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ACETYLCHOLINE RECEPTOR INACTIVATION IN TORPEDO CALIFORNICA ELECTROPLAX MEMBRANE VESICLES. DETECTION OF TWO PROCESSES IN THE MILLISECOND AND SECOND TIME REGIONS.

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SUMMARY: The rates of inactivations of the acetylcholine receptor-controlled ion flux were measured with membrane vesicles prepared from the electric organ of Torpedo californica. A flow quench technique with a time resolution of 2 msec was used. The vesicles were preincubated with carbamylcholine for various periods of time and then the rate of $^{86}Rb^{+}$ influx was measured. The influx rate decreased progressively with the length of preincubation time. This decrease (inactivation) in influx rate occurred in two different time zones. A fast inactivation process with a half-time of ∿300 msec resulted in at least a 60-fold decrease of ion flux rate. The remaining ion flux activity decreased to an undetectable level in a slow inactivation process with a half-time of 6-7 seconds. Previously, only one inactivation process has been observed by ion flux measurements with receptor-rich vesicles from Electrophorus electricus or Torpedo species.

INTRODUCTION

The inactivation (desensitization) of the acetylcholine receptor due to exposure to acetylcholine or carbamylcholine (agonists) was first observed by Katz and Thesleff(1) in electrophysiological experiments with muscle cells. In their experiments the electrical signal due to agonists first increased and then returned to the original value, even with the agonist concentration remaining constant. Later, receptor inactivation was observed in measurements of acetylcholine receptor-controlled ion flux in membrane vesicles prepared from the

ABBREVIATIONS

TPS, Torpedo physiological saline; PMSF, Phenylmethylsulfonylfluoride; NEM, N-ethyl maleimide; Carb, carbamylcholine.

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electric organs of Torpedo species (2-5) and E.electricus (6-10). In studies with E.electricus membrane vesicles, one inactivation process has been observed that follows an exponential rate law and depends on ligand concentration (8, 9, 12). A slow receptor-mediated ion flux persists after inactivation with a rate that is independent of additional preincubation time but dependent upon carbamylcholine concentration (7-9).

A single inactivation process has also been observed in Torpedo sp. This process is much slower than the one observed in E.electricus vesicles and ion flux apparently ceases completely after preincubation with carbamylcholine (2-5). A two-step desensitization process has been proposed for the Torpedo receptor (5) to account for discrepancies in the dissociation constant for carbamylcholine determined in the msec versus the sec time region.

Ion flux measurements in the msec time region using rapid mixing and quenching techniques provide a way to resolve the processes of ion translocation and ligand-induced inactivation of the receptor in membrane vesicles (7-10). The kinetics of inactivation can be investigated directly by measuring radioactive ion influx for a constant period of time after preincubation of membrane vesicles with ligand for various periods of time (9, 12). Here we report the discovery of two distinct receptor inactivation processes in Torpedo membrane vesicles.

MATERIALS AND METHODS

Membrane vesicles were prepared from electroplax tissue of Torpedo californica as described (15). Final membrane fractions were suspended in Torpedo Ringer's (250 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, 0.02% NaN_3, 10 mM Tris pH 7.6). For comparison Torpedo vesicles were prepared by a different method (13) and suspended in Torpedo physiological saline (250 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM NaP_i pH 7) (5). Tissue homogenization in both procedures was carried out in the presence of appropriate protease inhibitors (10^{-4} M PMSF, 1 mM EDTA, 5 mM NEM). Membrane vesicles were stored in liquid N_2 at a protein concentration of 10 mg/ml. Membrane protein concentration was initially determined by the Lowry method (15) then routinely adjusted prior to influx measurements by monitoring turbidity (O.D. at 600 nm). The [^{125}I]α-bungarotoxin binding assay described previously (16) gave an average value of ∿1000 pmoles toxin bound per mg protein for native Torpedo vesicles. The quench flow apparatus and the techniques used to make the rapid $^{86}Rb^+$ influx measurements have been described (7-10).

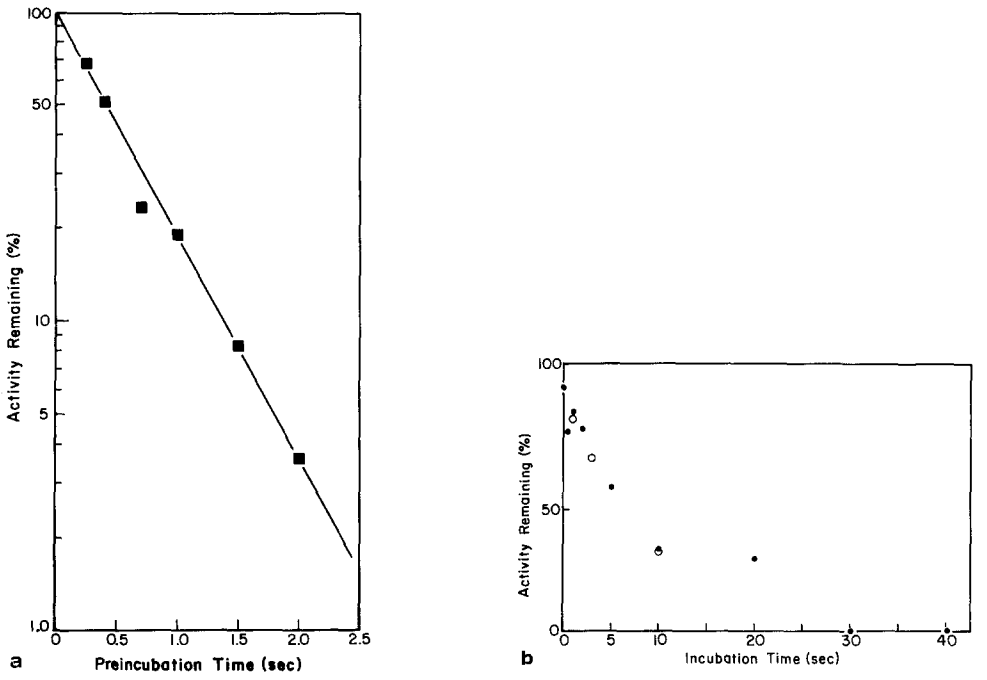

Figure 1: Inactivation of $^{86}Rb^{+}$ influx in Torpedo membrane vesicles, pH 7.0, 1°C.

(a) Fast inactivation in TPS. Preincubation of vesicles with 10 mM Carb for times indicated on the abscissa was followed by incubation with $^{86}Rb^{+}$ solution containing 10 mM Carb for 10 msec to assess flux activity. Activity remaining is defined as ($[^{86}Rb^{+}]$ in vesicles after preincubation)/($[^{86}Rb^{+}]$in vesicles without preincubation). The observed rate coefficient for this inactivation, obtained from the slope of the semi-logarithmic plot is 2 sec^{-1}. The solid line was computed using a least squares computer program and gave a correlation coefficient of 0.99.

(b) Slow inactivation. Using the experimental procedure described in Fig. 1a, two different experimental conditions were tested. Using membranes prepared in TPS, pH 7.0, according to Sobel et al. (13), vesicles were preincubated with 10 mM Carb for various times and then exposed to $^{86}Rb^{+}$ solution for 2 sec (○). In another experiment vesicles prepared in Torpedo Ringer's, pH 7.6, by the procedure of Delegeane and McNamee (14) were preincubated in 1 mM Carb and influx was measured for 2.5 sec (●). Under both sets of conditions $t_{1/2}$ for Carb-induced inactivation is 6-7 sec.

RESULTS AND DISCUSSION

Figure 1a illustrates the fast inactivation of Torpedo receptor in the presence of a saturating concentration of carbamylcholine. Membrane vesicles were preincubated with 10 mM carbamylcholine for various times then exposed to $^{86}Rb^{+}$ for 10 msec before quenching, to assay the ion flux activity ($[^{86}Rb^{+}]$ in vesicles after preincubation)/($[^{86}Rb^{+}]$ in vesicles without preincubation). The inactivation process obeys a first order rate law over 95% of the reaction

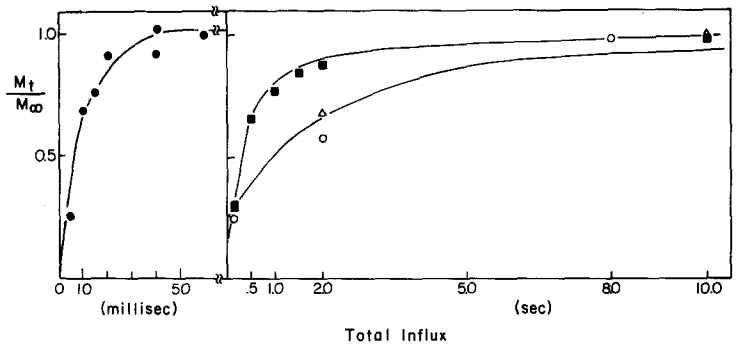

Figure 2: Time courses of $^{86}Rb^+$ influx in TPS, pH 7.0, 1°C, in presence of 1 mM Carb (●) and after constant preincubation for 2 sec (■) or 10 sec (○, △) with 10 mM Carb. M refers to vesicle-entrapped $^{86}Rb^+$ and subscripts represent the time of measurement. The $t_{\frac{1}{2}}$ values for $^{86}Rb^+$ influx are 5 msec (1 mM Carb), 300 msec (2 sec preincubation 10 mM Carb) and 1.0 sec (10 sec preincubation 10 mM Carb).

and is characterized by a rate coefficient of about 2 sec^{-1}. The rate constants for the fast inactivation process in Torpedo and E.electricus vesicles (7-9) are similar at the same ligand concentration. In Torpedo vesicles the second slower inactivation is observed in a different time region (Fig. 1b). When $^{86}Rb^+$ influx is measured over a longer time interval (2 or 2.5 sec) inactivation is characterized by a $t_{\frac{1}{2}}$ value of 6-7 sec in 10 mM and 1 mM carbamylcholine. The ion flux activity determined by 2.5 sec $^{86}Rb^+$ influx is completely abolished by preincubation with 10 mM carbamylcholine for 30 sec (Fig. 1b). This observation stands in contrast to the finding in E.electricus vesicles that a slow rate of ion flux remains even after 1 hr of preincubation with carbamylcholine (9). Prior to inactivation the influx in presence of 10 mM carbamylcholine was complete within 10 msec and was too fast to be measured accurately. In presence of 1 mM carbamylcholine (Fig. 2,○) the influx kinetics could be measured and was characterized by a $t_{\frac{1}{2}}$ value of about 5 msec. When influx after preincubation of the vesicle suspension with 10 mM carbamylcholine for 2 sec was measured (Fig. 2) the observed $t_{\frac{1}{2}}$ value was 300 msec. The rate has therefore decreased by at least a factor of 60 during 2 sec of preincubation. In the following 8 sec of preincubation the influx rate decreases by only a factor of 3, from a $t_{\frac{1}{2}}$ value of 300 msec to one of 1.0 sec. This slower

decrease in ion flux rate reflects the slow inactivation process that occurs after completion of the fast inactivation process. The ion flux which occurs after a 10 sec incubation with ligand demonstrates the presence of the residual receptor activity remaining after the fast inactivation process is over.

Here we show that ion flux in Torpedo vesicles shares two major features in common with E.electricus vesicles: an inactivation in the msec time region followed by slow receptor-controlled ion flux. Previously, only the slow inactivation process which occurs in Torpedo receptor, and that apparently abolishes ion flux completely, has been observed (2-5). The simplest assumption, consistent with our measurements and those of single channel currents in muscle cells (17) is that the same receptor molecules are inactivated in two successive stages occurring in two different time zones.

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