Effects of Thio-Group Modifications of *Torpedo californica* Acetylcholine Receptor on Ion Flux Activation and Inactivation Kinetics†

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ABSTRACT: The effects of thio-group modifications on the ion permeability control and ligand binding properties of the acetylcholine receptor were measured in reconstituted membranes prepared from purified *Torpedo californica* acetylcholine receptor and soybean lipids (asolectin). A quench flow device was used to obtain subsecond time resolution for agonist-stimulated cation influx using carbamylcholine chloride (Carb) as the ligand and $^{86}Rb^+$ as the cation. The effects of disulfide reduction with dithiothreitol (DTT), affinity alkylation with [4-(*N*-maleimido)benzyl]trimethylammonium ion and bromoacetylcholine, and nonspecific alkylation with *N*-ethylmaleimide and *N*-benzylmaleimide were examined. Activation, fast inactivation, and slow inactivation rates were measured on the chemically modified membranes. The flux results were compared with similar measurements on native membranes, and the role of vesicle size, heterogeneity, and influx time on ion flux results was analyzed. Major conclusions are that the binding sites that react with affinity labels are the same sites that mediate ligand-activated ion flux and that blockade of one of the two ligand binding sites is sufficient to block about 95% of the ion flux response. The main effect of DTT reduction is to shift the EC_{50} values for activation and slow inactivation to higher Carb concentrations, consistent with a decrease in binding affinity for Carb. The EC_{50} value for fast inactivation was not affected by DTT. However, the maximum rate of ion flux activation and the maximum rate of fast inactivation were decreased 2-fold after DTT treatment.

The acetylcholine receptor (AcChR)[1] is an integral membrane protein that represents one of the best characterized neurotransmitter receptors and transmembrane ion channels (Karlin, 1980; Changeux, 1981; Steinbach, 1980). Membrane vesicles can be prepared from electroplax tissue of marine rays (*Torpedo* species) that contain up to 50% AcChR by weight, and recently, techniques have been developed that allow solubilization of AcChR in detergent and reincorporation of the highly purified receptor protein into liposomes of defined lipid composition [for reviews, see McNamee & Ochoa (1982) and Anholt (1981)]. The purified receptor protein complex composed of five tightly associated subunits ($\alpha_2\beta\gamma\delta$) displays both the ligand binding and cation channel functions expected of AcChR. The carbamylcholine chloride (Carb) stimulated activation and inactivation properties are unaltered by purification and reconstitution (Wu & Raftery, 1981; Lindstrom et al., 1980; Walker et al., 1982), and the behavior of single channels is normal (Schindler & Quast, 1980; Tank et al., 1983; Suarez-Isla et al., 1983).

Despite the impressive progress that has been made in characterizing AcChR structure and function, many aspects of the molecular mechanism of AcChR function, particularly those aspects relating to the coupling of ligand binding to ion flux, remain unclear.

Specific chemical modifications of AcChR have been used extensively as one way to elucidate the structure and function of the receptor in many systems. The effects of thio-group reagents including dithiothreitol (DTT), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and various alkylating agents have been well documented by electrophysiological and binding studies (Karlin, 1974, 1980). Such studies have resulted in specific proposals about the functional consequences of disulfide and sulfhydryl modification. Several groups have suggested that thio-group modification inhibits the process of desensitization and can lock the receptor in an active or a desensitized state (Moore & Raftery, 1979; Miller et al., 1979; Barrantes, 1980). DTT has been shown to reduce a disulfide bridge in the vicinity of the acetylcholine binding site and causes a decreased affinity for acetylcholine analogues (Walker et al., 1981). In *Torpedo*, DTT treatment also cleaves a disulfide bridge that links two receptor monomers (M_r 250 000) through their δ subunits [see Karlin (1980)]. In one study, this disassociation of receptor dimers had no effect on function (Anholt et al., 1980). A few groups have reported direct perturbation of the ion channel by thio-group modification (Ben Haim et al., 1975; Steinaker, 1979).

Possibly the most powerful approach has focused on the use of covalent affinity labels that are specific for the agonist binding sites (Karlin, 1974, 1980) or the local anesthetic binding sites (Oswald et al., 1981). Silman & Karlin (1969) demonstrated in *Electrophorus electricus* electroplax that AcChR could be permanently activated by covalent attachment of a potent receptor agonist, bromoacetylcholine (BAC). A variety of affinity reagents (both agonists and antagonists) synthesized by Karlin and co-workers have provided basic insights into the nature, localization, and function of the agonist binding sites (Karlin, 1980; Cox et al., 1979). However, affinity labeling studies have resulted in some controversy over the relationship between affinity label sites and toxin sites. Early studies reported that [4-(*N*-maleimido)benzyl]trimethylammonium ion (MBTA) reacted with one and only one of the toxin sites and that BAC and MBTA labeling was mutually exclusive (Damle & Karlin, 1978). Functional studies of affinity-labeled AcChR extended this interpretation to conclude that activation could occur by agonist binding at a single site (Delegeane & McNamee, 1980).

Recent studies reveal that the two toxin sites are quite similar and that under some conditions both sites can be affinity labeled (Wolosin et al., 1980). Each site also represents

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[1] Abbreviations: AcChR, acetylcholine receptor; Carb, carbamylcholine chloride; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); BAC, bromoacetylcholine; MBTA, [4-(*N*-maleimido)-benzyl]trimethylammonium iodide; MOPS, 4-morpholinepropanesulfonic acid; ^{125}I-α-BGT, ^{125}I-labeled α-bungarotoxin; NEM, *N*-ethylmaleimide; NBM, *N*-benzylmaleimide; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; Me_2SO, dimethyl sulfoxide.

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a reversible agonist binding site (Neubig & Cohen, 1979). Two agonist binding sites are likely to be functionally relevant since the concentration dependence for channel activation reveals a quadratic dependence on ligand concentration (Dionne & Stevens, 1978; Cash & Hess, 1980) and positive cooperativity with a Hill coefficient approaching 2 (Neubig & Cohen, 1980; Walker et al., 1982). However, the observation by Karlin (1969) that DTT treatment dramatically decreased the cooperativity of channel activation suggests that after reduction the two sites may be "uncoupled" and may independently trigger channel opening (Delegeane & McNamee, 1980; Lester et al., 1980).

We report here an in-depth description of the effects of DTT, affinity alkylation, and nonspecific sulfhydryl alkylation on the ion flux properties of AcChR in native and reconstituted membranes. Related experiments have been carried out by Blanchard et al. (1982), and the results are compared and analyzed. Our new results provide insights into the mechanisms of activation and desensitization as well as the involvement of disulfides and sulfhydryls in AcChR function.

Materials and Methods

Torpedo californica rays were obtained live from the Bodega Bay Marine Station of the University of California. The electroplax tissue was removed and stored frozen in liquid nitrogen before use. Native *Torpedo californica* membranes were prepared by the method of Delegeane & McNamee (1980). Reconstituted membranes were prepared from purified receptor and asolectin (Associated Concentrates, Woodside, NY) by using a cholate dialysis procedure (Epstein & Racker, 1978; Walker et al., 1982). The dialysis buffer (buffer A) contained 100 mM NaCl, 10 mM MOPS, 0.1 mM EDTA, and 0.02% NaN_3, pH 7.4.

Electrophorus electricus electric eels were obtained live from World Wide Scientific Animals, Miami, FL. Native eel membranes were prepared from freshly dissected tissue by using the Kasai & Changeux (1971) procedure.

Binding Assays. Equilibrium binding and the rate of binding of ^{125}I-labeled α-bungarotoxin (^{125}I-α-BGT, Amersham Corp.) were measured as described previously (Walker et al., 1981a). Binding of Carb was determined by competition assays using the ^{125}I-α-BGT rate binding procedure (Walker et al., 1981a).

Ion Flux Assays. Influx of $^{86}Rb^+$ (New England Nuclear, Boston, MA) into membrane vesicles was measured at 0 °C by manual mixing of 1 volume (50 μL) of membranes with 1 volume of $^{86}Rb^+$ solution (100 μCi/mL in buffer A) with and without Carb. Influx was quenched after 15 or 30 s by addition of 1 volume of 15 mM *d*-tubocurarine (Calbiochem-Behring; La Jolla, CA). For native membranes, a 125-μL aliquot of the reaction mixture was filtered within 15 s through an HAWP0025 (0.45-μm) Millipore filter and washed (Walker et al., 1981). For reconstituted membranes, a 125-μL aliquot was applied to an ice-cold 2-mL disposable column containing Dowex 50W-X8 (50–100 mesh) and eluted with 3 mL of 175 mM sucrose (Walker et al., 1982). Samples were counted without scintillation fluid in a Beckman LS200 counter with a window setting of 50–600.

Rapid flux measurements of activation and inactivation kinetics were carried out in a quench flow device developed by Cash & Hess (1981) under conditions described previously (Walker et al., 1982). Typically, a push time of 100 ms and a 0.225-mL volume of membranes were used for each data point.

Chemical Modifications. Native *Torpedo californica* membranes were adjusted to 3–4 mg of protein/mL in buffer B (250 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 4 mM $CaCl_2$, 10 mM Tris, and 1 mM EDTA, pH 7.6). Reduction was started by the addition of 0.01 volume of 100 mM dithiothreitol (in H_2O), and the membrane suspension was incubated at 25 °C for 30 min. Reduced membranes were then treated with 0.01 volume of 300 mM DTNB, 300 mM NEM, or buffer B only. NEM (Sigma Chemical Co.) was dissolved in Me_2SO. DTNB was prepared in buffer B at pH 7.0 and then titrated to pH 7.6 with 100 mM Tris, pH 8.4.

Labeling with MBTA or BAC was modified from the procedure of Delegeane & McNamee (1980). MBTA was synthesized according to the procedures outlined by Karlin (1977), and BAC was synthesized according to Damle et al. (1978). A 1-mL sample of DTT-treated membranes was diluted in buffer B and centrifuged at 150000*g* for 30 min to remove DTT. The pellet was resuspended in ~1 mL of buffer B and immediately treated with 10 μL of 10 mM MBTA (in 10^{-4} M HCl) for 20 min at room temperature. Membranes were washed by dilution in buffer B and centrifuged to remove unreacted MBTA.

An alternate method was used to achieve more complete labeling. An aliquot of membranes (~1 mL) was diluted to 0.5 mg/mL in buffer B and adjusted to pH 8.0 by adding 100 mM Tris, pH 8.4. Reduction was carried out at 1 mM DTT for 30 min at room temperature, then the pH was adjusted to 7.0 by adding 100 mM Tris-HCl. MBTA or BAC (in water) was then added to give the final concentration desired. Whenever BAC was used, membranes were pretreated with 10^{-4} M eserine (Sigma Chemical Co.) for >30 min to inhibit acetylcholinesterase. After 10 min of alkylation, membranes were diluted in buffer B and centrifuged to remove unreacted MBTA or BAC. Pellets were resuspended to the desired protein concentration depending on whether flux or toxin binding was to be measured.

For nonspecific alkylation, 10 μL of 1 M *N*-benzylmaleimide (NBM; ALFA Products) or 10 M *N*-ethylmaleimide (NEM) prepared in Me_2SO was added to 1 mL of membranes. Membranes were incubated for 10 min at room temperature and then kept on ice. Iodoacetate was prepared in 10 mM sodium phosphate and neutralized by adding 1 M NaOH.

Chemical modifications of *reconstituted* membranes were carried out under conditions similar to those for native membranes except that reconstituted membranes were not diluted prior to modification and buffer A was used for washing rather than buffer B. Detailed descriptions for individual experiments can be found in the figure legends.

Results

Ion Flux Response. Acetylcholine receptor containing membranes responded to AcChR activators with a large uptake of $^{86}Rb^+$ into a population of sealed vesicles. The magnitude of the response was determined by the difference in vesicle-entrapped cpm after 30 s of flux in the presence and absence of Carb. For native *Torpedo* membranes, the response reached a maximum value at 10^{-3} M Carb while the concentration that gave a 50% response (EC_{50}) was (5.3 ± 2.8) × 10^{-5} M Carb. For reconstituted membranes containing purified receptor, the EC_{50} value was (1.3 ± 0.3) × 10^{-5} M Carb when measured under similar but not identical conditions. The 30-s flux response in both preparations often decreased at very high Carb concentrations (>10^{-3} M). When flux was measured at 25 °C rather than on ice, the fall-off in flux response at high concentration was greater, and a slight shift in the EC_{50} value to 2–3-fold lower Carb concentrations was observed (Figure 1).

Each membrane preparation was characterized by its spe-

Table I: Toxin Binding and Ion Flux Properties of *Torpedo* Membranes[a]

membrane	^{125}I-α-BGT binding[b] (nmol/mg of protein)	flux response[c] (μL/mg of protein)	equilibrium volume[d] (μL/mg of protein)	relative flux response[c] (μL/nmol of AcChR)
native	0.9	0.34	0.86	0.4
reconstituted (nonfrozen)	8.0	10.5	39.8	1.3
reconstituted (freeze-thaw cycle)	8.0	26.9	105	3.4

[a] Native *Torpedo* membranes were prepared by the method of Delegeane & McNamee (1980). Reconstituted membranes were prepared with purified AcChR and asolectin and stored at 0 °C or frozen in liquid nitrogen and thawed before use. All values given are for a single native and reconstituted preparation but are typical of values from a large number of different preparations. [b] Toxin binding was measured in Triton X-100 by using the standard ^{125}I-α-BGT equilibrium binding assay. Protein was determined by the procedure of Lowry et al. (1951). [c] Influx of $^{86}Rb^+$ in response to 1 mM Carb was normalized to the total $^{86}Rb^+$ concentration. For native membranes, a filtration procedure was used, and for reconstituted membranes, an ion-exchange procedure was used (see Materials and Methods). [d] Equilibrium volume was determined after incubating membranes with $^{86}Rb^+$ for greater than 24 h (native membranes) or 48 h (reconstituted membranes) before separating external from membrane-enclosed $^{86}Rb^+$.

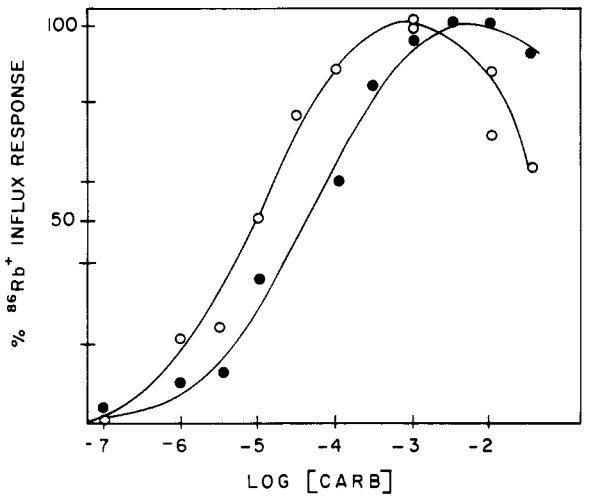

FIGURE 1: Dose–response curves for the 30-s integrated $^{86}Rb^+$ influx response. Native *Torpedo* membranes (3 mg/mL protein) were assayed for $^{86}Rb^+$ influx amplitude at 0 (●) and 25 °C (○) as described under Materials and Methods. Carb concentrations were varied by serial dilution of a stock Carb solution.

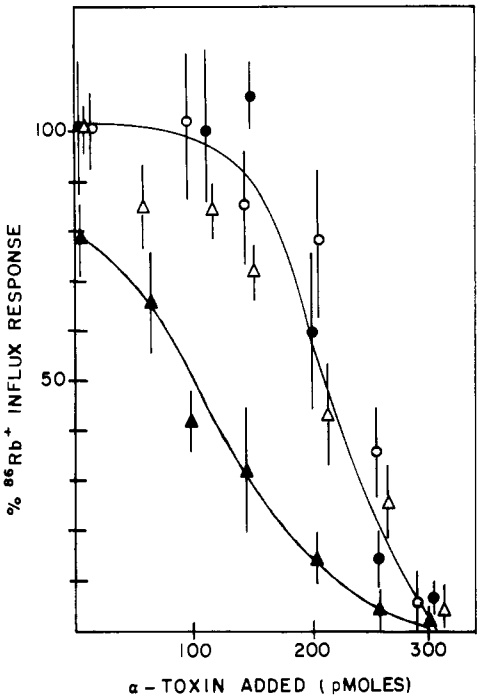

FIGURE 2: Titration of $^{86}Rb^+$ influx with α-toxin. Aliquots (200 μL) of native membranes were equilibrated with increasing concentrations of *Naja naja siamensis* α-toxin overnight at 0 °C. $^{86}Rb^+$ influx was measured for each aliquot with and without 5 mM Carb for 30 s at 0 °C by filtration on Millipore filters. Data are presented for three different membrane preparations and two different flux temperatures: (●) JW7 P2B; (○) JW5 P_2B; (△) JW20 III; (▲) JW20 III with flux measured at 25 °C. Error bars represent standard deviations of duplicate determinations.

cific ^{125}I-α-BGT binding (nanomoles per milligram of protein) and by its normalized flux amplitude to $^{86}Rb^+$ uptake (microliters of vesicle volume per milligram of protein). From these measurements, a direct comparison could be made between native and reconstituted preparations based upon the number of tracer ions apparently transported per AcChR (i.e., microliters of vesicle volume per nanomole of toxin sites). Reconstituted membranes appeared to transport 2–3 times more ions per receptor than native membranes (Table I). However, each reconstituted AcChR has more internal volume associated with it because of a lower surface density of receptors and larger vesicles. Reconstituted vesicles were even further enlarged by freezing in liquid nitrogen (Ochoa et al., 1983; Anholt et al., 1982), and the number of ions transported per receptor increased further. In both native and unfrozen reconstituted vesicles, the maximum uptake of $^{86}Rb^+$ was limited by vesicle size and therefore did not directly reflect intrinsic receptor properties.

Titration of Flux Response with Toxin. Pretreatment of native *Torpedo* membranes with *Naja naja siamensis* α-toxin to block a significant fraction of the ^{125}I-α-BGT sites did not decrease the maximum flux response (Figure 2). This observation, originally reported by Moore et al. (1979), has led to the conclusion that spare receptors are characteristic of native *Torpedo* membranes (Neubig & Cohen, 1980). The amount of spare receptors in a membrane preparation was determined here by measuring the decrease in maximum $^{86}Rb^+$ influx amplitude as increasing amounts of toxin were added. In native preparations, flux amplitude was unaffected until greater than 40% of the toxin sites were blocked (Figure 2). Reconstituted membranes showed less of this spare receptor phenomenon (Figure 3A) and showed almost none after a freeze–thaw cycle (Figure 3B). The spare receptor problem was dealt with by use of a quench flow technique to improve the time resolution of the $^{86}Rb^+$ influx assay. A 100-ms influx response was measured in the quench flow apparatus after treatment of reconstituted membranes with increasing amounts of *Naja naja siamensis* toxin. Inhibition of flux was nonlinear (Figure 3C) and could be fit to eq 1, where y represents the

$$cpm_{toxin} = cpm_{no\ toxin}(1 - y)^2 \quad (1)$$

fractional saturation of external toxin sites and $(1 - y)^2$ describes the fraction of receptor monomers that have both toxin sites free [assuming toxin binds randomly; see Moore et al. (1979)].

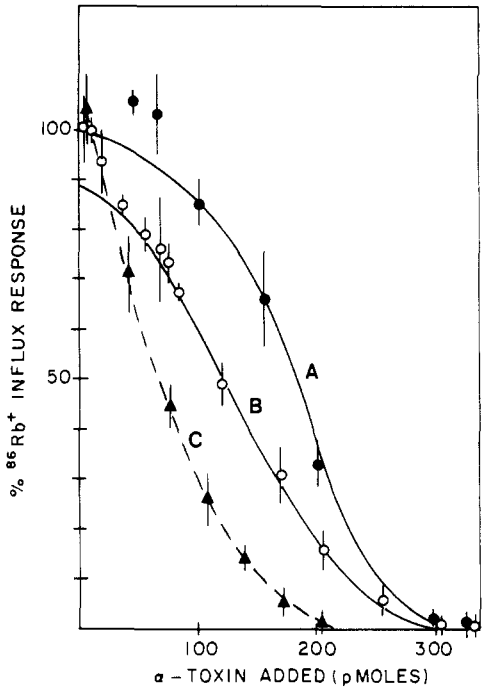

FIGURE 3: Membranes reconstituted with asolectin were equilibrated with α-toxin as described above. A 30-s $^{86}Rb^+$ influx amplitude was determined in response to 1 mM Carb at 0 °C and plotted as a function of the amount of α-toxin added. Data were plotted for unfrozen membranes (●) and for membranes frozen in liquid nitrogen and then thawed (○). $^{86}Rb^+$ influx of unfrozen membranes was measured at 100 ms in the presence of 0.5 mM Carb in the quench flow apparatus to approximate the influx *rate* (▲). The dashed line represents a theoretical curve from eq 1, which assumes that one bound toxin blocks an ion channel. Error bars represent standard deviations of triplicate determinations for line C and duplicate determinations for lines A and B.

Table II: Summary of Kinetic Parameters Based on $^{86}Rb^+$ Flux Rates in AcChR-Reconstituted Membranes[a]

parameter[b]	unreduced[c]	DTT reduced[d]
activation		
max rate (s^{-1})	100	46
EC_{50} for Carb (M)	3.0×10^{-4}	1.5×10^{-3}
Hill coeff	1.8	1.2
inactivation, fast		
max rate (α) (s^{-1})	5.3	2.8
EC_{50} for Carb (M)	1.3×10^{-3}	1.5×10^{-3}
Hill coeff	1.1	1.1
inactivation, slow		
max rate (β) (s^{-1})	0.6	0.5
EC_{50} for Carb (M)	2.5×10^{-4}	6.0×10^{-4}
Hill coeff	1.1	1.1

[a] By use of a quench flow technique, the concentration dependence of three district process (channel activation and two inactivations) was measured. The membranes (nonfrozen) were assayed by an ion-exchange procedure (see Materials and Methods). [b] Maximum rates of $^{86}Rb^+$ activation influx were determined from the data of Figure 4A,B. Maximum rates of inactivation (α and β) were measured at 10 mM Carb (Figure 5A, B). EC_{50} values are midpoints of the concentration dependence curves from Figures 4B and 5A,B which show typical results for a single membrane preparation. Similar results were obtained by using a different membrane preparation. Hill coefficients were obtained from slopes of double log plots as shown in Figure 5B, insert. [c] Data from Walker et al. (1982). [d] Reduction of membranes was started by the addition of 0.01 volume of 100 mM DTT (dissolved in H_2O) and continued for 30 min at 25 °C (see Materials and Methods).

The close fit of the data in Figure 3C to eq 1 suggests (1) that the 100-ms response was not limited by vesicle volume (and perhaps approximates the rate of influx) and (2) that one bound toxin molecule was sufficient to inactivate each receptor monomer.

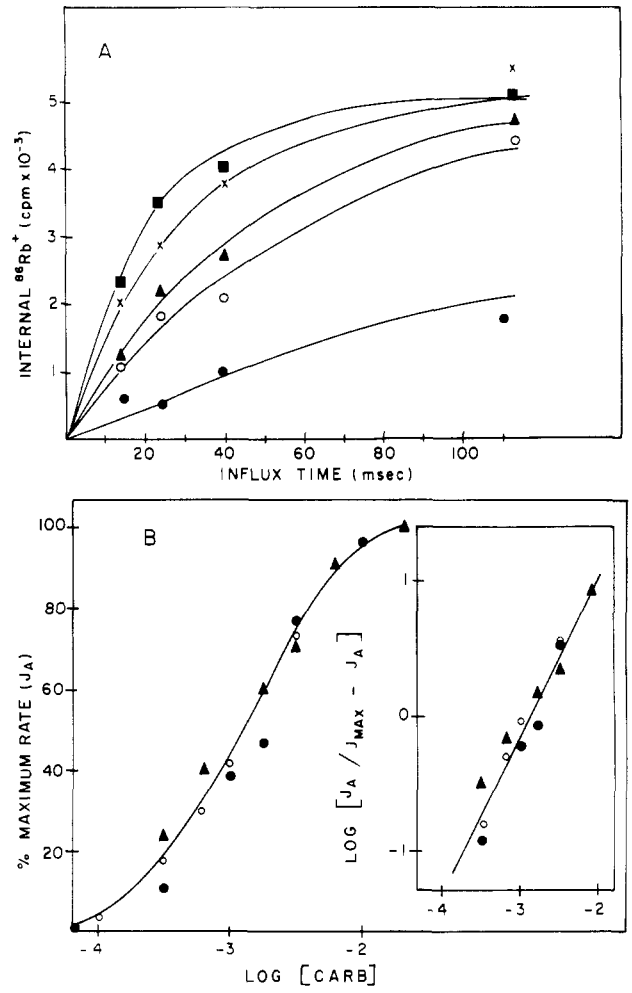

FIGURE 4: $^{86}Rb^+$ influx rates in DTT-treated membranes. Reconstituted membranes were treated with 1 mM DTT as described under Materials and Methods. (A) $^{86}Rb^+$ influx rates were measured in the quench flow device by determining the $^{86}Rb^+$ content of vesicles at four different times as shown. Rates were determined for five concentrations of Carb after a base-line influx measured in the absence of Carb was subtracted. The base line was less than 1000 cpm and was flat over 110 ms. (●) 0.3 mM Carb; (○) 1 mM Carb; (▲) 1.8 mM Carb; (×) 5 mM Carb; (■) 10 mM Carb. (B) Influx rate constants (J_A) were plotted as a function of Carb concentration. The inset shows a Hill plot with a slope (n_H) of 1.2 ± 0.1. (●) Data from part A; (▲, ○) data from a separate experiment with a different preparation of reconstituted membranes also treated with DTT.

Effects of DTT on Flux Properties. As shown above, the 30-s flux response was at best a qualitative measure of functional receptors because of volume limitations, spare receptors, and unresolved desensitization. However, influx *rates* measured with a quench flow technique provide a quantitative measure of channel activity without these complications. We have reevaluated the effects of DTT on receptor-mediated flux properties, including the fast desensitization process, by using quench flow techniques.

Figure 4 and Table II summarize the effects of 1 mM DTT treatment on the influx rate constant for Carb activation measured with $^{86}Rb^+$ in the quench flow device. The dissociation constant for Carb increased from 1.5×10^{-4} to 9×10^{-4} M, and the maximum rate decreased by a factor of 2 compared to unmodified membranes. The Hill coefficient obtained from a double logarithm plot decreased from a value of 1.8 ± 0.1 measured previously (Walker et al., 1982) to 1.2 ± 0.1. These observations are consistent with previous interpretations of 30-s flux measurements that a major effect

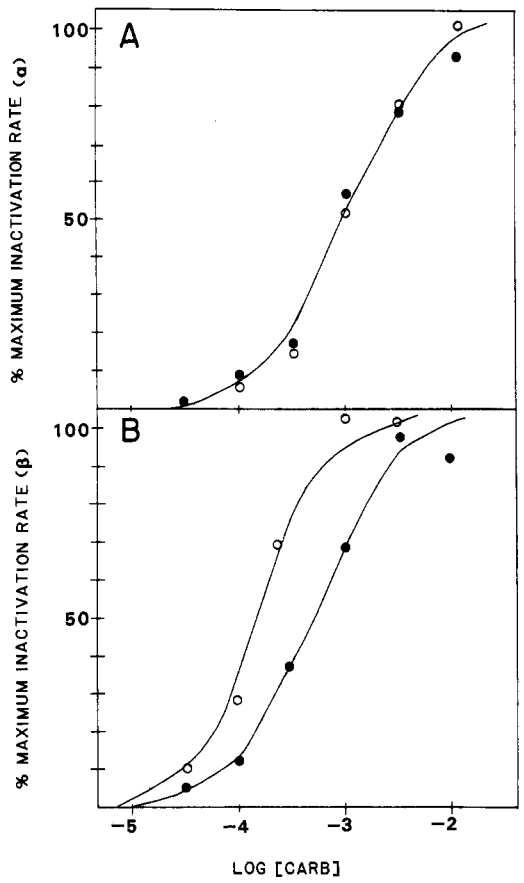

FIGURE 5: Effect of DTT on the inactivation of flux. (A) Fast inactivation of the 25-ms $^{86}Rb^+$ influx response was measured in the quench flow apparatus as described (Walker et al., 1982). The maximum inactivation rate was determined at 20 mM Carb and was 5.3 s^{-1} for unmodified membranes and 2.8 s^{-1} for DTT-reduced membranes. (○) Unmodified reconstituted membranes; (●) membranes after 1 mM DTT treatment. (B) Slow inactivation of the 15-s $^{86}Rb^+$ influx amplitude was measured in reconstituted membranes with a manual assay. Rate constants (β) were determined by linear regression analysis of data obtained as shown in Walker et al. (1982). (○) Unmodified reconstituted membranes, $K_{app} = 2 \times 10^{-4}$ M, n = 1.1, $k_{max} = 0.6$ s^{-1}; (●) DTT membranes, $K_{app} = 6 \times 10^{-4}$ M, n = 1.1, $k_{max} = 0.5$ s^{-1}. Solid lines represent theoretical curves obtained from $v/V_{max} = [A]^n/([A]^n + K^n)$ where V_{max} was obtained at 10 mM Carb, [A] is the Carb concentration, and n is the Hill coefficient.

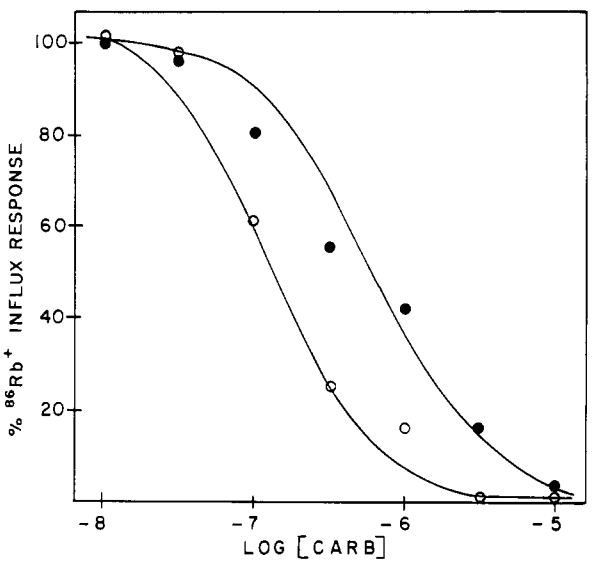

FIGURE 6: Equilibrium desensitization of the $^{86}Rb^+$ influx response. Reconstituted membranes diluted to 1 μM in α-toxin sites were incubated with various concentrations of Carb for 60 min on ice. Each sample was tested for $^{86}Rb^+$ influx in response to 0.1 mM Carb. Solid lines represent a fit of the data to an equation of the form % response = $[A]_f/(K_D + [A]_f)$ where $[A]_f$ is the free agonist concentration and K_D is the equilibrium dissociation constant. An iterative computer program was used that takes into account that a significant fraction of the added Carb is bound because of the high receptor concentration. (○) Unmodified membranes, $K_D = 8 \times 10^{-8}$ M; (●) DTT-modified membranes, $K_D = 4 \times 10^{-7}$ M.

of DTT was to decrease receptor affinity by 6–10-fold (Walker et al., 1981a). Interestingly, this decrease in affinity measured here was observed in the "ultra-low"-affinity state, which is characteristic of the resting receptor. In the previous studies, the affinity decrease was detected for the "low" and "high" states. A DTT-induced decrease in the maximum rate of ion translocation was also revealed by measuring influx rates (Table II).

DTT treatment did not prevent desensitization of the receptor after equilibrium exposure to agonists (Walker et al., 1981a). The specific effects of DTT on receptor desensitization were investigated in more detail here since two phases of desensitization occur in *Torpedo* (Walker et al., 1981b). The well-documented slow phase (seconds to minutes) of desensitization originally observed in electrical measurements (Katz & Thesleff, 1957) is preceded by a fast phase (milliseconds) recently detected by a rapid mixing quench flow technique (Hess et al., 1982; Walker et al., 1981b, 1982). Qualitatively, DTT did not alter the two-phase inactivation of receptor-mediated flux. Fast inactivation was detected after DTT treatment, and the dependence of the rate on Carb concentration was unaltered (Figure 5A). The maximum rate of inactivation was decreased 2-fold (see Table II). In contrast, the dependence of slow inactivation rates on Carb concentration was altered by DTT treatment (Figure 5B). The half-maximal rate required about a 3-fold higher Carb concentration, again indicating a decrease in receptor affinity. When the extent of inactivation was measured after 60 min of preincubation with Carb, reconstituted *Torpedo* membranes (like native) could be fully desensitized, but flux inactivation required a 5-fold higher Carb concentration after DTT treatment (Figure 6). Thus, two different aspects of desensitization were altered by DTT treatment in a similar fashion. The slow phase of inactivation and the extent of inactivation at equilibrium both required higher Carb concentrations to achieve values comparable to those for unmodified membranes.

Affinity Alkylation. Treatment of receptor with 1 mM DTT exposes sulfhydryl groups near the agonist binding sites that provide covalent attachment sites for the affinity reagents BAC and MBTA. Under certain conditions, [^{3}H]MBTA and [^{3}H]BAC have been shown to bind specifically and irreversibly to half of the toxin sites (Damle & Karlin, 1978; Damle et al., 1978). This half of the sites reactivity was further shown to be due to a preexisting difference between the two toxin sites rather than due to negative cooperativity in MBTA binding (Damle & Karlin, 1978). More recently, it was demonstrated that the stoichiometry of [^{3}H]BAC sites to ^{3}H-α-toxin sites could be 1:2 or 1:1 depending on reaction conditions (Wolosin et al., 1980). This finding complicates an interpretation of affinity labeling studies (Deleageane & McNamee, 1980) and led us to further investigate these important reactions.

(*A*) *Effect of the Concentration of the Alkylating Agent.* The dependence of alkylation on the concentration of BAC is illustrated in Figure 7A. Specific alkylation was determined by the inhibition of ^{125}I-α-bungarotoxin binding. Greater than 80% of the toxin sites were blocked at a BAC concentration of 10 mM if the DTT concentration was maintained at 1 mM during alkylation. Inhibition was distinctly biphasic with 50% of the sites blocked at 10 μM BAC. By comparison, when

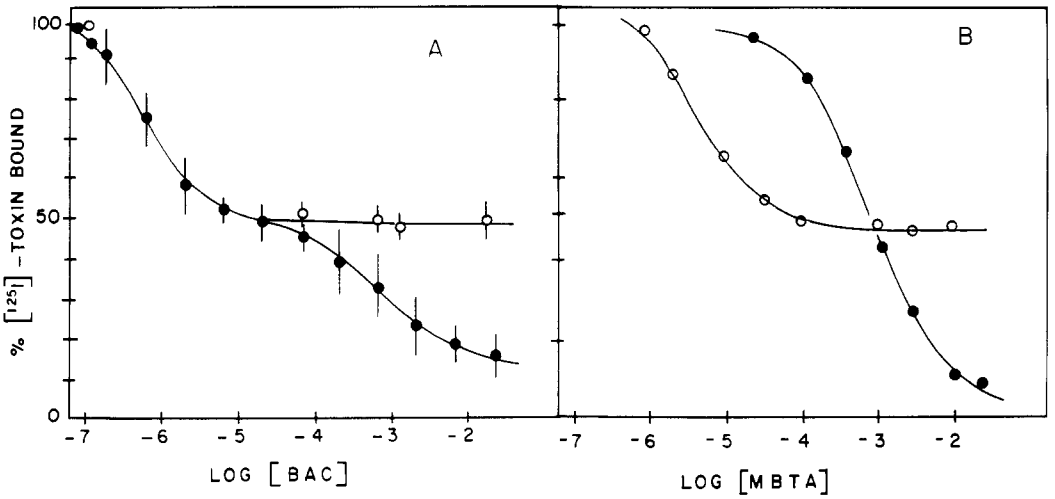

FIGURE 7: (A) Inhibition of ^{125}I-α-bungarotoxin binding by alkylation with bromoacetylcholine (BAC). Native *Torpedo* membranes (1 μM in toxin sites) were treated with 1 mM DTT for 30 min at room temperature in MDB–Tris, pH 8.0. The pH was then adjusted to 7.0 with Tris-HCl, and aliquots were treated with increasing concentrations of BAC for 10 min at room temperature (●). Alkylation was stopped by dilution and centrifugation of membranes to remove unreacted BAC. Specific α-toxin binding was determined for each sample by using the equilibrium ^{125}I-α-BGT binding assay. Error bars represent standard deviations of data from five separate labeling experiments. (○) Membranes were centrifuged after DTT treatment to remove DTT. Pellets were resuspended in MDB–Tris and treated with increasing concentrations of BAC as described above followed by the equilibrium ^{125}I-α-BGT binding assay. (B) Inhibition of ^{125}I-α-BGT binding by DTT–MBTA treatment. Native *Torpedo* membranes (0.5 mg/mL) were treated with 1 mM DTT for 30 min at room temperature. Half of the membranes were diluted in MDB–Tris and centrifuged to remove DTT. The pellet was resuspended in MDB–Tris to 0.5 mg/mL and divided into 200-μL aliquots for MBTA labeling. Each membrane fraction was treated with various concentrations of MBTA (in 10^{-4} M HCl) for 10 min at room temperature and then diluted and centrifuged to remove unreacted MBTA. Each pellet was then resuspended and assayed for ^{125}I-α-BGT binding. (○) DTT diluted to about 1 μM after reduction; (●) DTT maintained at 1 mM during affinity labeling.

MBTA was investigated, inhibition of toxin binding was not biphasic, and greater than 90% of the sites were blocked at high MBTA concentration if the DTT concentration was kept at 1 mM (Figure 7B). In the presence of DTT, very high MBTA concentrations were required to obtain any labeling. In this case, the competing reaction between DTT and MBTA is clearly decreasing the effective concentration of MBTA available for modification of the receptor.

(B) Effect of DTT Concentration on the Extent of Alkylation. In some alkylation procedures, DTT concentrations were decreased by dilution or by centrifugation in order to minimize alkylation of excess DTT. As illustrated in Figure 7A,B, when the DTT concentration was decreased to 1 μM the inhibition of toxin binding for both BAC and MBTA was quite different. Specifically, half of the sites were no longer susceptible to alkylation even at high concentrations of alkylating agent. The concentration of DTT used during the initial reduction step was then varied to investigate the possibility that disulfides at each site displayed different reactivities. As the DTT concentration was varied from 10^{-6} to 10^{-2} M, the extent of BAC labeling increased hyperbolically to reach a maximum at 10^{-3} M DTT as if a single class of disulfides was being reduced (data not shown). Thus, the biphasic reaction with BAC was apparently not due to selective reduction of one of the disulfide pairs associated with the BAC binding sites. The most likely explanation is that one pair of sulfhydryl groups reoxidized rapidly when the DTT concentration was reduced.

(C) Flux Response in Affinity-Alkylated Membranes. Alkylation of *Torpedo* membranes by the method of Delegeane & McNamee (1980) gave membranes with half the number of ^{125}I-α-BGT sites compared to unmodified membranes. Native *Torpedo* membranes alkylated with MBTA (after DTT) displayed a full 30-s $^{86}RB^+$ influx response, and the dose–response curve was similar to that observed after DTT alone (i.e., a 10-fold increase in the EC_{50} value). Measurements of ion flux rates with a quench flow technique revealed that influx rates in response to 1 mM Carb were decreased by a factor of 20 for both DTT (Figure 8A) and DTT–MBTA (Figure 8B) modified membranes. Typically, influx was measured in the presence of 1 mM DTT for the reduced sample, but the alkylation procedure involved diluting the DTT to 1 μM prior to affinity labeling. As shown above, the extent of modification (and presumably the oxidation state of the crucial disulfides) depended on the final DTT concentration.

Several lines of evidence suggest that the flux behavior of DTT and DTT–MBTA was really quite different. The effects of DTT alone on ion flux activation rates could be overcome to a large extent by higher Carb concentrations (see section B under Affinity Alkylation), and the effects of DTT were explained mainly by a decrease in the affinity of the receptor for Carb. In MBTA-modified membranes, the 20-fold decrease in flux rate was not overcome at all by higher Carb concentrations, suggesting an impaired response unrelated to agonist affinity. Receptors labeled with MBTA at half of the toxin sites were activated with at best 5% of the efficiency of unmodified receptor. We cannot rule out the possibility that 5% of the receptors have escaped MBTA modification under these conditions. Furthermore, the sulfhydryl oxidizing agent DTNB completely reversed the effects of DTT on ion flux properties, whereas DTNB had no effect on the flux properties of DTT–MBTA-modified membranes [data not shown; see also Delegeane & McNamee (1980)].

Another approach was used for BAC. Native and reconstituted *Torpedo* membranes were reduced and alkylated with concentrations of BAC varying between 10^{-7} and 10^{-2} M, under conditions which gave the biphasic labeling curves shown in Figure 7A. Carb-stimulated $^{86}Rb^+$ influx was then measured for each membrane sample by using a manual 30-s assay. For reconstituted membranes, the influx response decreased even when the extent of BAC modification was low (10–20%); complete inhibition of flux was observed in the range of 50–60% alkylation (Figure 9). Inhibition of Carb-stimulated flux in native membranes required higher concentrations of BAC (presumably because of the spare receptor phenomenon) although complete inhibition again occurred in the region of

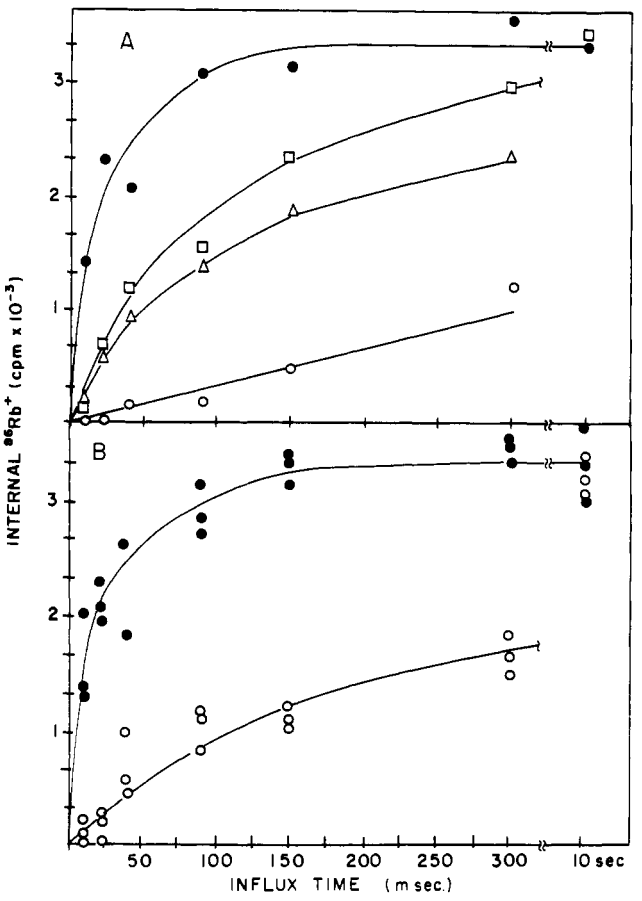

FIGURE 8: (A) Time course of $^{86}Rb^+$ influx in DTT-reduced membranes. Native *Torpedo* membranes were treated with 1 mM DTT for 30 min at room temperature. Influx was measured in the quench flow apparatus. Each data point is the average of triplicate determinations. (●) Native (unmodified) membranes at 1 mM Carb (J_A = 54 s^{-1}); DTT-treated membranes at 1 mM Carb (○) (J_A = 0.9 s^{-1}), at 5 mM Carb (△) (J_A = 6.1 s^{-1}), and at 10 mM Carb (□) (J_A = 9.5 s^{-1}). (B) Time course of $^{86}Rb^+$ influx in MBTA-modified membranes. Native *Torpedo* membranes were reduced and alkylated with MBTA by the method of Delegeane & McNamee (1980). Influx was measured in the quench flow apparatus, and all data points are shown. (●) Native (unmodified) membranes at 1 mM Carb, J_A = 61 s^{-1}; (○) MBTA-modified membranes at 1 mM Carb, J_A = 3.2 s^{-1}.

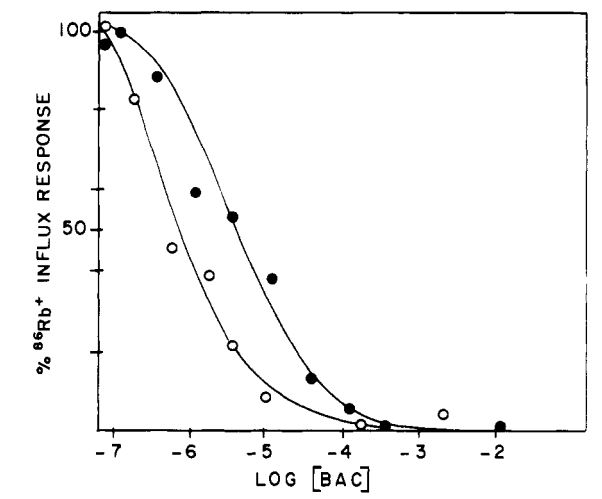

FIGURE 9: Inhibition of $^{86}Rb^+$ influx amplitude by alkylation with BAC. Native (●) and asolectin-reconstituted membranes (○) were treated with 1 mM DTT and increasing concentrations of BAC as described in Figure 6A. After removal of unreacted BAC, the membranes were tested for the 30-s $^{86}Rb^+$ influx amplitude response at 0 °C in the presence and absence of 1 mM Carb. In the absence of Carb, membrane vesicles showed only the passive influx of $^{86}Rb^+$ even after both sites had BAC molecules covalently attached.

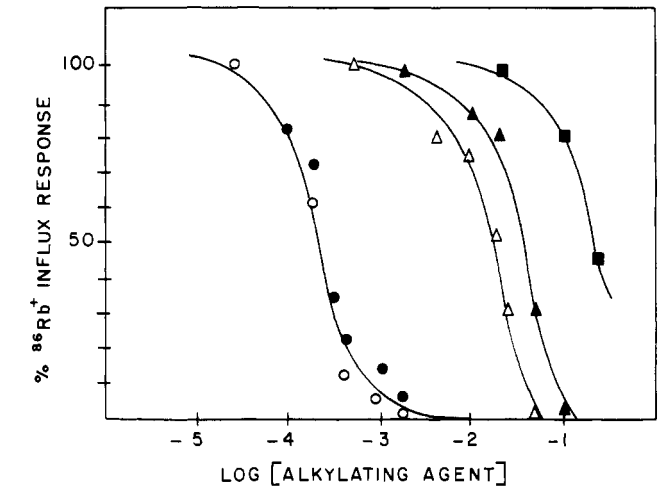

FIGURE 10: Inhibition of $^{86}Rb^+$ influx by alkylation with *N*-benzylmaleimide (●), *N*-ethylmaleimide (▲), and iodoacetate (■) and after DTT reduction by *N*-ethylmaleimide (○) and iodoacetate (△). 150-μL aliquots of native membranes (4 mg of protein/mL) were treated with 15 μL of alkylating agents (10 min, room temperature) to give the final concentrations shown on the abscissa; 25-μL aliquots of membranes were assayed for $^{86}Rb^+$ influx amplitude ± 0.1 mM Carb. Standard DTT treatment involved 1 mM DTT for 30 min at room temperature.

50–60% alkylation. Clearly, if the extent of modification was monitored closely, the functional response of the receptor was impaired long before both toxin sites were occupied by affinity labels. It is worth noting here that passive flux rates (no Carb) were unaltered by the extent of BAC modification, even though BAC is a potent receptor activator. Thus, *Torpedo* receptors with BAC molecules covalently attached (either one or two per receptor) behaved as though they were desensitized.

Nonspecific Alkylation. After incubation with 1 mM DTT, treatment of *Torpedo* membranes with 3 mM *N*-ethylmaleimide (NEM) caused inhibition of the $^{86}Rb^+$ influx response (Walker et al., 1981a) and dramatic changes in Carb and toxin binding properties, including abolition of the agonist-induced affinity transition (Barrantes, 1980; Walker et al., 1981a). NEM at a final concentration of 3 mM had no effect on receptor function prior to DTT treatment or after the DTT–DTNB sequence. A well-known consequence of DTT treatment is to reduce a disulfide in the vicinity of the agonist binding sites (Karlin, 1980). Alkylation of the resultant sulfhydryl groups may explain the dramatic effects observed on both Carb and toxin binding properties. However, alkylation at those sites may not be the direct cause of flux inhibition. Recently, Huganir & Racker (1982) have shown that some maleimide derivatives can inhibit receptor function without DTT pretreatment. In that study, the inhibitory potency increased with increasing hydrophobic character so that *N*-benzylmaleimide (NBM) caused inhibition at 10-fold lower concentrations than *N*-ethylmaleimide. It was of interest to us to determine if the inhibition of flux by maleimides was affected by DTT treatment.

(A) Alkylation without DTT Pretreatment. Figure 10 confirms the inhibition of $^{86}Rb^+$ influx in *Torpedo* membranes by NBM, NEM, and iodoacetate both with and without DTT treatment. It should be noted that 100-fold higher NEM concentrations were required to inhibit flux without the DTT step. NEM or NBM treatment did not decrease the number of ^{125}I-α-BGT sites measured by the standard equilibrium toxin binding assay in detergent or by the rate of toxin binding without detergent. The Carb binding properties were exam-

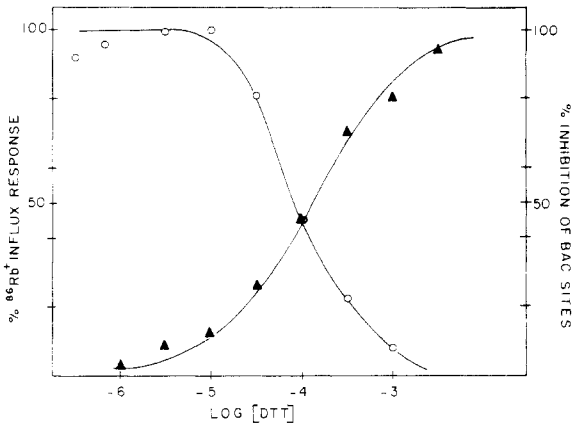

FIGURE 11: Effect of DTT concentration on DTT–NEM modification. 200-μL aliquots of native *Torpedo* membranes were treated with 20 μL of DTT to give the final concentration shown. After 30 min at room temperature, 20 μL of 100 mM NEM was added and incubated for 10 min at room temperature. 25-μL aliquots were assayed for the 30-s $^{86}Rb^+$ influx amplitude ± 0.1 mM Carb (○). An aliquot of membrane after the successive DTT–NEM treatment was subjected to 1 mM DTT (30 min) followed by 30 mM BAC (10 min). Membranes were then diluted to 0.05 μM for ^{125}I-α-BGT binding (▲). The availability of α-BGT sites served as an indirect measure of the sites occupied by BAC.

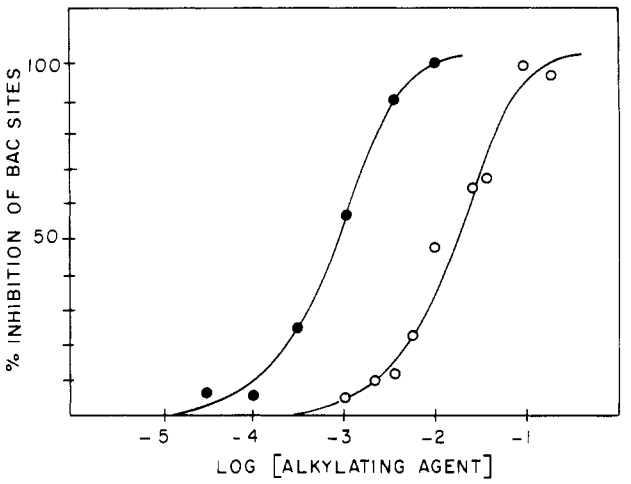

FIGURE 12: Inhibition of affinity alkylation by NEM (●) and iodoacetate (○). 50-μL aliquots of native *Torpedo* membranes were reduced with 5 μL of 100 mM DTT for 30 min. Each aliquot was treated with 5 μL of alkylating agent (10 min, room temperature) to give the concentration shown on the abscissa. To each aliquot was added 5 μL of 300 mM bromoacetylcholine (10 min, room temperature). Membranes were diluted to 0.05 μM in 100 mM NaCl–10 mM MOPS–0.2% Triton X-100, pH 7.4, and 2 × 50 μL aliquots were assayed for ^{125}I-α-BGT binding.

ined in detail by using the toxin competition assay (Walker et al., 1981a). In contrast to DTT–NEM membranes (Walker et al., 1981a), membranes modified with either 10 mM NBM or 100 mM NEM displayed no abnormal binding properties. ^{125}I-α-BGT binding occurred with a second-order rate constant of 1.1×10^5 M^{-1} s^{-1}, and Carb dissociation constants were 10 μM (no preincubation) and 0.04 μM (with Carb preincubation), in good agreement with values obtained in unmodified receptor. Furthermore, membranes alkylated by NBM (or NEM) could be reduced with DTT and alkylated with BAC at the agonist sites. The perturbation caused by NBM (inhibition of flux) could not be correlated with impaired ligand binding properties, suggesting a site of action away from agonist binding sites. In preliminary experiments, high concentrations of Carb did not protect the receptor from NBM inhibition.

(B) Effects of Varying DTT and NEM Concentrations. To investigate the DTT–NEM reaction in detail, DTT and NEM concentrations were varied independently, and the extent of modification was assessed. The presence of sulfhydryl groups near the recognition site was quantitated by affinity labeling. Complete affinity labeling required 1 mM DTT, and complete inhibition of labeling was accomplished with high NEM concentrations. The dependence of NEM alkylation on DTT concentration is given in Figure 11. The extent of NEM alkylation was determined by the decrease in the extent of BAC labeling. Modification of active-site sulfhydryls with NEM showed the same dependence on DTT concentration as did the BAC modification. An analogous series of experiments was carried out at a constant DTT concentration while NEM and iodoacetate concentrations were varied. Inhibition of BAC labeling by increasing concentrations of NEM or iodoacetate was monotonic, suggesting a single class of reactive sulfhydryls (Figure 12).

(C) Ion Flux Response in Partially Alkylated Membranes. A 30-s influx response was obtained for alkylated membranes under conditions where the flux response was roughly proportional to the number of active receptors. In three distinct cases where the extent of alkylation was manipulated by varying the concentration of either DTT, NEM, or iodoacetate, the inhibition of the $^{86}Rb^+$ influx response occurred in the same concentration range where BAC labeling was inhibited. In all three cases, inhibition of flux correlated with alkylation of those sulfhydryls near the active sites for agonist binding. These sites required DTT pretreatment to be exposed. Reactive groups (presumably sulfhydryls) that were alkylated by higher NEM concentrations (and by lower concentrations or more hydrophobic maleimides) most likely represent a distinct inhibitory site on the receptor molecule. Inhibition of ion flux at these sites did not require DTT treatment and did not prevent normal binding of the cholinergic ligands Carb, BAC, and toxin.

Modification of Eel Membranes. In preliminary comparative experiments using isolated membrane vesicles from eel, we found that thio-group modification had the following similar consequences in eel and *Torpedo*: (1) 1 mM DTT treatment did not inhibit the $^{86}Rb^+$ influx response to 10 mM Carb; (2) 1 mM DTT treatment followed by 3 mM NEM inhibited the flux response completely; and (3) neither treatment altered the number of ^{125}I-α-BGT sites measured in detergent. Furthermore, both toxin sites on the eel receptor could be occupied by affinity labels. When reduced eel membranes were treated with 10 mM BAC, greater than 90% of the toxin sites were protected by covalently attached BAC. However, eel membranes were *not* completely desensitized after both sites were affinity alkylated with BAC (in marked contrast to *Torpedo* membranes). BAC-modified eel membranes displayed an influx rate (when diluted into $^{86}Rb^+$ alone) that was 5–10-fold faster (Figure 13) than the passive influx rate was measured in the presence of a large excess of an AcChR inhibitor (i.e., 10^{-4} M curare).

After preincubation with 10 mM Carb for 60 min, a slow influx process could still be detected in eel membranes, and this slow influx rate is associated with equilibrium-desensitized receptor (Aoshima et al., 1981). DTT treatment had no effect on this process, and DTT treatment followed by BAC resulted in eel membranes which displayed a similar slow agonist-stimulated influx rate.

Discussion

The thio-group modifications used here on both native and reconstituted membranes containing *Torpedo californica*

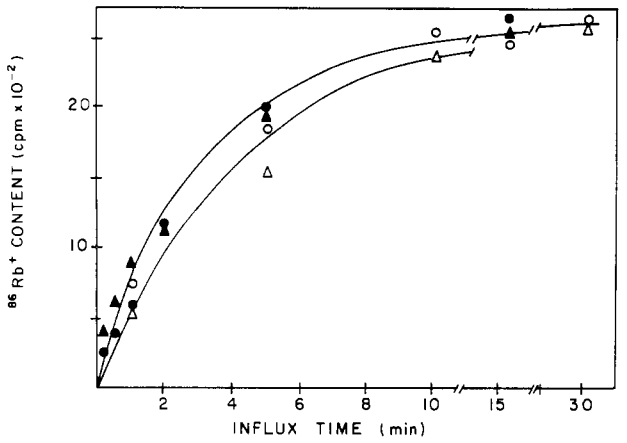

FIGURE 13: Effect of DTT treatment and DTT–BAC treatment on desensitization in *Electrophorus* membranes. 1.5 mL of membranes (0.8 mg of protein mL) was adjusted to pH 8.0 and treated with 1 mM DTT (30 min, room temperature). The pH was then adjusted to 7.0. Control membranes (native, untreated) and DTT membranes were preincubated with 10 mM Carb at 0 °C for 1 h. Each was tested for flux by addition of 1.5 mL of 100 μCi/mL $^{86}Rb^+$ + 10 mM Carb. At various times, 0.5-mL aliquots were removed and diluted into 1.7 mL of 5 mM curare, and 15 s later 2 × 1.0 mL aliquots were filtered on Millipore filters. Passive flux was determined for each membrane sample by omitting Carb at both the preincubation and flux steps. The slow flux rate constant of native (●) and DTT-treated (▲) membranes in the presence of Carb was 0.35 min^{-1}. The same experiment was carried out as described above except that after DTT treatment membranes were preincubated with 10 mM BAC (1 h, 0 °C). Membranes were pretreated with 10^{-4} M eserine to inhibit acetylcholinesterase. The slow flux rate constants for native (○) and DTT-treated (△) membranes in the presence of BAC were 0.25 min^{-1}. Solid lines represent first-order plots of the form $cpm_t = cpm_f(1 - e^{-k_I t})$ with k_I values for flux rate constants given above. DTT–BAC membranes were diluted and centrifuged to remove unreacted BAC. ^{125}I-α-BGT binding showed that 93–96% of the toxin sites were blocked by BAC. When diluted into $^{86}Rb^+$, the DTT–BAC membranes displayed an influx rate faster than the passive rate (K = 0.1 min^{-1}).

acetylcholine receptor provide new insights into the coupling between ligand binding and ion permeability control. A key result is the 20-fold decrease in Carb-stimulated ion flux *rates* measured by quench flow techniques under conditions in which exactly half of the agonist binding sites are reacted with the covalent affinity label MBTA. This result is consistent with other observations that indicate that the two agonist sites must be occupied by activating ligands to get a significant flux response (Neubig et al., 1982; Sine & Taylor, 1980; Lindstrom et al., 1980). The rate measurements are more accurate than previous flux *amplitude* measurements that indicated that MBTA-labeled receptor could mediate significant ion flux responses (Delegeane & McNamee, 1980). As demonstrated here and also shown previously by others (Moore et al., 1979; Lindstrom et al., 1980), the amplitude measurements are extremely sensitive to spare receptors so that a small fraction of active receptors can effectively lead to complete vesicle filling during the relatively long times (10 s) used for the amplitude measurements. The residual flux observed after MBTA reaction could be due to inefficient channel operation or to the presence of a small number of unreacted receptors. The dependence of alkylation efficiency on the reaction conditions is clearly illustrated by the results. The ability to label either one or two toxin sites with the affinity labels extends the usefulness of the labels and offers an opportunity to examine interactions between the two sites.

Similar results were obtained with BAC, but the results are complicated by the fact that BAC, an activating ligand, can trigger receptor desensitization and thus block ion flux by an independent mechanism. There is no evidence that reversibly bound MBTA or covalently reacted MBTA triggers desensitization. In fact, the agonist-induced transitions in agonist binding affinity are preserved in the MBTA-labeled membranes at the site *not* labeled by MBTA (Delegeane & McNamee, 1980). These transitions are generally associated with the desensitization process. Thus, MBTA-labeled membranes are presumably not desensitized.

A necessary prerequisite for the affinity alkylations is prior reduction with a disulfide reducing agent such as dithiothreitol (DTT). The effects of DTT alone were examined previously by ligand binding and flux amplitude measurements (Walker et al., 1981a). The main effect of DTT treatment was a decrease in the apparent binding affinity for agonists in both the low-affinity and high-affinity states. DTT did not prevent desensitization, contrary to some initial reports (Moore & Raftery, 1980). The effects of DTT observed here are generally consistent with those observed by Blanchard et al. (1982) in which fluorescent techniques were used to detect ligand binding and ion influx. The quench flow experiments reported here on reconstituted membranes provide supporting evidence for the DTT effect on ligand binding. There is a 2-fold decrease in the maximum ion flux rate, indicating that the function of the ion channel itself was not drastically affected by DTT. However, the Hill coefficient for the ligand dependence of channel activation rates decreased from 1.8 to 1.2. Interestingly, the shift to lower binding affinity was observed for the ultra-low-affinity state detectable only by quench flow techniques. The effects of DTT on inactivation (desensitization) rates were also examined. The Carb concentration dependence of the fast inactivation process was not affected by DTT, but the dependence of the slow phase was increased to about 4-fold higher Carb concentrations. The maximum values for both fast and slow inactivation rates were decreased slightly by DTT treatment (see Table II).

The affinity alkylation results provide good evidence that the toxin binding sites with which the alkylating agents compete are the same sites that control channel activation and inactivation. Recently, Raftery and colleagues have proposed that the activation site is a physically distinct third site (Dunn & Raftery, 1982; Dunn et al., 1983). The hypothesis is based primarily on fast fluorescence responses of a covalently attached probe. The probe responds to the binding of agonists in the high ligand concentration range characteristic of receptor activation (the ultra-low-affinity state), but the probe is insensitive to the desensitization state of the receptor. Prior reaction of the AcChR with BAC at the two "regular" sites also does not block the fluorescence change. If one accepts the premise that AcChR is in a nondesensitized state after reaction with MBTA (as examined previously under Discussion), then MBTA-labeled membranes should be activatable by agonists at high concentrations if activation occurs at a separate class of site(s). Our new results indicate that MBTA-labeled membranes are essentially inactive, thus providing circumstantial evidence against a physically distinct activation site. The direct correlation by Neubig et al. (1982) between ligand binding changes and the slow phase of desensitization is consistent with the results presented here for chemically modified membranes.

The lack of a DTT effect on the EC_{50} value of the *fast* inactivation process is consistent with our previous suggestion (Walker et al., 1982) that a distinct low-affinity site may be involved in some aspects of desensitization.

The effects of the nonspecific alkylating agents also indicate that additional inhibitory sites may exist on the receptor. In the presence of DTT, the nonspecific alkylating agents, such

as NEM and iodoacetate, appear to react with the same sulfhydryls as the affinity reagents, albeit at higher concentrations. However, these same agents can also block ion flux even in the *absence* of DTT reduction at much higher concentrations. *N*-Benzylmaleimide, a hydrophobic alkylating agent, blocks AcChR-mediated flux at relatively low concentrations without prior DTT reduction. These results suggest that modification of a free sulfhydryl readily accessible to hydrophobic agents can inhibit AcChR flux independently of any blocking of ligand binding to the well-characterized toxin binding sites.

Experiments are now in progress to determine the relationship between sulfhydryl modification at the additional site(s) and the process of fast inactivation. It will be especially interesting to extend the comparisons with native and reconstituted eel membranes, since the eel shows only a fast inactivation process and also retains some flux properties even after long-term exposure to agonists.

Acknowledgments

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Registry No. Ru, 7440-17-7; Carb, 51-83-2; MBTA, 40748-21-8; BAC, 17139-54-7; NEM, 128-53-0; NBM, 1631-26-1.

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