APPARENT COOPERATIVE EFFECTS IN ACETYLCHOLINE RECEPTOR-MEDIATED ION FLUX IN ELECTROPLAX MEMBRANE PREPARATIONS

George P. Hess,† John P. Andrews, and Gary E. Struve

Section of Biochemistry
270 Clark Hall
Cornell University
Ithaca, N.Y. 14853 U.S.A.

Received February 17,1976

Summary: The kinetics of acetylcholine receptor-mediated flux of ^{22}sodium ions from microsacs has been measured in the presence of activators (carbamylcholine and decamethonium) and an inhibitor (d-tubocurarine) of neural transmission. The dependence of the first-order rate constant, k_{obs}, for ^{22}sodium ion efflux on either decamethonium or carbamylcholine concentration does not exhibit cooperativity. The apparent cooperativity observed by Kasai and Changeux in dose-response curves for ^{22}sodium flux from the same preparation is adequately accounted for by the contribution which efflux from non-excitable microsacs, the main component of the preparation, makes to the measurements. d-Tubocurarine was found to be a non-competitive inhibitor of decamethonium-activated ^{22}sodium efflux. The results of the kinetic measurements are in agreement with equilibrium measurements of the interaction of decamethonium with the same microsac preparation, i.e. adherence to a classic Langmuir binding isotherm and separate binding sites for activators and inhibitors of neural activity. The results indicate a direct relationship between ligand binding and receptor-mediated ion flux. How these two processes contribute to electrophysiological measurements is not apparent.

The binding of chemical mediators to the membrane-bound acetylcholine receptor initiates changes in the permeability of neural membranes to inorganic ions, and thus in their electrical potential (1,2). The relationship between these processes is not known. Investigations using the electroplax of Electrophorus electricus indicated marked cooperativity in electrophysiological measurements (3,4), and competitive interactions between activators and inhibitors of neural activity (4,5,6). Studies of the binding of effectors to the receptor in electroplax membrane preparations gave no evidence (7-12) for the marked cooperativity observed in electrophysiological measurements and indicated (10-12) the existence of separate binding sites for activators and inhibitors.

†To whom reprint requests and correspondence should be addressed.

In this paper we present a quantitative comparison between the binding process and the resulting acetylcholine receptor-mediated changes in permeability to sodium ions in electroplax membrane preparations. We have measured the effect of an activator of neural transmission, decamethonium, on ^{22}Na efflux in presence and absence of d-tubocurarine, an inhibitor. We also present data on the carbamylcholine-induced efflux of $^{22}Na^+$, measured under the conditions of Kasai and Changeux (13-15), and an analysis of the data in terms of both receptor-mediated and unspecific flux of $^{22}Na^+$. The results indicate a direct relationship between binding of ligands and receptor-mediated ion flux.

Kasai and Changeux (13-15) have elegantly demonstrated that microsacs from electroplax membranes exhibit acetylcholine receptor-mediated ion flux. We (16) have recently developed an approach by which the receptor-mediated flux of $^{22}Na^+$ from electroplax microsac preparations can be analyzed without the measurements being obscured by efflux from non-excitable microsacs, the main component of the membrane preparation (16). In our experiments the efflux followed a single exponential decay. The following relationship between the observed first-order rate constant for $^{22}Na^+$ efflux, k_{obs}, and carbamylcholine concentration was established: $k_{obs} = k' \frac{L}{L + K_D}$ (eq.1). L represents the molar concentration of effector and K_D is the apparent dissociation constant of the receptor-ligand complex. k' is the maximum observable efflux rate at infinite ligand concentration and has been defined previously (16). In Figure (1a) k_{obs} values, obtained in the absence and presence of 0.2 μM and 0.5 μM d-tubocurarine, at pH 7.0 and 4°C, are plotted as a function of decamethonium concentration according to a linear form of equation (1). In order to demonstrate that our results are reproducible and independent of membrane preparation or eel, we measured efflux in the absence of d-tubocurarine from microsacs prepared from five different eels. The coordinates of the solid lines were computed by a linear least-squares method. The ordinate intercept of the line determined in absence of d-tubocurarine gives a k' value of 0.15 ± 0.01 min^{-1}. The slope of the line gives a value for K_D for decamethonium of 0.5 ± 0.15 μM. In equilibrium binding experiments (11), K_D was

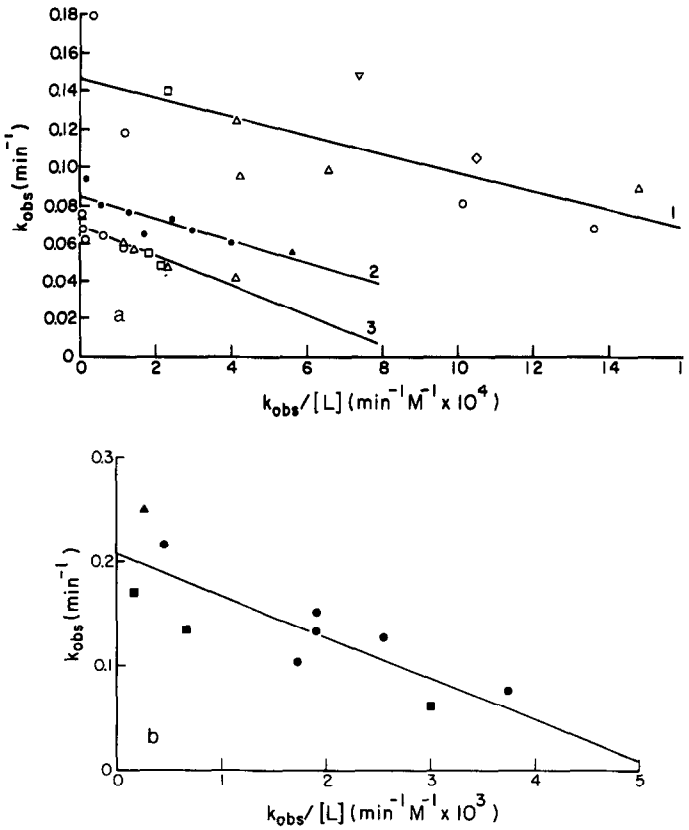

Figure 1: Acetylcholine receptor-mediated ^{22}Na flux from microsacs, pH 7.0, 4°C. The electroplax membranes were prepared essentially as described by Kasai and Changeux (13-15). In the experiments illustrated in (a) the microsacs were equilibrated with 10 mM NaCl, 90 mM KCl, and 0.4 M sucrose, and efflux was measured in a solution of the same composition, with the addition of 1 mM phosphate buffer, pH 7.0. In the experiment illustrated in (b) the microsacs were equilibrated with a solution of 10 mM NaCl and 0.4 M sucrose, and efflux was measured in a solution of 170 mM KCl, 2 mM $CaCl_2$, 1 mM phosphate buffer, pH 7.0. The equilibration with 0.24 μM $^{22}NaCl$ (stock solution 1 mCi/ml) was done under conditions which allow one to measure acetylcholine receptor-mediated efflux only (16). $k_{(obs)}$ was evaluated as described previously (16). A linear least-square computer program, giving the standard error of slope and intercept, was used in the analysis of efflux measurements. The amount of $^{22}Na^+$ retained by the microsacs was determined by a Millipore filter assay. Materials were obtained as indicated in (16). Different symbols represent membrane preparations from different eels.

(a) The dependence of k_{obs} on decamethonium concentration in absence (curve 1) and presence of 0.2 μM (curve 2) and 0.5 μM (curve 3) d-tubocurarine. The data were plotted according to a linear form of eq. (1), $k_{obs} = k' - k_{obs} \times K_D \times L^{-1}$. The slopes of the lines are proportional to K_D, and the intercepts to k' in absence of d-tubocurarine, and to k'_I in presence of the inhibitor. $k'_I = k'K_I (I_0 + K_I)^{-1}$ in presence of a non-competitive inhibitor; I_0 is the molar concentration of the inhibitor and K_I the dissociation constant of the inhibitor from its complex.
Curve (1): $K_D = 0.5 \pm 0.15$ μM; $k' = 0.15 \pm 0.01$ min^{-1}; Curve (2): $K_D = 0.6 \pm 0.1$ μM; $k'_I = 0.08 \pm 0.01$ min^{-1}; Curve (3): $K_D = 0.8 \pm 0.1$ μM; $k'_I = 0.07 \pm 0.002$ min^{-1}.
(b) The dependence of k_{obs} on carbamylcholine concentration. The data were plotted as in (a). $K_D = 40 \pm 9$ μM; $k' = 0.21 \pm 0.02$ min^{-1}.

found to be 0.3 ± 0.2 μM at pH 7.0 and 4°C. Under the same experimental conditions, but with carbamylcholine as ligand, the value of k' was 0.17 ± 0.02 min^{-1} (16). The effect of d-tubocurarine on decamethonium-induced $^{22}Na^{+}$ efflux is typical of non-competitive inhibitors. The ordinate intercept of the lines, representing the efflux rate at infinite concentrations of decamethonium, is affected, indicating that decamethonium can not displace d-tubocurarine from its sites. Within experimental error, the slope of the lines is not affected. This means that decamethonium binds as well to the receptor in presence of d-tubocurarine as in its absence. The relationship between k' in presence and absence of inhibitor, assuming a simple non-competitive inhibition, is given in the Figure legend. The dissociation constant for d-tubocurarine calculated from this relationship is 0.3 μM. Direct measurements of the binding process with membranes prepared by the same method gave a value of 0.2 μM and indicated separate binding sites for inhibitors and activators of neural activity (10-12).

We have found no evidence for cooperativity in decamethonium-induced $^{22}Na^{+}$ efflux (Fig.1(a)), in agreement with results obtained previously with carbamylcholine (16). With the exception of $^{22}Na^{+}$, the components of the internal and external media of the microsacs were at equilibrium at the start of the efflux measurements. The experimental conditions chosen by Kasai and Changeux (13-15) for efflux measurements were such that NaCl inside the microsacs was opposed by a high concentration of KCl outside. In order to see whether the resulting counterflow of opposing ions was responsible for the cooperative phenomena observed in their experiments but not in ours, we investigated the carbamylcholine-induced $^{22}Na^{+}$ efflux using their conditions. The results of this experiment are shown in Figure 1(b). The fit of the data, when plotted according to a linear form of equation (1), does not provide evidence for a cooperative process.

In Figure 2(a) the percent of $^{22}Na^{+}$ remaining in the microsacs is plotted on a logarithmic scale as a function of time. τ_0 and τ represent the time at which the microsacs have lost 50% of their initial $^{22}Na^{+}$ content in absence and presence of 1×10^{-3} M carbamylcholine respectively. Kasai and Changeux (13-15)

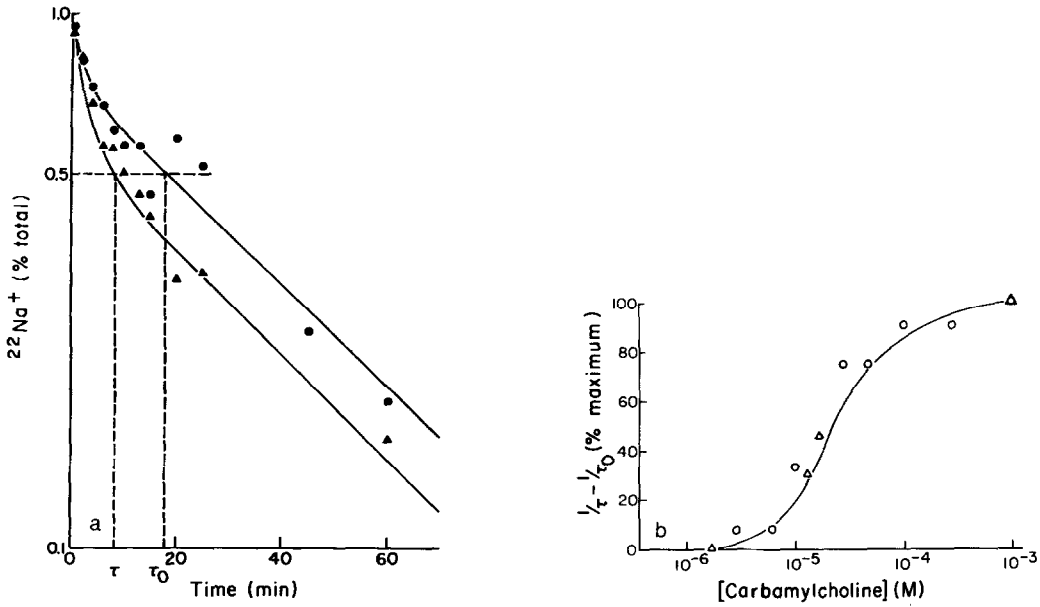

Figure 2: $^{22}Na^+$ flux from microsacs measured according to the procedure of Kasai and Changeux (13-15), pH 7.0, 4°C.
The microsacs were incubated overnight with a solution of 10 mM NaCl, 0.24 μM $^{22}NaCl$, and 0.4 M sucrose. Efflux in absence or presence of carbamylcholine was initiated by diluting the microsacs 50-fold with a solution 170 mM KCl, 2 mM $CaCl_2$, 1 mM phosphate buffer, pH 7.0.

(a) The data are plotted on a semi-logarithmic scale as log fraction of $^{22}Na^+$ retained by the microsacs at zero time versus time. The time for half-equilibration of the microsacs with the dilution buffer is indicated by τ_0 for the experiment in absence of carbamylcholine, and by τ for the experiment in presence of 10^{-3}M carbamylcholine.
●, Efflux in absence of carbamylcholine. The observed time-course of the reaction was fitted to the sum of two exponentials:
$[^{22}Na^+]_t = [^{22}Na^+]_{t=0} (\alpha e^{-k_1 t} + \beta e^{-k_2 t})$. The solid line was computed using the following parameters which were evaluated by use of a non-linear least-square computer program: $\alpha = 0.26$, $\beta = 0.74$, $k_1 = 0.49$ min^{-1}, $k_2 = 0.02$ min^{-1}.
▲, efflux in the presence of 1×10^{-3} M carbamylcholine. The parameters of the line were computed by use of eq. (2) and were evaluated as described in the text. Typically, after diluting the membrane preparation to initiate efflux, the experimental points were scattered. This affects only the evaluation of k_1 which does not contribute to the calculation of τ values (see text). The value of k_2 is based on all experiments with the same membrane preparation and on measurements in the 1 hr to 5 hr time period which are not shown for aesthetic reasons.
(b) Dependence of $1/\tau - 1/\tau_0$ on carbamylcholine concentration. The different symbols represent experimentally determined points obtained on different days. The solid line was computed by use of eq.(2) and the parameters given in the text. For reasons of comparison and convenience, a semilogarithmic scale is used.

---

defined an excitability factor $(\frac{\tau_0}{\tau} - 1)$, and found that its value varied from preparation to preparation. Our values fell within the range reported (13-15, 17). In this experiment the value was 1.0. The time-course of the $^{22}Na^+$ efflux in

absence of ligand falls into two clearly separable steps (Fig. 2(a)). The coordinates of the upper curve in Figure 2(a) were computed by fitting the time-course of the efflux to the sum of two exponentials (see the legend to the Figure). A non-linear least squares computer program was used to evaluate the exponentials pertaining to the fast and slow steps, k_1 and k_2. The values obtained were 0.49 min^{-1} and 0.02 min^{-1}.

The following equation was used to evaluate the efflux data in presence of effectors:

$$[^{22}Na^+]_t = [^{22}Na^+]_{t=0} (\alpha e^{-k_1 t} + \beta e^{-k_2 t} + \gamma e^{-k_{obs} t}) \quad \text{(eq. 2)}.$$

The procedures used for obtaining the parameters of the equation and the assumptions made are described below. (1) The k_1 and k_2 values were obtained from experiments in which effector was not present (Fig. 2(a) upper curve) for the following reasons: The slope of the lines pertaining to the slow phase of the $^{22}Na^+$ efflux is essentially the same in presence and absence of carbamylcholine (Fig. 2(a)), indicating that this activator has no observable effect on that phase of the reaction, and that the value of k_2 is the same in both experiments. When the microsacs were incubated with $^{22}Na^+$ for 20 minutes and the efflux was measured immediately, only the fast phase of the efflux was seen and carbamylcholine had no effect (16). From this we deduced that the efflux rate constant, k_1, was also not affected by carbamylcholine. (2) The coefficients α, β, and γ were determined as follows: the ordinate intercepts, obtained by extrapolating the progress curves of the slow phase of the reaction, gives the β values in absence (0.74) and in presence (0.56) of carbamylcholine. The difference between these values (0.18 in this case) varied from preparation to preparation and corresponded, within experimental error, to the fraction of $^{22}Na^+$ efflux mediated by the acetylcholine receptor, investigated under conditions where only specific efflux was measured (16). We assigned the value of 0.18 to γ in equation (2), and, therefore, α is 0.26. (3) At any given ligand concentration the k_{obs} values were calculated from the k' (0.21 min^{-1}) and K_D (40 μM) values determined in the experiment shown in Figure 1(b).

Using these independently determined parameters and equation (2) we found

good agreement between calculated and experimentally determined τ_0 and τ values. Figure 2(b) illustrates the dependence of $(1/\tau - 1/\tau_0)$ on carbamylcholine concentration, and the relationship between calculated and experimentally determined values. In agreement with the data of Kasai and Changeux (13-15) the graph (Fig. 2(b)) shows a sigmoidal rather than a hyperbolic curve and gives an apparent Hill coefficient of 1.5. However, no assumption of cooperativity was made in the calculation. The shape of the curve can be understood if one calculates the contribution each of the processes makes to the half-equilibration time; at all effector concentrations, 26% of the original $^{22}Na^+$ content of the microsacs is lost in the α-process. At low carbamylcholine concentrations (5 μM), 20% is contributed by the β process, and only 4% by the specific γ process; at high ligand concentrations (1 mM) the β process contributes 8%, and the γ process 16%, or 4 times more than at low ligand concentration. Thus ligand-receptor interaction is much less effective in determining τ values at low ligand concentrations than it is at high concentrations. The observed effect is similar to a cooperative phenomenon in which the affinity of the ligand-binding sites increases with increasing ligand concentration.

A phenomenon known as desensitization has been observed in experiments with membrane-bound receptor (4,6,18). In desensitization, the effector-induced increase in the permeability of the cell membrane to inorganic ions decreases with time even though the effector is present. A comparison of the $^{22}Na^+$ content of the microsacs before addition of effector, and the zero time value determined from the rate of specific effector-induced ^{22}Na efflux indicates the absence of a significant fast release of $^{22}Na^+$ which occurs prior to the process which we measure. The electrophysiological experiments which indicated cooperativity (3) involved exposure of the receptor to ligand concentrations for equal or longer times than we used. Therefore, desensitization does not appear to be responsible for the difference in the effector concentration-response curves observed in ion flux and electrophysiological measurements with electroplax membranes.

The Kasai and Changeux membrane preparations (13-15) are an important ad-

vance because they allow one to investigate receptor-ligand interaction and permeability changes under specified conditions in the same membrane preparation. The comparison between these two processes required the development of methods (10-12, 16) which allowed one to study quantitatively the specific properties of the receptor which is only a very small component of the membrane preparation. The absence of cooperativity in receptor-mediated efflux of sodium ions, and the non-competitive interaction between d-tubocurarine and an activator of neural transmission, indicate a direct relationship between ligand binding and receptor-mediated ion flux. How the two measured processes contribute to electrophysiological measurements is an interesting problem for future work.

Acknowledgments: This work was supported financially by Grant NS 08527 awarded by the National Institutes of Health and Grant BMS 72-01908 awarded by the National Science Foundation. J.P.A. was the recipient of U.S. Public Health Service Fellowship No. NS 05107; G.S. received No. NS 05095.

REFERENCES

1. Katz, B., and Miledi, R. (1972) J.Physiol. (London) 224, 665-699.
2. Nachmansohn, D., and Neumann, E. (1975) "Chemical and Molecular Basis of Nerve Activity" (Academic Press, New York).
3. Changeux, J.-P., and Podleski, T.R. (1968) Proc.Nat.Acad.Sci.USA 59, 944-950.
4. Lester, H.A., Changeux, J.-P., and Sheridan, R.E. (1975) J.Gen.Physiol. 65, 797-816.
5. Higman, H.B., Podleski, T.R., and Bartels, E. (1963) Biochim.Biophys.Acta 75 187-193.
6. Sheridan, R.E., and Lester, H.A. (1975) Proc.Nat.Acad.Sci.USA 72, 3496-3500.
7. Eldefrawi, M.E., Eldefrawi, A.T., and O'Brien, R.D. (1972) Mol.Pharmacol. 7, 104-110.
8. Weber, M., and Changeux, J.-P. (1973) Mol.Pharmacol. 10, 15-34.
9. Cohen, J.B., and Changeux, J.-P. (1973) Biochemistry 12, 4855-4864.
10. Bulger, J.E., and Hess, G.P. (1973) Biochem.Biophys.Res.Commun. 54, 677-684.
11. Fu, J.-j.L., Donner, D.B., and Hess, G.P. (1974) Biochem.Biophys.Res.Commun. 60, 1072-1080.
12. Hess, G.P., Bulger, J.E., Fu, J.-j.L., Hindy, E.F., and Silberstein, R.J. (1975) Biochem.Biophys.Res.Commun. 64, 1018-1027.
13. Kasai, M. and Changeux, J.-P. (1971) J.Membr.Biol. 6, 1-23.
14. Kasai, M. and Changeux, J.-P. (1971) J.Membr.Biol. 6, 24-57.
15. Kasai, M. and Changeux, J.-P. (1971) J.Membr.Biol. 6, 58-80.
16. Hess, G.P., Andrews, J. P., Struve, G.E., and Coombs, S.E. (1975) Proc.Nat. Acad.Sci. USA 72, 4371-4375.
17. McNamee, M.G., and McConnell, H.M. (1973) Biochemistry 12, 2951-2958.
18. Katz, B., and Thesleff, S. (1957) J.Physiol. (London) 138, 63-80.