

exhibit an apparent temperature dependence which is the opposite of that expected.

## CONCLUSION

In deuterium oxide solvent the channel closing rate is speeded up so that the mean channel lifetime is smaller. A possible explanation is that the p*K*'s of some groups involved in channel gating change in D<sub>2</sub>O, making the open state less stable.

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# ACTIVATION AND INACTIVATION KINETICS OF *TORPEDO CALIFORNICA* ACETYLCHOLINE RECEPTOR IN RECONSTITUTED MEMBRANES

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The function of nicotinic acetylcholine receptors (AChR) in fish electric organs and at vertebrate neuromuscular junctions can be conveniently divided into four subfunctions: *a*, specific ligand binding; *b*, coupling of binding to cation permeability increases; *c*, ion permeation; and *d*, desensitization, a process by which ion channels are inactivated in the prolonged presence of activating ligands (1). Two recent advances in AChR research provide a unique opportunity to characterize both the biochemical and ion permeability properties of the AChR in a single, well-defined membrane environment. Reliable methods for reconstitution of AChR ion permeability control properties in lipid vesicles have been developed (1, 2), and quench-flow techniques to measure the initial rates of ion fluxes across membrane vesicles in the millisecond time domain have been successfully applied to AChR-containing membranes (3, 4).

We provide here quantitative measurements of both activation and inactivation kinetics of AChR-mediated cation flux in reconstituted vesicles containing purified *Torpedo californica* AChR. We also show that treatment of AChR with the disulfide reducing agent dithiothreitol (DTT) alters the ligand affinity of the AChR without altering channel properties. These results, together with previous work from our laboratory (5, 6), show that the purified AChR protein retains four important functional characteristics: *a*, a high efficiency of cation translocation; *b*, a fast inactivation process in the subsecond time domain;

*c*, a slower inactivation process in the seconds time domain; and *d*, a sensitivity to specific chemical modifications.

## MATERIALS AND METHODS

Methods for isolation of membranes from *Torpedo californica* *electroplax*, extraction and purification of AChR in cholate by affinity chromatography, and reconstitution of AChR with soybean lipids (Asolectin, Associated Concentrates, Woodside, NY) were as described by Walker et al. (5). Ion flux was measured using the quench-flow techniques developed by Cash and Hess (4) as modified by Walker et al. (5). Trapped cations were detected following ion-exchange chromatography (2, 5). The activating ligand was carbamylcholine chloride (Carb), the quenching reagent was d-tubocurarine chloride (Curare), and the radioactive tracer cation was 86-Rb<sup>+</sup>.

## RESULTS AND DISCUSSION

Reconstituted membranes containing 1 mg/ml purified AChR (6-8 nm  $\alpha$ -bungarotoxin binding sites/mg of protein) and 20 mg/ml soybean lipids were tested for ion flux using the quench-flow technique as described by Walker et al. (5). A typical value for  $J_a$ , the maximum flux rate constant at saturating Carb concentrations, was 100 s<sup>-1</sup>. Such a value corresponds to the movement of 10<sup>4</sup> ions/AChR channel/s as expected for fully functional AChR under the ionic conditions of the assay.

The effects of DTT reduction on the initial flux rates

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were obtained by pretreating membranes with 1 mM DTT at pH 8 for 20 min. Values of  $J_a$  vs. log of the Carb concentration are shown in Fig. 1 A. The main effects of DTT reduction were a shift in the dose response curve to higher Carb concentrations with no effect on the maximum flux rate and a decrease in the calculated Hill coefficient from 1.8 to 1.2 (Fig. 1 B). Although the low Hill coefficient for DTT-reduced membranes suggests a loss of cooperative interactions, there is no direct evidence that the fundamental mechanism for channel opening involving the binding of two ligands has been altered. The results confirm and extend previous conclusions about DTT effects inferred from ligand binding and flux studies carried out using slow manual assays (6).

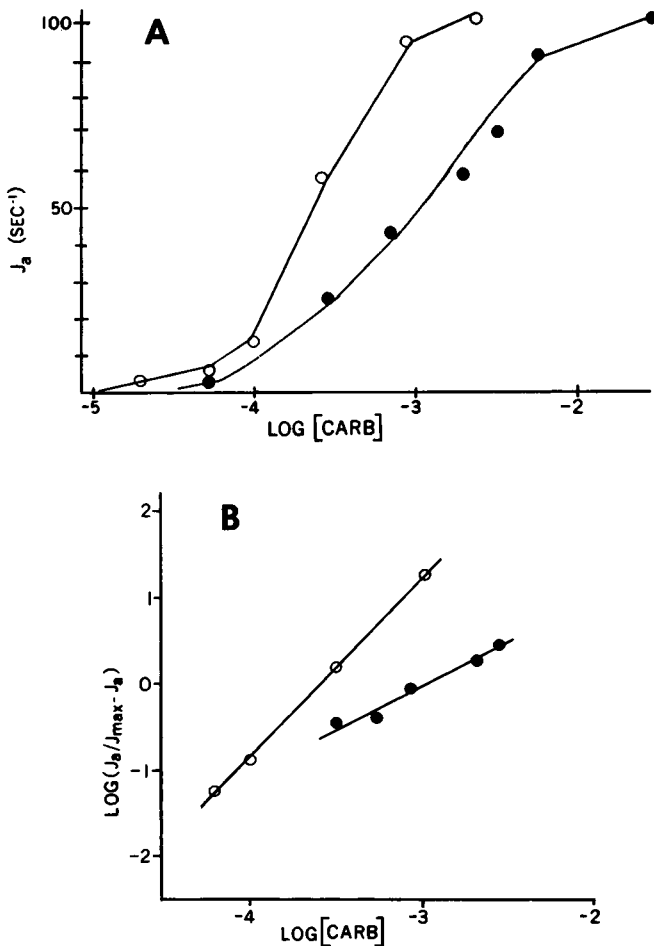


FIGURE 1 Influx rate constants ( $J_a$ ) were obtained by measuring  $^{86}\text{Rb}^+$  uptake at different times for the different Carb concentrations using a rapid mixing quench flow technique. Influx was initiated by mixing 0.225 mls of vesicle suspension with an equal volume of  $^{86}\text{Rb}^+$  solution and was quenched by addition of 0.225 mls of 45 mM Curare. The data were fit by first-order equations of the form:  $\text{cpm}(t) = \text{cpm}(\infty)(1 - \exp[-J_a \cdot t])$ . A, values of  $J_a$  for both untreated ( $\circ$ ) and DTT-reduced membranes ( $\bullet$ ) were plotted vs. the log of the Carb concentration and then B, replotted using the Hill equation. For untreated membranes the half-maximal flux rate occurred at 0.3 mM Carb and the Hill coefficient was 1.8. For DTT-treated membranes the half-maximal rate was at 1.3 mM Carb and the Hill coefficient was 1.2.

Inactivation of the flux response was measured by pretreating membranes with various Carb concentrations in the quench-flow device before addition of  $^{86}\text{Rb}^+$  and a saturating Carb concentration. Preincubation times ranged from 10 ms to 10 s and influx times were either 18 ms (when measuring fast inactivation) or 15 s (when measuring slow inactivation). Rate coefficients for both the slow and the fast processes were measured as a function of the Carb concentration used during the preincubation. Fig. 2 shows the first-order plots of fast inactivation rates at several different Carb concentrations for DTT-treated membranes. The results for DTT-treated membranes were indistinguishable from previous results with untreated membranes (6). For slow inactivation, the DTT-treated membranes gave slightly smaller values for the rate of inactivation at all Carb concentrations.

Neither the fast nor the slow inactivation process showed cooperativity in its Carb concentration dependence. In contrast to channel opening, the binding of a single agonist may be able to trigger desensitization as documented by Aoshima et al. (7) for native eel receptor membranes. The shift of the fast-inactivation Carb concentration dependence to values higher than those required for channel activation suggests that fast inactivation may still be increasing in rate even after the activation binding sites are saturated. The possible existence of separate binding

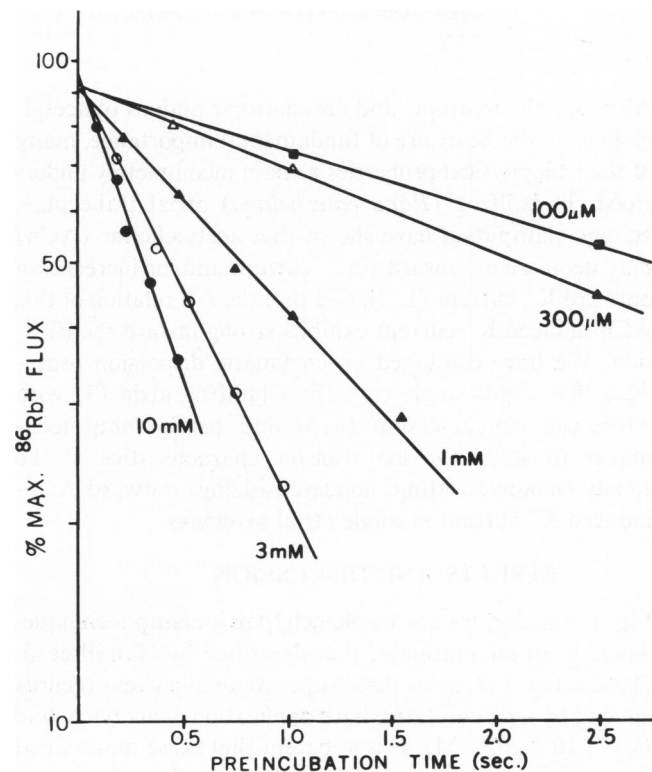


FIGURE 2 Vesicles were preincubated with different Carb concentrations for the times shown and then  $^{86}\text{Rb}^+$  influx was allowed to proceed for 18 ms before quenching. The maximum rate of inactivation was  $5.3 \text{ s}^{-1}$  ( $t_{1/2} = 130 \text{ ms}$ ) at 10 mM Carb.

sites for activation and fast desensitization in *Torpedo* receptor is being further investigated.

The effects of different ligands, lipids, and anesthetics on the kinetic parameters are being explored in an effort to work out the relationships among the activation and inactivation pathways. The chemical modification techniques begun here will be extended to include subsequent alkylation with affinity reagents. In addition, the quantitative relationship of ligand binding and ligand affinity changes to the flux kinetics is being analyzed.

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# ACETYLCHOLINE-INDUCED $K^+$ CURRENT IN AMPHIBIAN ATRIAL CELLS

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Although the inotropic and chronotropic actions of acetylcholine on the heart are of fundamental importance, many of their biophysical properties remain incompletely understood. In bullfrog (*Rana catesbeiana*) atrial trabeculae, voltage-clamp data have shown that acetylcholine (ACh) may decrease an inward  $Ca^{++}$  current and/or increase an outward  $K^+$  current (1, 2), and that the  $I-V$  relation of this ACh-induced  $K^+$  current exhibits strong inward rectification. We have combined an enzymatic dispersion procedure that yields single cells from bullfrog atria (3) with whole-cell voltage-clamp (4-5) and patch-clamp techniques to study the ion transfer characteristics of the steady (noninactivating, nondesensitizing) outward ACh-induced  $K^+$  current in single atrial myocytes.

## RESULTS AND DISCUSSION

Fig. 1 A illustrates our whole-cell/patch-clamp technique, which is an adaptation of that described by Hamill et al. (1982). Fig. 1 B shows three superimposed current records elicited by noncumulative bath application of acetylcholine ( $10^{-7}$ ,  $10^{-6}$ ,  $10^{-4}$  M). It is apparent that these maintained outward currents, induced by ACh, are dose-dependent and do not desensitize. Additional measurements (not shown) indicate that these currents can be inhibited completely by atropine ( $3 \times 10^{-8}$  M).

Fig. 2 summarizes results which define the reversal potential, and hence the ionic nature, of the ACh-induced current. Short (100 ms) "puffs" of ACh were delivered repeatedly from a pressure-phoresis device and the membrane potential (holding potential) was clamped in the range  $-80$  mV to  $-120$  mV. The data in the left-hand panel show a reversal potential near  $-105$  mV in 2.5 mM  $K^+$  Ringers; the results in the right-hand panel show that this reversal potential shifts 58 mV/10-fold change in  $(K^+)_{\circ}$  when  $(K^+)_{\circ}$  is increased in the range 2.5-50 mM. Thus, the classical muscarinic receptor-mediated increase in  $K^+$  "permeability" (6) can be demonstrated in individual, enzymatically-dispersed atrial cells.

Subsequent whole-cell voltage-clamp and patch-clamp experiments have attempted to define the ion transfer (current-voltage,  $I-V$ ) characteristics of this ACh-induced  $K^+$  current. The data in Fig. 3 show that the  $I-V$  relation for the ACh-induced  $K^+$  current exhibits strong inward rectification, in agreement with earlier data obtained from atrial trabeculae (1, 2). In isolated atrial cells we consistently find that ACh-induced  $K^+$  current strongly resembles the previously identified background inwardly-rectifying  $K^+$  current,  $i_{K_1}$ , in that both currents (a) have a high selectivity for  $K^+$ ; (b) exhibit prominent inward rectification positive to the reversal potential; and (c) are inhibited