Acetylcholine-receptor-mediated ion flux in electroplax membrane preparations

(sodium/potassium/microsacs/*Electrophorus electricus*/carbamylcholine)

GEORGE P. HESS*, JOHN P. ANDREWS, GARY E. STRUVE, AND SUSAN E. COOMBS

Section of Biochemistry, 270 Clark Hall, Cornell University, Ithaca, New York 14853

ABSTRACT   The kinetics of acetylcholine-receptor-mediated sodium efflux from electroplax microsacs of *Electrophorus electricus* has been analyzed. This led to the discovery that only a small fraction of the observed efflux is affected by chemical effectors such as carbamylcholine. Experimental conditions were chosen so that the receptor-mediated flux could be analyzed without the measurements' being obscured by efflux from the nonexcitable microsacs. Near equilibrium the efflux follows a single exponential decay. The apparent first order rate constant for sodium-22 efflux was determined as a function of effector concentration and is considerably higher than previously estimated. The process does not show cooperativity under the experimental conditions, in agreement with the binding isotherms of effectors and the same membrane preparation. The presence of potassium ions inhibits the receptor-mediated sodium flux. It is suggested that interaction of inorganic ions with the receptor may play an important role in the cooperative effects observed in electrophysiological experiments.

Nerve impulse generation and transmission involve permeability changes of the neural membrane to Na^+ and K^+ (1). Initiation of increased ion flow across the membrane by the binding of chemical effectors to a specific membrane-bound receptor protein plays an important role in bioelectric phenomena (2–4). The effector–receptor interactions are believed to provide the basis for integrated neural responses. The underlying molecular mechanism, by which the interaction of chemical mediators with the receptor regulates the flux of inorganic ions, is unknown. In this paper we report the study of a fundamental process in the acetylcholine-receptor-mediated ion flux in electroplax microsacs, which is closely related to chemical mediator-receptor interaction.

Electroplax membrane preparations from *Electrophorus electricus* were chosen for the experiments because they appeared to be uniquely suited for finding a correlation between interactions of chemical mediators with the receptor and changes of the membrane permeability to inorganic ions. Considerable insight into the chemical properties of these membranes, and the electrophysiological properties of single electroplax, comes from the work of Nachmansohn and colleagues (2–6).

The acetylcholine receptor of *E. electricus* has been isolated in several laboratories and is being characterized (e.g., 7–11). The interaction of effectors with the receptor in electroplax microsac preparations has been studied by equilibrium and kinetic methods (e.g., 12–20,†,‡). Kasai and Changeux have demonstrated that electroplax microsacs exhibit

receptor-mediated ion flux (12, 19, 20). The observed complexity of the efflux, the apparent inefficiency of the effector-induced efflux, and the variability of the efficiency from preparation to preparation suggested that the parameters measured by these authors reflected only partially the underlying primary process. For these reasons we reinvestigated the efflux of sodium ions from the microsacs, and found that the receptor-mediated flux, which constitutes only a small component of the total observed flux, is obscured by efflux from the non-excitable microsacs.

MATERIALS AND METHODS

Electric eels were obtained from World Wide Scientific Animals, Ardsley, N.Y. The microsacs were prepared, and ion flux determined, essentially as described by Kasai and Changeux (19). Protein concentration and acetylcholinesterase activity were determined by the methods of Lowry *et al.* (21) and Ellman *et al.* (22), respectively. The concentration of α-bungarotoxin sites was determined with ^{125}I-labeled α-bungarotoxin (23) according to the procedure of Bulger and Hess (16). Carbamylcholine chloride (= carbamoylcholine chloride) and *d*-tubocurarine chloride were obtained from the Sigma Chemical Co. and ICN-K & K, respectively. All other chemicals were reagent grade and were obtained from either the Fisher Scientific Co. or the Mallinkrodt Chemical Co. A least squares computer program was used to evaluate the efflux rate coefficient k_{obs} (see Eq. 1).

RESULTS

Fig. 1a depicts the results of an experiment performed under the experimental conditions of Kasai and Changeux (19, 20). The microsacs were incubated overnight with 0.6 M sucrose, 10 mM NaCl, and 73 nm $^{22}NaCl$ (1 mCi/ml). At zero time, the incubation mixture was diluted to 70-fold with a solution that was 170 mM KCl, 2 mM $CaCl_2$, 1 mM phosphate buffer, pH 7.0. The amount of $^{22}Na^+$ retained by the microsacs was determined by a Millipore filter assay. The lower and upper curves were obtained in the presence and absence of 10^{-4} M carbamylcholine chloride, respectively. An excitability factor, which was used to calculate ion flux, has been defined by Kasai and Changeux (19) as $(\tau_0/\tau) - 1$, where τ and τ_0 represent the half time for complete equilibration of the microsacs with the dilution buffer in the presence and absence of effector, respectively. Our value of 1.1 is in good agreement with the values of 1 to 4 obtained by Kasai and Changeux (19), and by McNamee and McConnell (24). The solid lines in Fig. 1a were obtained by analysis of the efflux data using a non-linear least square computer program to fit the data to the sum of two exponentials. A good fit of the data is obtained in the absence and presence of carbamylcholine. Except for a very rapid initial release of $^{22}Na^+$ in the presence of effector, the calcu-

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lated curves appear to be identical. This analysis of the data suggested to us that the active microsacs constitute only a small fraction of the vesicle preparation, and that only the fast initial flux contributes to the decrease in the half-equilibration time. Experiments showed that the active vesicles of a microsac preparation were much less permeable to sodium ions in the absence of an effector than the chemically nonexcitable microsacs were (see Fig. 1b). Advantage was taken of these deductions and experimental conditions were chosen which allowed us to investigate the relevant process. In the experiment shown in Fig. 1b, the vesicles were equilibrated for 12 hr with 0.4 M sucrose, 10 mM NaCl, and 90 mM KCl. Just prior to efflux measurements, ^{22}NaCl was added and equilibration was allowed to proceed in the absence (lower curve) and presence (upper curve) of 10^{-4} M carbamylcholine for 20–40 min. At the end of this time, the vesicle preparation was diluted 100-fold with a solution of the same composition as the incubation solution, and the efflux was measured. The data indicate that in this short time the microsacs incubated in the presence of carbamylcholine (circles) took up more ^{22}Na$^+$ than those incubated without the effector (triangles). Furthermore, a difference in ^{22}Na$^+$ content was maintained for a number of hours. Addition of carbamylcholine to the microsacs incubated in the presence of carbamylcholine causes a rapid release of ^{22}Na$^+$ and the experimental and control curves become identical. The amount of ^{22}Na$^+$ inside the excitable microsacs is about 15% of the ^{22}Na$^+$ present initially. This rapid efflux is abolished by the presence of 2 μM d-tubocurarine. At constant concentrations of carbamylcholine the decrease in efflux rate depends on the concentration of d-tubocurarine. Similar results have been reported by Kasai and Changeux (19, 20). Addition of carbamylcholine to the microsacs incubated in the absence of carbamylcholine does not cause a rapid release of ^{22}Na$^+$ (Fig. 1b). Evidently, the excitable microsacs do not incorporate significant amounts of ^{22}Na$^+$ under these conditions. Our procedures make it possible, therefore, to analyze in detail the receptor-mediated ion flux of the excitable microsacs without the measurements' being obscured by efflux from nonexcitable microsacs, which are the main component of the preparation.

The experimental conditions chosen by Kasai and Changeux (12, 19, 20) for quantitative measurements, selected presumably to mimic physiological conditions, were such that concentrations of sodium chloride inside the microsacs were opposed by large concentrations of KCl outside the vesicles. In our preliminary investigations we tried to avoid the difficulties involved in analyzing exchange diffusion processes (25) inherent under such experimental conditions, and chose conditions in which the microsacs were near equilibrium with the external medium. Under these conditions, the differential equations pertaining to the system simplify.

The efflux experiment Fig. 2a was initiated several hours before addition of carbamylcholine to allow the sodium ions of the non-excitable microsacs to equilibrate with the medium. Zero time refers to the time of addition of carbamylcholine to the microsac preparation. As can be seen, the data for carbamylcholine-induced ^{22}Na$^+$ efflux follow a single exponential decay:

$$[^{22}\text{Na}^+]_t = [^{22}\text{Na}^+]_{t=0}e^{-k_{obs}t} \qquad [1]$$

In this equation [^{22}Na$^+$] represents the concentration of radioactive ions inside the microsacs. k_{obs} is an overall flux rate constant which reflects the movement of ions in both directions across the microsac membrane. The back diffusion of
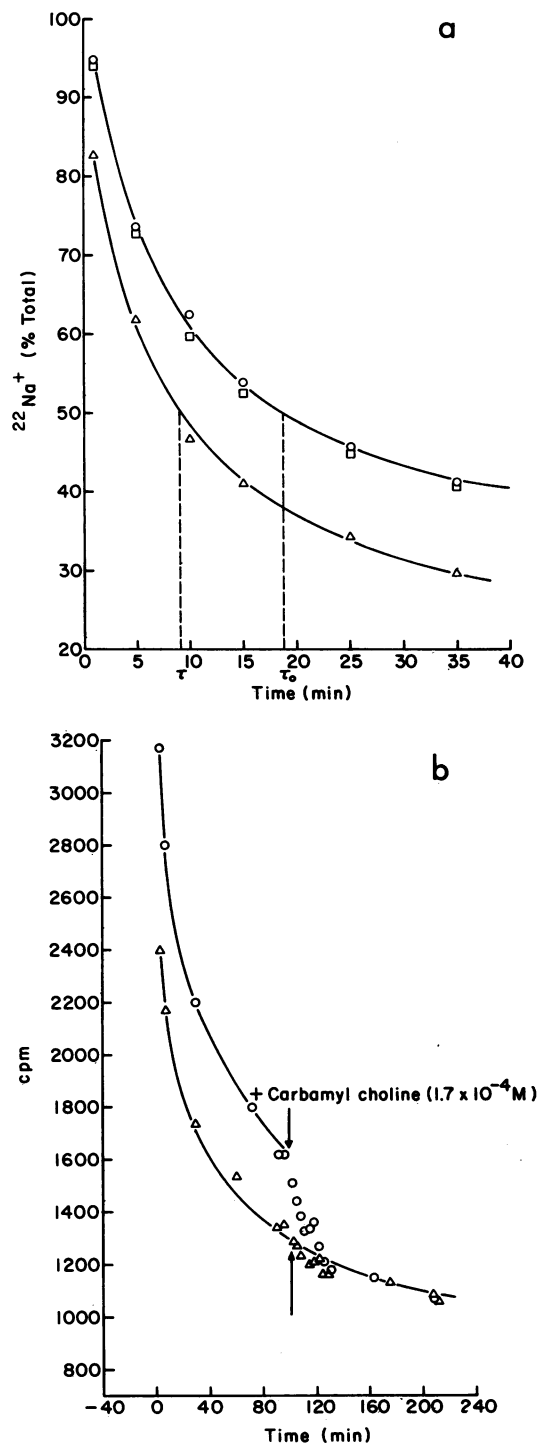

FIG. 1. (a) ^{22}Na$^+$ efflux from *E. electricus* electroplax membrane vesicles, diluted 70-fold according to the procedure of Kasai and Changeux (19), at pH 7.0 and 22°. The time for half-equilibration of the microsacs with the dilution buffer is given for the control curve (τ_0) and carbamylcholine-mediated flux curve (τ). O, efflux in the absence of effector (control); △, efflux in the presence of 1×10^{-4} M carbamylcholine; □, superposition by a parallel shift of the effector-mediated flux curve upon the control curve (see the Appendix). (b) Permeability of microsacs incubated in the presence and absence of carbamylcholine for 40 min and then diluted 100-fold, pH 7.0 and 4°. O, efflux of ^{22}Na$^+$ from microsacs incubated with 10^{-4} M carbamylcholine, with further addition of carbamylcholine as indicated; △, efflux of ^{22}Na$^+$ from microsacs incubated in the absence of carbamylcholine, with further addition of carbamylcholine as indicated.
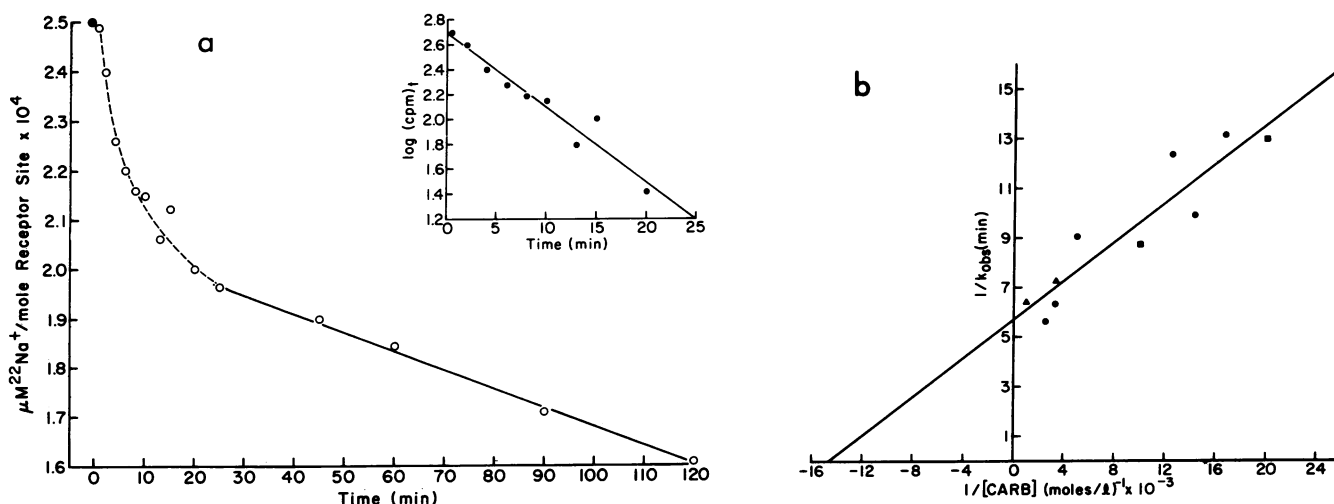
FIG. 2. (a) $^{22}Na^+$ efflux as a function of time. The microsacs containing 10 mM NaCl and 90 mM KCl were diluted 50-fold into a dilution mixture of identical salt composition, pH 7.0, 4°. O, experimentally determined points; broken line, computed according to Eq. 1 (see the *text*); solid line, computer fit based on all experiments with the same membrane preparation. *Inset* is a typical first order plot for $^{22}Na^+$ efflux in the presence of 2×10^{-4} M carbamylcholine. k_{obs} was determined from the slope of the linear least squares fit of the experimental points. The value of the slope was calculated to be -0.06 ± 0.007 min^{-1}, and k_{obs} has a value of 0.14 ± 0.02 min^{-1}. (b) Double reciprocal plot (35) of carbamylcholine-mediated $^{22}Na^+$ efflux from *E. electricus* microsacs. All determinations of k_{obs} were performed at 4°, pH 7.0, 10 mM NaCl, 90 mM KCl. The microsacs were equilibrated, and the flux measured, under conditions of equal osmolarity and ionic strength. Each symbol represents membrane preparations from different eels. The linear least squares fit of the experimental points gave a slope of $3.9 \pm 0.6 \times 10^{-4}$ M min and an intercept of 5.7 ± 0.6 min.

$^{22}Na^+$ and its concentration at $t = \infty$ can be neglected, however, because the volume of the external solution is large compared with the internal volume of the microsacs. The broken line was computed on the basis of Eq. 1. The solid point in Fig. 2a gives the concentration of $^{22}Na^+$ inside the microsacs at zero time. In this and all other experiments reported here, the calculated zero time value was found to be in excellent agreement with the experimentally observed values, indicating that we have not missed a very rapid initial process which contributes to the measurements. The inset to Fig. 2a contains a log $[^{22}Na^+]_t$ versus time plot for the efflux of $^{22}Na^+$ at a carbamylcholine concentration of 2×10^{-4} M. The coordinates of the line were computed by the method of least squares, giving a value for k_{obs} of 0.14 ± 0.02 min^{-1}. k_{obs} values were obtained at different carbamylcholine concentrations at pH 7.0, 4°. Assuming that the rate of sodium efflux is directly proportional to the concentration of the receptor–ligand complex, and that each complex contributes identically to the rate process, the following relationship is consistent with the data:

$$k_{obs} = k' \frac{L}{L + K_D} \qquad [2]$$

L represents the molar concentration of the effector, and K_D is the dissociation constant of the receptor–ligand complex. Assuming a simple mechanism for receptor-mediated ion flux,

$k' = $ (number of ions transported per unit time)

(number of specific receptor-formed ion gates)$^{-1}$

$\times f(R_0)$,   [3]

where $f(R_0)$ represents the maximum number of specific ion gates as a function of the molar concentration of receptor sites per unit weight of membrane protein.

Fig. 2b is a plot of $(k_{obs})^{-1}$ versus [carbamylcholine]$^{-1}$. From the intercept and slope of this plot one can calculate K_D and k', respectively. The values for k_{obs} shown in Fig. 2b

were obtained with membrane preparations from three different eels. The amount of $^{22}Na^+$ released in response to effectors, as well as the excitability factor of Kasai and Changeux (19), varied from preparation to preparation, presumably reflecting variation in the number of excitable microsacs. Within experimental error, the k_{obs} values which we have determined are identical and independent of the membrane preparation for a given concentration of effector. The value of K_D, $7 \pm 0.8 \times 10^{-5}$ M at pH 7.0 and 4°, calculated from these data, is similar to the electrophysiologically determined value at the same pH and temperature. It is important to notice from the data shown in Fig. 2b that there is apparently no evidence for cooperativity, in contrast to the dose–response curves obtained in electrophysiological measurements (6, 26) and in the flux measurements of Kasai and Changeux (19). Our data do agree well with the binding isotherms obtained in experiments with chemical mediators and the receptor bound to the same membrane vesicle preparation (17, †). A value for k' of 0.17 ± 0.02 min^{-1} was obtained from the intercept.

The data in Fig. 3 indicate that potassium ions have a small but definite effect on the effector-induced sodium efflux. The experiments were performed at a constant ionic strength but varying mole fractions of NaCl and KCl. It is to be noted that at high concentrations of KCl a 10-fold increase in NaCl concentration does not affect the value of k_{obs}. As the NaCl concentration is increased by only 50% but the KCl concentration decreased 10-fold, k_{obs} increases. A simple explanation of these data is that potassium ions inhibit the receptor-mediated efflux of sodium ions.

DISCUSSION

The Kasai and Changeux (19) membrane preparations, which allow one to investigate in the same preparation receptor–ligand interaction and permeability changes under specified conditions, are an important advance. Their experimental conditions and calculation of excitability values yielded dose–response curves similar to those observed in
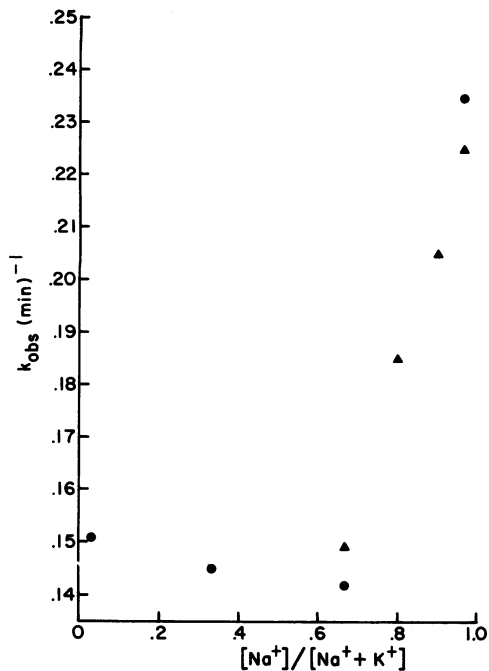
FIG. 3.   Dependence of k_{obs} on the mole fraction of NaCl at 5 × 10^{-4} M carbamylcholine, 4°, pH 7.0. All experiments were performed at a total salt concentration of 150 mM. Membrane preparations from different eels are represented by different symbols.

electrophysiological experiments. In the *Appendix* we show how this can occur even though the chemical-effector-induced ion flux is obscured by the experimental conditions. The "excitability value" used by Kasai and Changeux (19) reflects both the flux rate constant for unspecific flux and that for a small fraction of excitable microsacs in the preparation (see the *Appendix*).

The data illustrated in Fig. 2b give no evidence for cooperativity in ligand–receptor interaction, in contrast to the dose–response curves observed in electrophysiological measurements. Single homogeneous binding isotherms, with decamethonium bromide as an effector, have been observed by us (17, †) in equilibrium measurements using the same microsac preparation as we used in these experiments. Furthermore, data concerning the binding of other effectors to these membrane preparations (12, 14) also do not exhibit the cooperativity seen in electrophysiological experiments. The similarity in the effect of ligands in efflux and binding experiments and the finding that the Na^+ ion efflux data can be fitted to a single exponential indicate an intimate relationship between the measured sodium efflux and receptor-effector interaction.

The observation by Kasai and Changeux of cooperativity in sodium efflux from microsacs and in electrophysiological measurements may arise in several ways. Among these are: (*1*) The sigmoidal shape of plots of $(\tau_0/\tau) - 1$ versus ligand concentration obtained by Kasai and Changeux (19) may, at least in part, be due to the complexity of the total process which they measure (see the *Appendix* and Fig. 1a) and to the counterflow of potassium ions. (*2*) The cooperative response observed in electrophysiological experiments does not require cooperative binding if the measured parameters reflect a process which occurs several steps after the binding process (e.g., 29). (*3*) Multistep processes, involving replacement of receptor-bound calcium ions by effectors, followed by a conformational change and creation of ion channels, have been suggested by Nachmansohn (3, 4) and Neumann

(30). Evidence for calcium binding to the isolated receptor has been obtained by Eldefrawi *et al.* (31). Indications of ligand-induced conformational changes of the membrane-bound receptor come from fluorescence studies (15) and from equilibrium and kinetic experiments of Hess *et al.* (16–18, †, ‡). The minimum mechanism proposed on the basis of equilibrium and kinetic measurements (18, †, ‡) is essentially the same as that proposed to account for the kinetic and regulatory properties of a number of allosteric enzymes which operate by ligand-induced conformational changes (32, 33). Such a mechanism can give hyperbolic binding isotherms, positive cooperativity, or negative cooperativity, depending on whether subunit interactions remain the same, increase, or decrease upon ligand binding (32, 33). Investigations of one of these enzymes by Kirschner *et al.* (34) showed that cooperative effects are seen only under specific conditions of pH and temperature. Further investigations of receptor-mediated ion flux are expected to reveal whether cooperative effects will be seen in microsacs under appropriate conditions of temperature and concentration of inorganic ions.

An important question which has been raised by Katz and Miledi (27) and by Rang (28) is whether or not the chemically induced ion flux observed in electroplax microsacs is relevant to electrophysiological measurements. Fluctuation analysis by Katz and Miledi (27) of acetylcholine-initiated noise of the membrane potential of frog neuromuscular preparations indicated a transfer of about 6 × 10^4 ions per channel per msec, a value very similar to one calculated by Nachmansohn (3, 4). As Katz and Miledi state, the experiments do not give information regarding the number of receptor binding sites involved in opening a channel, although this information could be obtained in principle by varying the effector concentration in the fluctuation experiments. Similarly, we do not have enough information to calculate the moles of ions moving through the membrane per unit time per mole of receptor site in the efflux experiments since we do not know $f(R_0)$ in Eq. 3.

Another problem is that the receptor-mediated ion transport involves only a small fraction of the microsacs present. Since receptor sites and intact vesicles appear to be prerequisites for efflux studies with excitable microsacs, the number of receptor sites per mg of membrane proteins most likely overestimates the pertinent number of sites. Until these questions are answered the data from efflux and electrophysiological measurements cannot be compared. We can, however, compare k' to the value of $(1/\tau) - (1/\tau_0)$ obtained by the method of Kasai and Changeux (19) (Fig. 1a) at room temperature. Assuming that the dissociation constant of the receptor–carbamylcholine complex calculated from flux measurements in 10 NaCl and 90 mM KCl (Fig. 2b) is approximately the same as in 145 mM NaCl and 5 mM KCl, we calculate a value for k' of 1 min^{-1} at room temperature from estimates of the temperature dependence of the rate constant (20). This is 20 times larger than the value for $(1/\tau) - (1/\tau_0)$ obtained in the type of experiment illustrated in Fig. 1a.

Only a few of the important problems which may be answered by investigating ion flux in excitable microsacs have been mentioned above. The data obtained so far suggest that systematic investigations of the receptor-mediated ion flux which we have characterized, using Kasai and Changeux membrane preparations, may be capable of giving considerable additional information about the mechanism of the regulatory processes in nerve impulse generation and transmission.

APPENDIX

The relationship between τ, the half-equilibration time of Kasai and Changeux (18, 19), and the k_{obs} value which we determine can be established from the data shown in Fig. 1a. The sodium efflux data shown were fitted to the sum of two exponentials in the absence of effector and to the sum of three exponentials in the presence of effector. We assume that both in the absence and in the presence of effector a fraction of the vesicles, α, shows a fast efflux rate k_1, and a fraction, β, shows a slow efflux rate k_2. We further assume that the fraction of "excitable" microsacs, γ, shows a slow efflux rate k_2 in the absence of effector, and an effector-mediated rate, k_{obs} (see Eq. 2 in the text) in the presence of a cholinergic ligand.

The equation pertaining to sodium efflux in the presence of an effector (Fig. 1a) to which we fit our data is:

$$[^{22}\mathrm{Na}]_t = [^{22}\mathrm{Na}]_{t=0}(\alpha e^{-k_1 t} + \beta e^{-k_2 t} + \gamma e^{-k_{obs} t}) \qquad [1A]$$

Using a nonlinear least square computer program we evaluate α, k_1, and k_2. We have evaluated γ in two ways: from the difference between the fraction of reaction in the slow phase in the presence and absence of effector; and by the direct determination of k_{obs}, k', and K_D, as described in the text (see Eq. 2 in the text). The following values were used to calculate the coordinates of the lines shown in Fig. 1a:

$$
\begin{aligned}
\alpha &= 0.35 & \beta &= 0.50 & \gamma &= 0.15 \\
k_1 &= 0.27\ \mathrm{min}^{-1} & k_2 &= 0.013\ \mathrm{min}^{-1} & k' &= 0.23\ \mathrm{min}^{-1} \\
& & & & K_D &= 4 \times 10^{-5}\ \mathrm{M}
\end{aligned}
$$

We can now calculate τ_0 and τ. $\tau_0 = 20$ minutes [τ_0(observed) = 19 min]. At carbamylcholine concentrations of 10^{-3} M, 10^{-4} M, and 5 $\times 10^{-6}$ M, $\tau = 8$ min, 9 min [τ(observed) = 9 min] and 16 minutes, respectively.

Analysis of the efflux curves in Fig. 1a shows that τ is dependent on effector concentration. All the coefficients and exponentials of Eq. 1A are involved in determining τ. At low ligand concentration, when k_{obs} is comparatively small, the term in equation 1A, $\beta e^{-k_2 t}$, becomes important, thus reducing the half-equilibration time τ. At high ligand concentration when $k_{obs} \approx k_1$, the term in equation 1A, $\alpha e^{-k_1 t}$, becomes important and increases the τ value. The excitability value of Kasai and Changeux [$(\tau_0/\tau) - 1)$] will, therefore, be high relative to k_{obs} at low ligand concentration, and low relative to k_{obs} at high ligand concentration. Membrane preparations are expected to contain various fractions of excitable microsacs. This will affect the coefficients of the efflux equation 1A and, therefore, the values of both τ_0 and τ. The experimental observations are (18, 19) that the excitability factor is constant for any given preparation, but varies from preparation to preparation by as much as 4-fold.

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