assessing the size of the excess receptor population. Using this approach, we predict that the dependence of the flux response on partial inactivation by α-bungarotoxin will drastically change if an essential AcChoR component is substantially removed from the membranes. Membranes from which M_r 43,000, M_r 90,000, and other polypeptides had been substantially removed by base extraction exhibited a flux response after random inactivation that was indistinguishable from that of untreated membranes. Therefore, those components which are substantially removed by base extraction do not appear to be essential for AcChoR-mediated ion flux.**

The acetylcholine receptor (AcChoR) from *Torpedo californica* has been shown to be composed of four polypeptides of M_r 40,000, 50,000, 60,000, and 65,000 after purification of detergent-solubilized electroplax membranes (1). However, enriched membrane preparations are more useful for functional studies (2). When prepared by centrifugation procedures these contain additional polypeptides, notably those of M_r 43,000 and ≈90,000 (3, 4). It is important to identify those peptide chains that contribute to the functioning of the receptor.

$^{22}Na^+$ efflux measurements provide a convenient "*in vitro*" assay (5–7) of the AcChoR-mediated cation permeability. The kinetics of this efflux process are too fast to be measured by filtration (7). Instead, a flux amplitude consisting of a time-integrated release of vesicle-entrapped $^{22}Na^+$ is measured after all flux processes have terminated. The observed variation in flux amplitude with agonist concentration indicates the presence of a time-dependent inactivation process (7, 8) such as desensitization.

Essentially identical flux amplitudes have been reported (3, 9) for native AcChoR membranes and for membranes from which polypeptides of M_r 43,000 and 90,000 were substantially removed by base extraction (3, 4, and unpublished data). The small fraction of these polypeptides that remains after base extraction, however, may produce a full flux response because a great excess of AcChoR-associated channels may be present on the vesicles above the level required to empty the entrapped $^{22}Na^+$ completely.

To determine whether base-extracted components are functional AcChoR components, we have used a partial inactivation technique to simulate the effect that removal of an essential AcChoR component would have on the ion flux. This method involves random inactivation of variable fractions of AcChoR channels by the irreversible binding of α-bungarotoxin (α-BuTx). The flux amplitude was determined for varying degrees of toxin occupancy before and after base extraction. The results indicate that the M_r 43,000, M_r 90,000, and certain other polypeptides substantially removed by base extraction are not essential for AcChoR-mediated ion flux and that the full response to agonist binding occurs with preparations composed of the four AcChoR polypeptides. In addition, the theory and technique of partial inactivation provides a method for analyzing ion flux results from any neurotransmitter receptor vesicles.

MATERIALS AND METHODS

AcChoR membranes were prepared as described. For base treatment, membranes (15–18 μM in α-BuTx sites) were diluted 1:10 into distilled water at room temperature and the pH was adjusted to 11 with 0.2 M NaOH. The membranes were stirred at 4°C for 1 hr and centrifuged at 39,000 × *g* for 45 min. The supernatant and a soft pellet that sedimented on top of the hard pellet were removed. The hard pellet was resuspended in 10 mM NaCl/10 mM Tris·HCl, pH 7.4 (buffer A), at 1.5–1.8 μM and the pH was again adjusted to 11. The membranes were immediately pelleted as before and resuspended (≈13 μM) in buffer A. α-BuTx binding was totally recovered in the various fractions. Also, the α-BuTx association rate constant and the $t_{1/2}$ of the affinity change induced by carbamoylcholine (CarbCho) were unaltered.

For partial toxin blockage, the membranes were diluted to 1.15 μM in 400 mM NaCl/1 mM EDTA/10 mM Na phosphate, pH 7.4, to decrease the α-BuTx binding rate. The reaction was initiated by rapid addition of an equal volume of α-BuTx in the same buffer to a well-stirred membrane suspension. The mixture was incubated at room temperature for 65 min to allow 99% completion of the reaction (measured bimolecular rate constant, $2.2 \times 10^4\ M^{-1}\ sec^{-1}$). Aliquots of the reaction mixture were withdrawn to determine the residual α-BuTx sites with ^{125}I-labeled α-BuTx. The membranes were washed with and resuspended in buffer A. Flux studies were carried out as described (7) except that (*i*) time points were 10 min after dilution and (*ii*) buffer A was used as the "dilution buffer."

Protein assays, α-BuTx site assays, gel electrophoresis, and gel scans were carried out as described (9).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: AcChoR, acetylcholine receptor; CarbCho, carbamoylcholine; α-BuTx, α-bungarotoxin; HTX, histrionicotoxin.

RESULTS

Theoretical Prediction of Ion Flux from AcChoR Vesicles. Consider the case of a homogeneous population of AcChoR vesicles equilibrated with carrier ions and with a radioactive tracer. Tracer efflux is initiated by dilution of the suspension into a large excess of isotonic buffer containing an agonist that increases the permeability to both tracer and carrier cations. The resulting ion efflux should be limited by ion transit through the open channel rather than by diffusion to the pore mouth (10).* In this case of pore-limited efflux, the open channel should at all times be saturated with a combination of carrier and tracer. Tracer ion flux will be nearly unidirectional, and carrier influx and efflux will approximately cancel. If k is the maximal ion transport rate per open channel and N and N_c are the number of tracer and carrier ions, respectively, present inside a single vesicle, then the number of tracer ions transported per unit time per channel is $kN/(N + N_c) \approx kN/N_c$. The number of open channels per vesicle is $\alpha(t)\rho S$ in which $\alpha(t)$ is the fraction of channels open at time t, ρ is the surface density of channels, and S is the surface area. The tracer efflux rate per vesicle is thus

$$-dN/dt = \alpha(t)\rho Sk\,(N/N_c). \quad [1]$$

Assuming that tracer efflux is slowed by a first-order channel inactivation process with a rate constant k_i (see *Discussion*),

$$\alpha(t) = \alpha_0 e^{-k_i t} \quad [2]$$

Combining Eqs. 1 and 2 and defining $k' = kS\alpha_0/N_c$,

$$-dN/dt = k'\rho N e^{-k_i t}.$$

Integrating from $t = 0$ to ∞,

$$\ln (N_\infty/N_0) = -(k'\rho/k_i).$$

Defining the flux response, R, as the fraction of tracer ions released by $t = \infty$ we have

$$R = 1 - (N_\infty/N_0) = 1 - e^{-\rho k'/k_i}. \quad [3]$$

This derivation resembles the analysis of Bernhardt and Neumann (8) except that the involvement of carrier ions in the tracer efflux has been explicitly considered.

Incubation of AcChoR vesicles with α-BuTx will decrease the effective surface density of channels. The relationship between the fraction of toxin sites occupied and the inactivation of the channels will depend on the specific model (see *Appendix*) assumed for the α-BuTx, agonist, and channel interactions. A most probable model is considered here.

T. californica AcChoR is a 13.7S dimer (11), probably with four α-BuTx sites and two high-affinity agonist sites at equilibrium (12–14). Each dimer is considered to be a fluxing unit because histrionicotoxin (HTX), which appears to interact with AcChoR channels, binds with a stoichiometry of one per four α-BuTx sites (2). Affinity labeling studies indicate that two α-BuTx sites, one on each monomer, are competitive with agonists and two α-BuTx sites are independent (15). Because α-BuTx binds to a single class of noninteracting sites (16), nonsaturating levels of α-BuTx will be statistically distributed among the four sites associated with each channel. Electrophysiological studies indicate that two agonist molecules must bind per channel for efficient induction of the transition to the conducting form (17–19). If y is the fraction of α-BuTx sites occupied, then the fraction of channels that remain capable of being activated by agonist can be shown to be $(1 - y)^2$. The effective surface density of channels is thus

$$\rho = \rho_0(1 - y)^2. \quad [4]$$

The relationship between ion flux (Eq. 3) and toxin occupancy, assuming k' and k_i are not affected by partial toxin binding, thus becomes

$$R = 1 - \exp(-A(1 - y)^2) \quad [5]$$

in which $A = \rho_0 k'/k_i$.

Observed Cation Efflux From AcChoR Membrane Preparations. In the absence of CarbCho, there was a $^{22}Na^+$ leakage from AcChoR-enriched membrane preparations which exhibited two exponential time constants (Fig. 1). Dilution into CarbCho-containing buffer produced an additional rapid release of $^{22}Na^+$ within 10 sec, as reported (7), followed by the two leakage components. The fast-leakage component was not affected by CarbCho and therefore it is unlikely to be associated with functional AcChoR. Vesicles that contain functional AcChoR leak $^{22}Na^+$ slowly. The CarbCho response was determined by the final level of $^{22}Na^+$ retained at the end of the activator-induced efflux (N_∞ in Eq. 3). This was measured 10 min after dilution to ensure that agonist-induced activation and inactivation was over and to allow complete discharge of the fast-leakage component. The contribution of the slow-leakage component was less than 5% during the 10-min incubation and can be ignored. A similar method was used by Hess and Andrews (6) for studies on eel electroplax except that addition of agonist was delayed until after the dilution incubation.

Effect of Partial Inactivation on the Observed Cation Efflux. We can determine the effect that removal of an essential AcChoR component would have on ion flux by using α-BuTx to partially inactivate the AcChoR. It is essential that partial inactivation occurs through a random distribution of α-BuTx binding over all vesicles. Because α-BuTx binding is an irre-

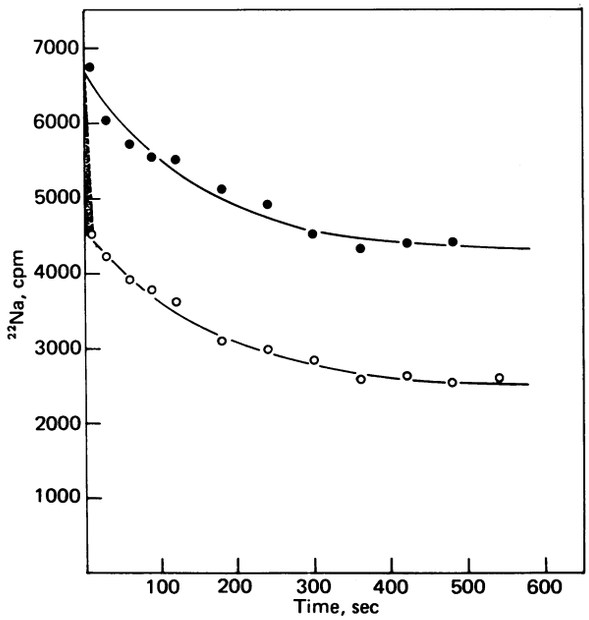

FIG. 1. $^{22}Na^+$ efflux from AcChoR-enriched *T. californica* membrane preparations. Vesicles equilibrated with $^{22}NaCl$ were isotonically diluted 1:20 into 0 μM (●) or 100 μM (○) CarbCho. Vesicle-entrapped $^{22}Na^+$ was determined at the indicated times after dilution. The curves (—) can be described by two single-exponential leakage components: $ae^{-0.00624t} + be^{-0.0001t}$ ($a = 2390$, $b = 4260$ for ●; $a = 2150$, $b = 2450$ for ○).

* If the efflux is limited by collision with the pore mouth rather than by transit through the channel, a similar analysis will hold with a different definition of constant A in Eq. 5.

versible process on the time scale of these experiments, the reaction was carried out under conditions that allowed complete mixing before significant binding occurred [≤4% bound within the ≈3 sec required for mixing (data not shown)]. Membrane preparations from *T. californica* contain a single class of noninteracting α-BuTx sites (16). In the preparations used here, a semilogarithmic plot of α-BuTx binding was linear up to >90% of the reaction (data not shown). Thus, the bound toxin will be homogeneously distributed over all AcChoR vesicles.

$^{22}Na^+$ efflux experiments performed on partially toxin-blocked membranes yielded response curves of the type illustrated in Fig. 2. Three response curves (no effectors, saturating CarbCho, and gramicidin) were determined for each of the toxin-blocked samples. Because the various toxin-treated samples were washed separately before flux measurements the CarbCho response was normalized to the total amount of vesicle entrapped $^{22}Na^+$, which is the amount released by gramicidin (unpublished data). The difference between the response to CarbCho and that to gramicidin at 0% toxin occupancy is most likely due to vesicles that do not contain functional AcChoR.

The CarbCho-induced $^{22}Na^+$ efflux responses of several AcChoR membrane preparations (Fig. 3) were plotted as a function of the fraction of toxin occupancy. Because the fraction of vesicles devoid of functional AcChoR may vary from preparation to preparation, the flux responses of the various toxin-blocked samples were normalized to the maximal response of that preparation. The plateau region of the curve indicates the presence of excess receptors over those necessary to remove the entrapped $^{22}Na^+$ completely. As the fraction of occupied toxin sites increased to >70%, the remaining receptors were no longer sufficient to remove the entrapped $^{22}Na^+$ before they were inactivated, resulting in a decreased flux response. The theoretical predictions of Eq. 5 fit the data well with $A = 37$ (Fig. 3, —).

These results allow us to predict the effect that partial removal of an essential AcChoR component would have on the flux response. For instance, removal of 98% of an essential AcChoR component (one per channel) would result in a 98% reduction of the effective surface density of channels and hence of the value of A in Eq. 5. Substituting $A = 0.74$ in Eq. 5, we obtain the predicted flux dependence (Fig. 3, -·-). The predicted line shape deviates drastically from that for native membranes and drops off at much lower levels of toxin occupancy because the number of excess receptors is greatly decreased. In addition, such a preparation should also exhibit a decreased flux amplitude at zero toxin occupancy. Because the relationship between the degree of toxin occupancy and channel inactivation is not linear (Eq. 4), the flux response of a toxin-free preparation in which 98% of the channels have been inactivated should be equivalent to that of an 86% toxin-blocked membrane. Such a preparation should never release more than 50% of the entrapped $^{22}Na^+$.

Neither of the results predicted above (line shape nor amplitude) were observed with the base-extracted membranes (ref. 3 and unpublished data) from which >98% of the M_r 43,000, M_r 90,000, and other polypeptides were removed (Fig. 4). An almost superimposable dependence of the flux response on the degree of toxin occupancy was observed for the untreated (□) and the base extracted (■) membranes (Fig. 3). Furthermore, we frequently observed release of >85% of the entrapped $^{22}Na^+$ of the base-extracted membrane vesicles. Therefore, the components that were removed in excess of 90% during the base treatment do not appear to be essential for receptor-mediated ion translocation.

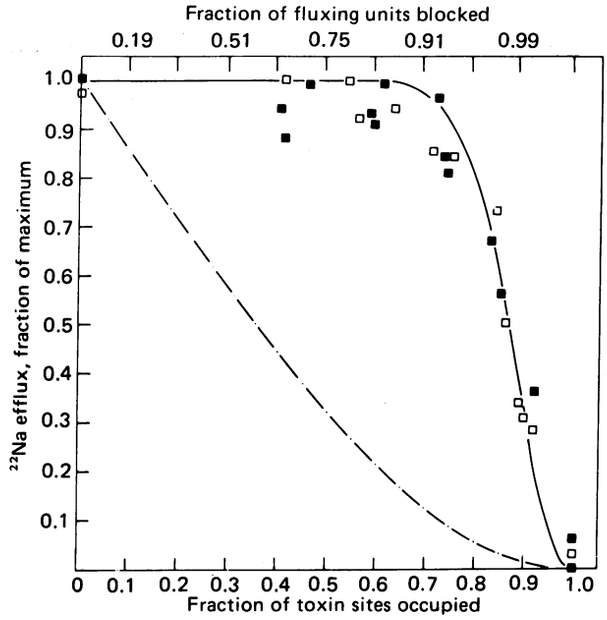

FIG. 3. Effect of partial inactivation on the CarbCho-induced $^{22}Na^+$ efflux. The flux amplitudes, as determined in Fig. 2, were expressed as percentage of maximal response for base-treated (■) or untreated (□) membranes and plotted as a function of the fraction of α-BuTx sites occupied. The data were fitted by a nonlinear least squares program to the normalized form of Eq. 5 [$R = (1 - \exp[-A(1 - y)^2])/(1 - \exp -A)$] yielding an A value of 37.4 (—). The flux response predicted for a preparation in which 98% of the channels have been inactivated (Eq. 5, $A = 0.02 \times 37.4 = 0.74$) is shown as -·-. The top axis represents the fraction of channel blocked at a given toxin occupancy (Eq. 4) for the model considered in the text. The flux amplitude at 0% toxin occupancy was normally 80–90% of the total entrapped $^{22}Na^+$ for base-extracted membranes and 50–60% for untreated membranes.

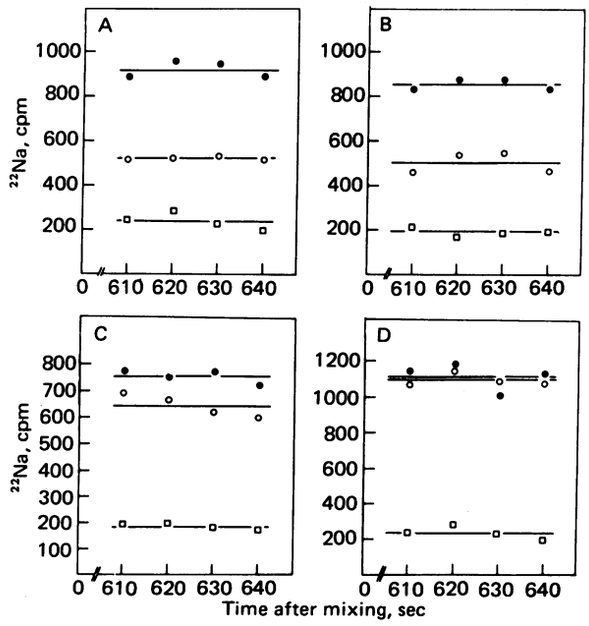

FIG. 2. $^{22}Na^+$ efflux from AcCHoR-membrane preparations partially inactivated by α-BuTx: Membranes treated with various amounts of α-BuTx (A, 0% toxin occupany; B, 56%; C, 89%; D, 100%) were loaded with $^{22}Na^+$. Flux assays were started 10 min after isotonic dilution into buffers containing 0 μM (●) or 100 μM CarbCho (○) or 5 μg of gramicidin (□) per ml. For a given sample, a flux amplitude was determined from the ratio of $^{22}Na^+$ released by CarbCho to that released by gramicidin.

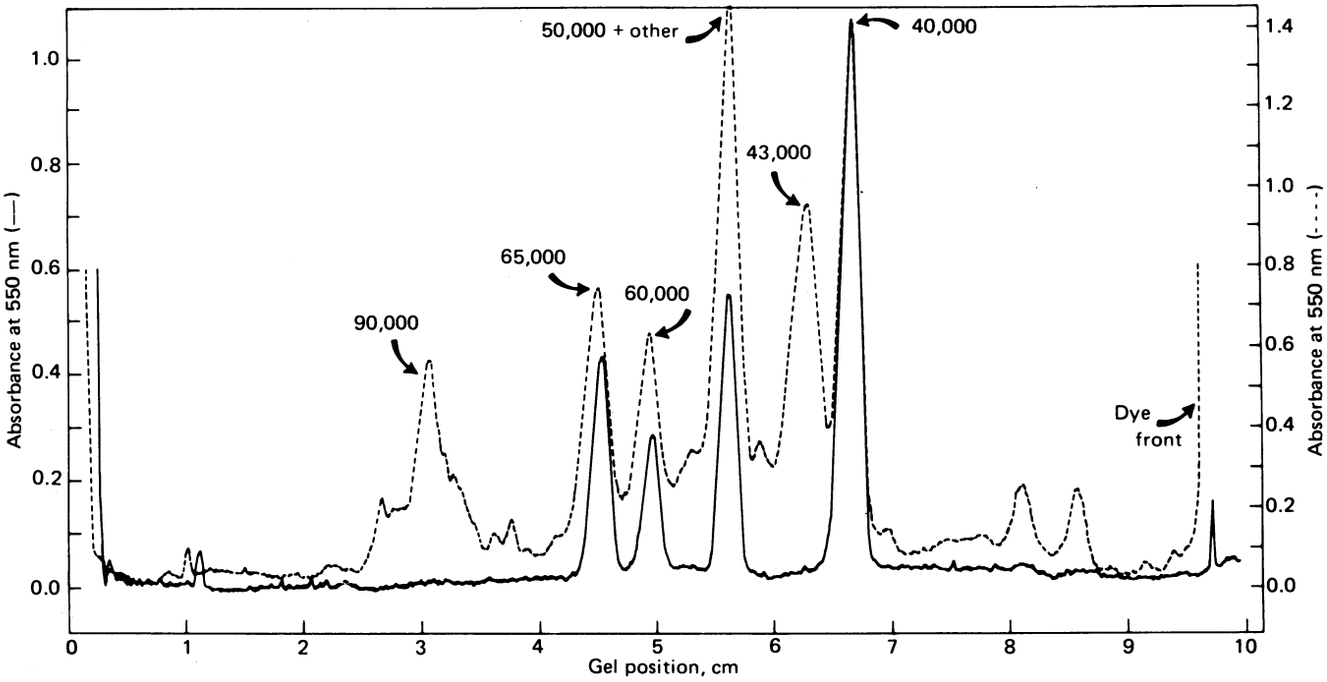

FIG. 4. Densitometer scans of Coomassie brilliant blue-stained sodium dodecyl sulfate/polyacrylamide gels of membranes before (- - -) and after (—) base extraction.

DISCUSSION

We have used partial inactivation by α-BuTx to assess the effect that removal of an essential AcChoR component would have on ion flux. The relationship between toxin occupancy and channel inactivation depends on the specific model assumed for the CarbCho, toxin, and channel interactions. In *Results* we have assumed a probable model based on current knowledge; five additional models are presented in the *Appendix*. All six models lead to the same conclusion: removal of a large fraction of an essential AcChoR component would drastically alter the vesicle flux–toxin occupancy response curve. Base extraction removes over 98% of the M_r 43,000, M_r 90,000, and certain other polypeptides without altering the curve shape. Thus, these polypeptides do not appear to be essential for AcChoR-mediated ion translocation. In addition, five of the six models require that no more than 50% of the entrapped $^{22}Na^+$ would be released by agonist after 98% inactivation of channels. Base-treated, toxin-free membranes depleted to >98% of nonreceptor peptides release >85% of the entrapped $^{22}Na^+$, in support of our interpretation.

Incomplete emptying of vesicle-entrapped $^{22}Na^+$ (7, 8) requires that a channel inactivation process [such as desensitization (20)] occurs which terminates the flux. We have assumed a single-exponential inactivation process which is consistent with any model in which a single overall rate constant dominates, such as the two-state model of Katz and Thesleff (20). In our analysis of the flux response this inactivation rate constant was assumed to be unaffected by base extraction. As demonstrated previously (3), base treatment does not alter the desensitization rate significantly. We have also assumed that fluxing vesicles are approximately homogeneous in size. In negative-staining electron micrographs, both base-treated and untreated membranes show a relatively homogeneous field of large AcChoR vesicles (unpublished observations).

The presence of a plateau region in the vesicle flux–toxin occupancy response curve (Fig. 3) indicates the presence of excess AcChoR over that necessary to release all entrapped $^{22}Na^+$. Taking a probable toxin–CarbCho–channel model as presented in *Results*, a decrease in the flux response occurs after approximately 95% (see Fig. 3, top axis) of the channels have been inactivated. Thus, 95% of the channels remain silent in current ion flux assays. Obviously, any interpretation of vesicle flux experiments must be approached cautiously due to this population of excess AcChoR channels. In recent work by Neubig *et al.* (3) removal of approximately 85% of the M_r 43,000 polypeptide by base extraction was shown to have no effect on the flux response. Assuming that their *Torpedo* vesicles resemble our preparation, we would predict that this extraction would have no effect on flux amplitudes (see Fig. 3) even if the M_r 43,000 polypeptide were an essential AcChoR component. Such flux amplitude studies (3, 9) yield only qualitative information, as noted by Neubig *et al.* (3).

At this time, the size of the excess AcChoR channel population is still uncertain. In the alternative models presented in the Appendix, A values ranging from 3 to 2000 were obtained which lead to predictions of excess AcChoR channel populations of <25% to >99.9% of the total. Future verification of a single model will permit an exact determination of the excess AcChoR channel population.

APPENDIX

We consider that the AcChoR is a dimer (11–13) with two α-BuTx sites (α and β) per monomer. Agonists bind to half the number of α-BuTx sites (12–14), presumably one to each monomer.† The relationship between channel blockage and toxin site occupation depends on (*i*) the competition between CarbCho and toxin binding and (*ii*) whether one or two agonists are required to activate a channel.

Three possible models similar to those described by Damle and Karlin (15) were considered for the CarbCho–toxin interaction. In these models, each monomer is considered to be an independent unit for CarbCho and toxin binding.

† If the numbers of agonist sites and α-BuTx sites are equal at two per monomer, then essentially the same analysis and A values presented for the six cases of Table 1 will hold.

Table 1. Carb–toxin–channel interaction models

Case	Model	$\rho(y)/\rho_0$	$A = \rho_0 k'/k_i$
1	I, RL_2 fluxes	$(1-y)^2$	37.4
2	I, RL, RL_2 flux	$1-y^2$	3.0
3	II, RL_2 fluxes	$(1-y^2)^2$	10.9
4	II, RL, RL_2 flux	$1-y^4$	0.85
5	III, RL_2 fluxes	$(1-y)^4$	1947.0
6	III, RL, RL_2 flux	$2(1-y)^2 - (1-y)^4$	18.9

The fraction of potentially active channels (ρ/ρ_0) remaining after partial toxin blockage was determined for each of the six cases as a function of y, the fraction of toxin sites occupied. This expression was substituted in Eq. 3 and a least squares fit to the experimental data yielded the A values presented. [In our studies, the agonists are present at or nearly at saturating levels because the same dependence of the flux response on toxin occupancy was obtained at higher concentration (500 μM) of CarbCho. In this case, α, the fraction of open channels, approaches a constant and only the ρ dependence on y needs to be considered.] For each model, we considered the case in which two Carb molecules per dimer are needed to open the channel (RL_2 fluxes) and the case in which either one or two CarbCho molecules can fully activate the channel (RL and RL_2 flux).

Model I. CarbCho binds only to one of the two monomer sites, say site α. Binding of toxin to this site (α) inhibits CarbCho binding, but binding of toxin to the other monomer site (β) has no effect on CarbCho binding.

Model II. CarbCho can bind to either of the two monomer sites (α or β), and binding to one site strongly inhibits further CarbCho binding to the same monomer by negative cooperativity. The occupation of both monomer sites (α and β) by toxin is required to inhibit CarbCho binding to the monomer.

Model III. CarbCho can bind to either site (α or β) of the monomer. When either (α or β) or both (α and β) of the sites are occupied by toxin, CarbCho can no longer bind to the monomer.

These models, combined with the assumption that binding of one or two agonists per dimer is necessary to activate a channel, yielded the six cases considered in Table 1. The fraction of potentially active channels (ρ/ρ_0) after partial inactivation is presented in Table 1 for these six cases. All six cases fit the experimental data reasonably well, yielding A values ranging from 0.85 to 2000. Case 4 can be eliminated because the fitted A value predicts that no more than 57% of the entrapped $^{22}Na^+$ can be released at 0% toxin occupancy which is inconsistent with the observed flux (see Fig. 3).

For the remaining five cases, 98% inactivation of channels would decrease the A value by 98% and lead to theoretical predictions of a vesicle flux–toxin occupancy curve that deviates strongly from the observed response of base-extracted membranes. Thus, the same conclusion is reached regardless of the case assumed. Base extraction, which substantially removes polypeptides of M_r 43,000 and 90,000, does not affect AcChoR-mediated ion flux.

The authors thank Drs. N. Davidson and H. Lester for valuable discussions, Steve Blanchard for the nonlinear least squares program we used, Valerie Purvis for illustrations and typing, and John Racs for purified membrane preparations. This research was supported by U.S. Public Health Service Grant NS-10294, by a grant and a fellowship (to P.R.H.) from the Muscular Dystrophy Association of America, and by a grant from the American Heart Association.

1. Raftery, M. A., Vandlen, R. L., Reed, K. L. & Lee, T. (1976) *Cold Spring Harbor Symp. Quant. Biol.* **40,** 193—202.
2. Elliott, J. & Raftery, M. A. (1979) *Biochemistry* **18,** 1868–1874.
3. Neubig, R. R., Krodel, E. K., Boyd, N. D. & Cohen, J. B. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 690–694.
4. Elliott, J., Dunn, S. M. J., Blanchard, S. & Raftery, M. A. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 2576–2579.
5. Kasai, M. & Changeux, J.-P. (1971) *J. Membr. Biol.* **6,** 1–23.
6. Hess, G. P. & Andrews, J. P. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 482–486.
7. Miller, D. L., Moore, H. P. H., Hartig, P. R. & Raftery, M. A. (1978) *Biochem. Biophys. Res. Commun.* **85,** 632–640.
8. Bernhardt, J. & Neumann, E. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 3756–3760.
9. Moore, H.-P. H., Hartig, P. R., Wu, W.C.-S. & Raftery, M. A. (1979) *Biochem. Biophys. Res. Commun.* **88,** 735–743.
10. Lauger, P. (1976) *Biochim. Biophys. Acta* **455,** 493–509.
11. Raftery, M. A., Schmidt, J. & Clark, D. G. (1972) *Arch. Biochem. Biophys.* **152,** 882–886.
12. Hamilton, S. L., McLaughlin, M. & Karlin, A. (1977) *Biochem. Biophys. Res. Commun.* **79,** 692–699.
13. Chang, H. W. & Bock, E. (1977) *Biochemistry* **16,** 4513–4520.
14. Moody, T., Schmidt, J. & Raftery, M. A. (1973) *Biochem. Biophys. Res. Commun.* **53,** 761–772.
15. Damle, V. N. & Karlin, A. (1978) *Biochemistry* **17,** 2039–2045.
16. Quast, U., Schimerlik, M., Lee, T., Witzemann, V., Blanchard, S. & Raftery, M. A. (1978) *Biochemistry* **17,** 2405–2414.
17. Adams, P. R. (1975) *Pflügers Arch.* **360,** 145–153.
18. Sheridan, R. E. & Lester, H. A. (1977) *J. Gen. Physiol.* **70,** 187–219.
19. Dionne, V. E., Steinback, J. H. & Stevens, C. F. (1978) *J. Physiol. (London)* **281,** 421–444.
20. Katz, B. & Thesleff, S. (1957) *J. Physiol. (London)* **138,** 63–80.