Acetylcholine-receptor-mediated ion flux in electroplax membrane microsacs (vesicles): Change in mechanism produced by asymmetrical distribution of sodium and potassium ions

(asymmetrical physiological distribution of ions/ion flux mechanism/excitability/"desensitization")

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ABSTRACT The kinetics of acetylcholine-receptor-mediated efflux of inorganic ions from electroplax microsacs of Electrophorus electricus in the presence of varying alkali metal ion concentrations on both sides of the membrane have been investigated. The efflux, a monophasic process when the ion distribution is symmetrical (the same concentrations and types of ions on both sides of the membrane), becomes a biphasic process, consisting of a very rapid initial release of ions followed by a slower first-order process, under conditions that resemble the physiological state of the neural membrane (potassium ions inside the microsacs and sodium ions on the outside). The initial phase of the efflux discriminates between calcium and sodium ions and is inhibited by potassium ions in the external solution. The rate constant associated with this phase is at least 40 times larger than the rate constant associated with the slower efflux. Both phases depend on the concentration of acetylcholine or carbamoylcholine, and are inhibited by receptor inhibitors (d-tubocurarine and α-bungarotoxin).

A simple model is proposed which relates the kinetics of the flux to ligand-induced conformational changes in the receptor. We also indicate the relationship between the biphasic kinetics of the flux observed in microsacs to "desensitization," the phenomenon in which, on addition of acetylcholine, the transmembrane voltage of muscle and nerve cells first increases and then decreases to its resting value within a few seconds.

The acetylcholine receptor is a membrane-bound protein of nerve and muscle cells which, upon binding of appropriate ligands, controls the flow of inorganic ions through the membrane (1, 2). The rates with which specific ions move across the membrane play a major role in determining the transmembrane voltage of these cells and whether a signal is propagated. Although the acetylcholine receptor has been isolated and is being characterized in many laboratories (for recent reviews see refs. 3 and 4), little is known about the relationship between the concentration of ligands and the rates with which specific inorganic ions move through the membrane. Attempts to make these measurements were made by Kasai and Changeux (5) on microsac preparations and they calculated (6) that 5 X 10^6 ions are transferred per receptor site per min in the presence of ligand. However, the value obtained with microsac preparations was less, by a factor of about 10^8, than the value determined by Katz and Miledi (7) and others (8–10) with cells by electrophysiological methods. We (11–12) have shown that one reason for the apparently low efficiency of the receptor-mediated process in the experiments of Kasai and Changeux is that the receptor-mediated flux of inorganic ions is from only a small percentage of the microsacs, and that the flux from microsacs that do not respond to acetylcholine dominates the kinetic measurements. Quantitative investigations of receptor-mediated fluxes have recently become possible through the development of microsac preparations (13) and kinetic techniques (11), which allow one to measure such fluxes without interference by fluxes that do not depend on acetylcholine. When the composition of the internal and external solutions is identical (90 mM KCl/10 mM NaCl/0.4 M sucrose) the receptor-mediated efflux of 22Na^+ and 86Rb^+ follows a single exponential rate law (11). The observed rate constant increases with ligand concentration, finally reaching a ligand concentration-independent value J (11). The number of inorganic ions that move through the membrane in the receptor-mediated process per min was calculated to be 10^5 (13). This is still lower, by a factor of about 100, than the value obtained with cells. The experiments reported in this paper were initiated to determine whether this discrepancy was due to potassium ions, since earlier experiments (11) suggested that they might inhibit the receptor-mediated flux of inorganic ions in microsac preparations.

MATERIALS AND METHODS

Electric eels were obtained from World Wide Scientific Animals, Ardsley, NY. The microsacs were prepared as described by Kasai and Changeux (5). Microsacs in sucrose-free solutions were prepared by pelleting the microsacs, prepared in sucrose, and resuspending them in eel Ringer's solution by the procedure of Fu et al. (14). Carbamoylcholine chloride and d-tubocurarine chloride were purchased from Sigma Chemical Co. and ICN-K & K, respectively. Acetylcholine bromide was obtained from Eastman Kodak. α-Bungarotoxin was prepared by the procedure of Bulger et al. (15). Neutralized stock solutions (1 mCi/ml) of 86RbCl, 22NaCl, and 45CaCl2, obtained from New England Nuclear, were used in microsac incubation mixtures. Tetram was the generous gift of R. D. O'Brien. All other chemicals were reagent grade and were obtained from either Fisher Scientific or Mallinckrodt. Protein concentrations and acetylcysteinesterase activities were determined by the methods of Lowry et al. (16) and Ellman et al. (17), respectively.

Ion flux measurements were made essentially as reported by Hess et al. (11). The microsac preparation, about 1.2–2.0 mg of protein per ml, was incubated overnight at 4° in appropriate solutions containing 22Na^+, 45Ca^2+, or 86Rb^+. The latter ion is thought to serve as an effective replacement for 42K^+ (18) and has the advantage of being a more stable isotope. At the beginning of each experiment the incubation mixture was diluted to a final concentration of 140–150 μg of protein per ml with appropriate solutions containing 1 mM phosphate buffer pH 7.0 at 4°. Before addition of activating ligand, the microsacs were allowed to remain in the dilution buffer for 20 min. By the end of this time the radioactive ions inside the unsppecific
**RESULTS**

Efflux of $\text{^{22}Na}^+$ and $\text{^{86}Rb}^+$ from membrane microsacs under physiological conditions is depicted in Fig. 1. The microsacs were incubated in an eel Ringer's solution that approximated the ion composition inside an electroray cell (20), and efflux was initiated by dilution of the incubation mixture into an eel Ringer's solution that approximated the extracellular ion composition (21). Unlike the single exponential efflux observed by Hess et al. (11) when the composition of the external and internal solutions was identical (Fig. 2, curve I), physiological conditions (a high concentration of potassium internally and of sodium externally) produced a very rapid initial efflux followed by a slower single exponential efflux at the concentration of ligand indicated. In the measurements shown in Fig. 1 the initial fast phase had ended before the first measurement could be made, 20 sec after addition of the ligand. To facilitate the study of the biphasic kinetics observed under physiological conditions, we used simplified incubation and dilution media containing only NaCl and KCl for the experiments shown in Fig. 2 and in subsequent experiments. The total ionic strength of Na$^+$ and K$^+$ was held constant at 100 mM both inside and outside the microsacs. In addition, all media contained 0.4 M sucrose, thereby eliminating the need to pellet the microsacs after the sucrose density gradient centrifugation. The results show that under these conditions also the type of inorganic ion in the external solution determines the flux kinetics. A single exponential efflux was observed when the internal and external solutions of the microsacs were 100 mM in KCl (Fig. 2, curve I), and biphasic efflux kinetics were observed when 100 mM KCl was inside and 100 mM NaCl outside the microsacs (Fig. 2, curve III). In this experiment the initial fast phase had ended before the first measurements could be made. The kinetics of the receptor-mediated efflux of Na$^+$ and K$^+$ were similar to those of Rb$^+$, but those of Ca$^{2+}$ (Fig. 2, curve II) differed. It can be seen in Fig. 2 that the initial fast process discriminates between Ca$^{2+}$ and Rb$^+$ on one hand and Na$^+$ and Rb$^+$ on the other. Under the same experimental conditions, when Rb$^+$ efflux was biphasic and 30% proceeded by an initial fast phase (Fig. 2, curve III), the Ca$^{2+}$ efflux followed a single exponential rate law and an initial fast phase could not be detected (Fig. 2, curve II).

To show that the conditions under which the fast process was observed did not cause physical disruption of the microsacs or changes in the amount of microsacs retained by the Millipore filter, we equilibrated the microsacs with 295 mM KCl and 10 mM sucrose. Either $\text{^{86}Rb}^+$Cl (40 $\mu$Ci/ml of incubation mixture) or $\text{^{14}C}+$sucrose (40 $\mu$Ci/ml of incubation mixture) was added to the incubation mixture. Efflux of $\text{^{86}Rb}^+$ or $\text{^{14}C}+$sucrose was then measured in a solution of 295 mM NaCl and 10 mM sucrose. Addition of 1 mM carbamoylcholine produced an initial fast phase in which 70% of the internal $\text{^{86}Rb}^+$ was exchanged for Na$^+$. No effect on the $\text{^{14}C}+$sucrose efflux was observed on addition of carbamoylcholine. In addition, it was shown that 1 mM d-tubocurarine inhibited the initial fast phase as well as the slow phase of the ligand-induced efflux. When 0.8 $\mu$M $\alpha$-bungarotoxin was added to the microsac preparation it did not affect the passive efflux of inorganic ions from microsacs, but completely abolished the effect of 1 mM carbamoylcholine.

Fig. 3 shows that K$^+$ in the external solution inhibits the initial fast phase of carbamoylcholine-induced efflux both of Rb$^+$ and Na$^+$. The ordinate of the graph indicates the fraction of the flux, $\alpha$, that occurs in the initial fast phase. As the mole fraction of Na$^+$ in the external solution increases, $\alpha$ increases.
The inhibitory effect of external $K^+$ on the initial fast phase depends also on the carbamoylcholine concentration, being greater at low than at high concentrations.

The effect of ligand concentration can be seen in Fig. 4 (open symbols). At low concentrations of carbamoylcholine (less than 1.5 times $K_b$, see Eq. 1), one observes, within the uncertainty of the measurements of ~15%, a slow efflux which obeys a single exponential rate law. At the highest carbamoylcholine concentrations used, 14 times $K_b$, about 65% of the efflux is due to the initial rapid process. The dashed line is defined by experimental measurements (15) of the reaction of [125I]-monooiodo-$\alpha$-bungarotoxin with the receptor. This reaction is also biphasic, and $\alpha$ also indicates the fraction of the reaction that proceeds by an initial rapid process.

DISCUSSION

Asymmetry of functional membrane-bound proteins such as Na$^+$-K$^+$ ATPase and cytochrome oxidase has been well documented (22, 23). The binding of ligands to the opiate receptor is markedly affected by sodium ions (24), and the affinity of ligands for the solubilized acetylcholine receptor varies with the ionic composition of the environment (25, 26). In this paper, we present evidence that the ionic composition of the medium bathing the extracellular portion of the membrane-bound acetylcholine receptor exerts major effects on the ion-translocating properties of the receptor (Figs. 1–3). The natural state of the membrane, with its asymmetrical distribution of Na$^+$ and K$^+$, gives rise to a biphasic receptor-mediated efflux with an initial very fast flux rate followed by a slower first-order rate process. A high concentration of Na$^+$ surrounding the external portion of the receptor enhances the fraction of efflux due to the initial rapid process (Figs. 1–3). Conversely, increases in the external K$^+$ concentration inhibit the fast process (Fig. 3).

There are at least two circumstances that would give rise to an initial fast efflux followed by a single exponential efflux:

(i) Two types of microsacs exist, one responding to low concentrations of ligand and characterized by slow efflux in inorganic ions, the other responding only to high concentrations of ligand and from which the efflux is fast. If this were the true situation, the total amount of radioactive ions involved in the efflux would depend on carbamoylcholine concentration. We found, however, that the amount of radioactive ions involved is the same whether the efflux is slow and monophasic or is biphasic.

(ii) The binding of ligands to the receptor results in the conversion of one receptor conformation into another, with one being associated with the fast phase of the efflux and the other with the slow phase. The conversion of one receptor conformation into another was indicated by the kinetics of the reaction of $\alpha$-bungarotoxin, a specific and irreversible inhibitor of the receptor (27), with the receptor in microsacs. The reaction was biphasic, an initial rapid phase followed by a slow phase (15, 28). The fraction of the toxin reaction that occurs in the initial fast phase depends on ligand concentration (Fig. 4, dashed line) in a similar manner and under similar experimental conditions as the fraction of the fast phase of the receptor-mediated efflux of inorganic ions from microsacs (Fig. 4, open symbols).

Several models, in which an equilibrium exists between at least two states of the receptor, have been proposed to explain (i) an increased permeability of the membrane to inorganic ions (29); (ii) "desensitization," a phenomenon observed in electrophysiological measurements with cells (30); and (iii) a number of ligand-binding experiments and kinetic measurements of the reaction of neurotoxins with the receptor (3, 15, 31–37). Our kinetic measurements of the receptor-mediated flux of inorganic ions indicate a relationship between flux rates and receptor conformations. A simple model, which relates the ligand-binding mechanism first proposed by Katz and Thesleff (30) to the kinetics of the receptor-mediated flux of inorganic ions through the membrane, is shown below.

$$
L_0 + R \underset{K_i}{\rightleftharpoons} LR \underset{J_i}{\rightarrow} J_i
$$

$$
[K^+] \overset{[Na^+]}{[Na^+] \leftarrow [K^+]}
$$

$$
L_0 + R' \underset{K_i}{\rightleftharpoons} LR' \underset{J_i}{\rightarrow} J_i
$$
In this model, \( L_0 \) represents the initial concentration of ligand, \( R \) and \( R' \) the concentrations of the two conformations of the receptor, \( K_1 \) and \( K_2 \) the receptor–ligand dissociation constants, and the vertical arrows the rate constants for the isomerization of receptor conformations. The initial fast efflux of inorganic ions, characterized by the rate constant \( J_1 \), is associated with the receptor conformation \( R \). The slow phase of the efflux, characterized by the rate constant \( J_2 \), is associated with the receptor conformation \( R' \). A feature of this model is that the interconversion between receptor conformations is in part a function of external \( \text{Na}^+ \) and \( \text{K}^+ \) concentration. We consider this a working model, which allows one to estimate the efficiency of the receptor-mediated translocation of inorganic ions and which serves as a basis for further experiments.

The equation for the efflux of inorganic ions, \([M^+]\), from microsacs, based on the model, is given by:

\[
\ln \left[ \frac{[M^+]_{t=0}}{[M^+]_i} \right] = \frac{J_1 L_0}{K_1 + L_0} \left( 1 - e^{-lt} \right) / \beta + \frac{J_2 L_0}{K_2 + L_0} t \quad [1]
\]

where \( \beta \) contains the ligand concentration-dependent rate constants for the isomerization of receptor forms.

Some of the predictions and assumptions made in the model and Eq. 1 are as follows. (i) The initial ligand concentration, \( L_0 \), is much larger than the concentration of the receptor and is considered to be constant, and the equilibration between the ligand and receptor is much faster than any subsequent steps. (ii) The initial phase of the efflux is fast compared to the second phase. Therefore, \( J_1 \) is much larger than \( J_2 \). (iii) When the exponential term in Eq. 1 decays, the efflux follows a single exponential rate law identical to one derived previously (11) when the composition of the solutions on either side of the microsacs was identical:

\[
[M^+]_t = [M^+]_{t=0} \Phi_{\text{exp}} = \left( \frac{J_2 L_0}{K_2 + L_0} t \right) \quad [2]
\]

where \( \Phi \) gives the fraction of the metal ions in the microsacs that are released in the process. The solid lines in Fig. 1 and 2 were computed according to Eq. 2. (iv) The second and slow phase of the efflux can occur only if the concentration of conformation \( R \) (associated with the initial rapid phase of the flux) becomes much smaller during the ligand binding process than the concentration of \( R' \) (associated with the slow phase of the flux). This requires \( K_1 \) to be much larger than \( K_2 \). (v) When \( K_1 \) is much larger than \( K_2 \) at low ligand concentrations, conformation \( R' \) will predominate and only a slow monophasic efflux is expected (Fig. 4). At high ligand concentrations two phases may be observed, depending on the effect of the inorganic ions in the external solutions on the equilibrium between receptor conformations \( R \) and \( R' \) and on the value of \( \beta \). If external \( \text{K}^+ \) shifts the equilibrium between \( R \) and \( R' \) all the way to the \( R' \) state, only the slow phase of the efflux will occur. If both conformations \( R \) and \( R' \) are initially present, but \( \beta \) is large, the initial fast phase of the efflux may involve a concentration of ions too low to be detected by the experimental technique used, and the efflux may appear monophasic (Fig. 4). (vi) Under the same experimental conditions the efflux may be monophasic for one metal ion, for instance, \( ^{45}\text{Ca}^{2+} \) (Fig. 2, curve II), and biphasic for another, for instance, \( ^{86}\text{Rb}^+ \) (Fig. 2, curve III), for at least two reasons. First, a different receptor may control the flux of \( ^{45}\text{Ca}^{2+} \). The values of \( J_2 \) and \( K_2 \) are, however, similar for \( ^{86}\text{Rb}^+ \) efflux in KCl solutions and \( ^{45}\text{Ca}^{2+} \) efflux in NaCl solutions, as shown by the parallel curves I and II. A second reason is that the \( J_1 \) values are different for different metal ions. If \( J_1 \) reflects the rate at which the metal ions move through the membrane and differs for \( \text{Ca}^{2+} \) and for \( \text{Rb}^+ \), the efflux may appear monophasic for one ion and biphasic for the other. This can be seen from Eq. 1, which shows that when \( L \) is greater than \( K_1 \), \( \alpha \) is determined by \( J_1/\beta \) (eqn) Eq. 1 can be used to calculate a lower limit for the value of \( J_1 \). Our earliest measurement was made 20 sec after the addition of ligand, by which time the initial fast phase of the efflux had ended. Accordingly, the half-time of the isomerization process had a maximum value of 4 sec, and \( \alpha \) had a value of about 0.65 at the highest ligand concentration used (Fig. 4). 1.2 mM carbamoylcholine. Assuming that this ligand concentration is sufficiently greater than \( K_1 \), the half-time for the isomerization process and \( \alpha \) can be used to calculate a lower limit for \( J_1 \) of 0.2 sec\(^{-1} \), a value that is 40 times larger than that of \( J_2 \). Our previous estimate of the number of ions transferred per unit time in the receptor-mediated process in microsacs (13), about 1/100 the value obtained from electrophysiological determinations, was based on measurements of the slow receptor-mediated efflux of inorganic ions from microsacs and the value of \( J_1 \). (ix) The model does not consider relatively slow changes in receptor inactivation observed in both ion flux experiments with microsacs (38) and ligand binding experiments (3, 37).

Now we can consider the implications of the biphasic kinetics of the receptor-mediated efflux of inorganic ions in microsacs with respect to studies of nerve and muscle cells. Measurements of the flux of \( ^{22}\text{Na}^+ \) from L-6 muscle cells upon addition of carbamoylcholine reveals a biphasic process (D. E. Moore, T. R. Podleski, and G. P. Hess, unpublished observations). Within a few seconds the observed efflux rates become equal to the rates observed in the absence of ligand. Since the transmembrane voltage of the cell is expected to depend to a large extent on the relative rates with which inorganic ions move through the cell membrane in the presence and absence of ligand, the increase in the transmembrane voltage of these cells, observed upon addition of ligand, is, therefore, expected to return to its resting value within a few seconds. The return of the acetylcholine-induced increase in the transmembrane voltage of nerve, muscle, and electrophyscs of \( E. \text{electricus} \) to the resting level is a well-documented phenomenon (30, 39, 40) called desensitization. In our view desensitization is a consequence of the characteristic biphasic kinetics of the receptor-mediated translocation of inorganic ions that results from a ligand-binding mechanism similar to that shown in the model, which is observed with many regulatory enzymes (41).

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