Ligand-Induced Interconversion of Affinity States in Membrane-Bound Acetylcholine Receptor from *Torpedo californica*. Effects of Sulfhydryl and Disulfide Reagents†

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ABSTRACT: The nicotinic acetylcholine receptor from *Torpedo californica*, in membrane-bound form, can undergo conversion from a state(s) of low affinity to one(s) of high affinity for carbamylcholine upon pretreatment with this ligand. The involvement of disulfide bonds in this conformational transition is suggested since treatment with a reducing agent, dithiothreitol, prevents the conversion. In addition treatment with *p*-chloromercuribenzoate similarly blocks the conversion to high affinity state(s) by the cholinergic ligand, while other reagents such as iodoacetic acid or iodoacetamide do not. These results suggest the possible involvement of sulfhydryl groups in the transition. Unmodified acetylcholine receptor in the high-affinity form(s) induced by carbamylcholine can revert to a form(s) of low affinity upon removal of the ligand. However, treatment with *p*-chloromercuribenzoate prior to such removal prevents the system from reverting to low affinity after removal of carbamylcholine. Those results again suggest that sulfhydryl groups may be important for the conformational transitions responsible for interconversion of affinity states of the receptor. They are also indicative of the possibility of stabilizing states of the receptor complex which should be useful for studies of its mechanism, and they suggest a possible chemical base for the conformational transitions.

The acetylcholine receptor (AcChR)[1] has been purified from *Torpedo californica* both in an enriched membrane preparation and in a detergent-solubilized, highly purified state (Duguid & Raftery, 1973; Schmidt & Raftery, 1973b; Raftery et al., 1975; Vandlen et al., 1976). Its biochemical properties are currently under intensive study with the aim of deducing molecular mechanisms for synaptic depolarization and other effects such as receptor desensitization.

It has long been known that the AcChR exhibits the phenomenon of desensitization in vivo (Katz & Thesleff, 1957). Prolonged application of the agonists acetylcholine (AcCh), carbamylcholine (Carb), or succinylcholine to frog sartorius end plates resulted in decreased effectiveness of these agonists. The AcChR was considered to change from an "effective" (low ligand affinity) to a refractory (high affinity) state in the presence of ligand. After complete removal of agonist, a slow recovery of the original activity was observed. Rang & Ritter (1970a,b) found that preincubation with certain agonists caused an increase in AcChR affinity for antagonists. Subsequent in vitro studies with AcChR-enriched membrane preparations have shown that preincubation with agonists induced a high-affinity form of the AcChR (Weber et al., 1975; Weiland et al., 1976, 1977; Lee et al., 1977; Quast et al., 1978). This in vitro ligand-induced affinity change thus appears to parallel in vivo desensitization in terms of many of its pharmacological properties. Although the ligand-induced affinity change of the AcChR has been studied in some detail and may in fact represent a reproduction in vitro of in vivo desensitization, little is known about the chemical interactions involved in this process.

Nicotinic AcChRs are known to contain a reactive disulfide bond(s) in the vicinity of the cholinergic ligand binding site(s) (Karlin & Bartels, 1966; Lindstrom et al., 1973; Ben-Haim et al., 1973). Electrophysiological studies have shown that reduction of this bond(s) inhibits the depolarizing effect of cholinergic agonists, while 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs_2) completely restores the membrane sensitivity to AcCh. The reduced receptor could be alkylated by *N*-ethylmaleimide (NEM) and could then no longer be reoxidized. The effect of reduction was found to be due to both a decreased apparent affinity of the receptor for AcCh (Ben-Haim et al., 1975; Schiebler et al., 1977) and a reduced conductance of single channels as revealed by noise analysis (Ben Haim et al., 1975). This disulfide bond has been shown to reside on the 40 000-dalton polypeptide of the *Torpedo* AcChR complex since affinity alkylation with the antagonist 4-(*N*-maleimido)benzyltrimethylammonium iodide (MBTA) (Weill et al., 1974) or the agonist bromoacetylcholine (Moore & Raftery, 1979) labels this particular receptor subunit.

In this communication, the effects of chemical modification of sulfhydryl groups and disulfide bonds on the interconversion of affinity states of the AcChR induced by cholinergic ligands were investigated. Reagents directed against both free sulfhydryl groups and disulfide bonds were found to inhibit the Carb-induced affinity change of AcChR-enriched membrane fragments. In addition, reversion of high-affinity AcChR to the low-affinity state(s) was blocked by the reagent *p*-chloromercuribenzoate (PCMB). These results are discussed in terms of two possible simple models which involve both cysteine and cystine residues in interconversion of the affinity states of the AcChR.

Experimental Section

T. californica was obtained locally. Lyophilized venom of *Bungarus multicinctus* was obtained from Sigma Chemical Co. DE-81 DEAE discs were from Whatman, Ltd. Scintillation counting was done in a Packard Tricarb liquid scintillation spectrometer (Model 3375) with 25% Triton X-100 and 0.55% Permablend III in toluene as scintillant.

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[1] Abbreviations used: AcCh, acetylcholine; AcChR, acetylcholine receptor; Carb, carbamylcholine; NEM, *N*-ethylmaleimide; PCMB, *p*-chloromercuribenzoate; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; DEAE, diethylaminoethyl; CM, carboxymethyl; α-BuTx, α-bungarotoxin; MBTA, 4-(*N*-maleimido)benzyltrimethylammonium iodide.

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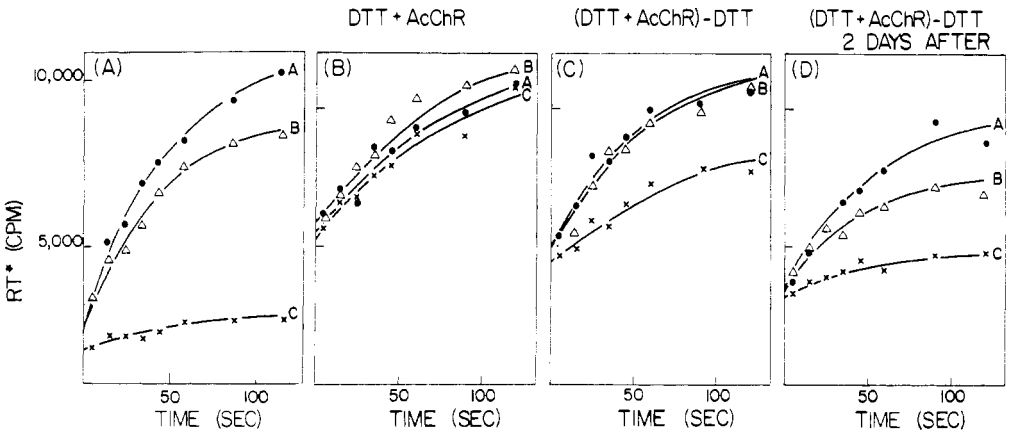


FIGURE 1: Effect of DTT and its subsequent removal on the Carb-induced AcChR affinity change. Curve A: (●) [^{125}I]-α-BuTx was added to a receptor membrane preparation [(4–8) × 10^{-8} M in *Torpedo* Ringer's solution] to a final concentration of 5 × 10^{-7} M at time zero, and the formation of the receptor–toxin complex was followed by DEAE disc assay. Curve B: (△) same as in curve A, except that 1 μM Carb was added with toxin at time zero. Curve C: (×) same as in curve A, except that the AcChR was preincubated with 1 μM Carb for 30 min prior to toxin addition. (A) Unmodified AcChR. (B) DTT-treated membrane-bound AcChR. (C) Membrane preparation which was modified as in (B) and dialyzed against *Torpedo* Ringer's solution for 16 h to remove DTT. (D) Membrane preparation which was treated as in (C) and stirred at 4 °C for 2 days after dialysis.

DTT, PCMB, Carb, and iodoacetamide were from Sigma Chemical Co. Iodoacetic acid was obtained from Matheson Coleman and Bell. All other chemicals were of the highest purity commercially available.

Membrane fragments were prepared by homogenizing *T. californica* electroplax from freshly killed animals or from electric organs stored at −80 °C as previously described (Duguid & Raftery, 1973; Reed et al., 1975). Buffer used throughout the preparation was 10 mM sodium phosphate, pH 7.4, 400 mM NaCl, 1–5 mM EDTA, and 0.02% NaN_3. α-BuTx was purified from lyophilized venom as described by Clark et al. (1972) and was labeled with ^{125}I by a procedure modified from that of Vogel et al. (1972). The monoiodo derivative was purified from other labeled species by CM-Sephadex chromatography. [^{125}I]-α-BuTx binding to AcChR was carried out by the DEAE filter disc assay method of Schmidt & Raftery (1973a).

All membrane fragment preparations were routinely assayed to determine whether they exhibited low or high affinity for Carb. The initial rate of [^{125}I]-α-BuTx binding was measured in the absence of agonist and in the presence of 1 μM Carb added either 30 min prior to or at the same time as [^{125}I]-α-BuTx, under conditions in which the toxin was in large excess over the AcChR toxin binding sites (Figure 1A). Membrane fragments were judged to be completely in the low-affinity form when the initial toxin binding rate observed in the absence of Carb (curve A) and when Carb and toxin were added simultaneously (curve B) were equal. Determination of the rates of Carb-induced isomerization of the AcChR and recovery of the AcChR from high- to low-affinity state were carried out as described previously (Lee et al., 1977). Apparent K_d values of Carb for the AcChR were determined by preincubating AcChR membrane suspensions with various concentrations of Carb and monitoring the initial rates of [^{125}I]-α-BuTx–AcChR complex formation by DEAE disc assay.

For chemical modification, membrane suspensions of AcChR containing 1–2 μM α-BuTx sites in Hepes Ringer's solution (250 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, 20 mM Hepes, pH 7.4, and 0.02% NaN_3) were allowed to react with 1 mM DTT or PCMB. The reaction vials were flushed with argon and allowed to stand at room temperature for 1 h. Alkylation of reduced AcChR was accomplished by adding 3 mM iodoacetamide or iodoacetic acid to the reduced membrane preparation, and the reaction was allowed to continue for another 2 h at 4 °C. The membrane fragments were then pelleted, washed, and resuspended in fresh *Torpedo* Ringer's solution. Stock solutions of reagents were made in 200 mM Tris buffer, pH 8.5.

Results

Effects of Sulfhydryl and Disulfide Reagents on the Conversion of Membrane-Bound AcChR from Low-Affinity to High-Affinity State(s) by Carb. (A) Reduction by DTT. Membrane-bound AcChR from *T. californica* underwent a reversible conformation change to a high-affinity form upon incubation with the agonist Carb (Figure 1A) as described previously (Lee et al., 1977; Weiland et al., 1976, 1977; Quast et al., 1978). Addition of DTT to a membrane preparation enriched in AcChR abolished the induction of this affinity change by 1 μM Carb (Figure 1B). However, upon removal of excess reducing agent, by centrifugation or dialysis, the AcChR gradually recovered its ability to undergo the affinity change as illustrated in Figure 1C,D; immediately after removal of excess DTT, preincubation of the AcChR with 1 μM Carb for 30 min reduced the initial toxin binding rate to approximately 50% of that in the absence of Carb (Figure 1C, curve C), and, after these membrane fragments were stirred in the cold for two days, complete recovery of the phenomenon was approached (Figure 1D, curve C). Neither the toxin binding rate nor the number of toxin binding sites was affected after these treatments. The half-time for conversion from low- to high-affinity state(s) in the presence of 1 μM Carb was determined for these "recovered" membrane fragments, by the toxin binding technique, giving a value of $t_{1/2}$ = 80 s. This value is in close agreement with that previously reported for unmodified membrane fragments (Lee et al., 1977).

(B) Reduction and Alkylation. When a DTT-reduced membrane preparation was alkylated with iodoacetic acid or iodoacetamide, a blockage of the affinity-state conversion induced by 1 μM Carb occurred (Figure 2) similar to that observed upon DTT treatment alone. However, in contrast to the results obtained with DTT alone, removal of excess reagents did not restore the ability of the AcChR to undergo the affinity change. Again, neither the toxin binding rate nor the number of toxin binding sites was substantially affected. Investigation of the affinity for Carb of a preparation which had been treated with DTT and iodoacetic acid revealed that

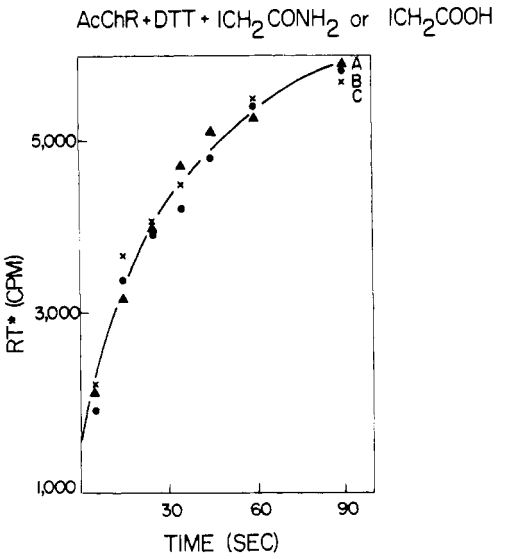


FIGURE 2: Effect of reduction and alkylation on the conversion of AcChR from low to high ligand affinity by Carb. Membrane-bound AcChR was reduced by DTT and alkylated with iodoacetic acid or iodoacetamide. The membranes were washed to remove the excess reagent and assayed for capability of ligand-induced affinity change by the method described in the legend to Figure 1.

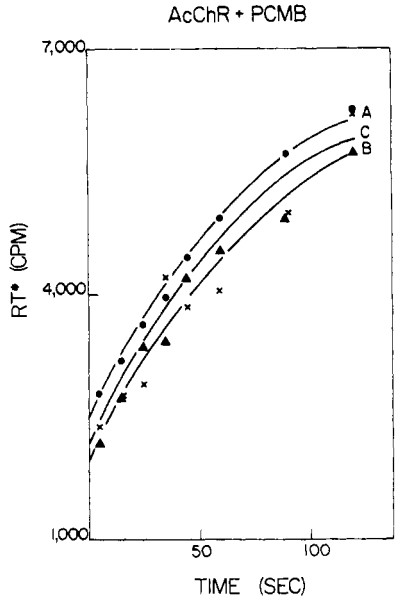


FIGURE 3: Effect of PCMB on the transition of AcChR from low- to high-affinity state. Membrane-bound AcChR was modified with 1 mM PCMB for 1 h at room temperature. The membranes were washed and assayed for ligand-induced affinity change as described in Figure 1.

a 50% reduction of the initial toxin binding rate was obtained at approximately 30 μM Carb. This value should be compared with the equilibrium value of 50–120 nM obtained with untreated preparations (Quast et al., 1978) and with the value of 30–50 μM obtained by the same authors for low-affinity membrane preparations. In control experiments it was found that iodoacetic acid or iodoacetamide alone (without prior reduction) at similar concentrations did not affect the affinity change induced by Carb.

(*C*) *PCMB.* Treatment of membrane-bound AcChR with 1 mM PCMB (without DTT treatment) also prevented the change in affinity induced by 1 μM Carb (Figure 3). This treatment did not affect the toxin binding rate or the number of toxin sites significantly. Values of K_d obtained for Carb by the [^{125}I]-α-BuTx binding technique indicated that the

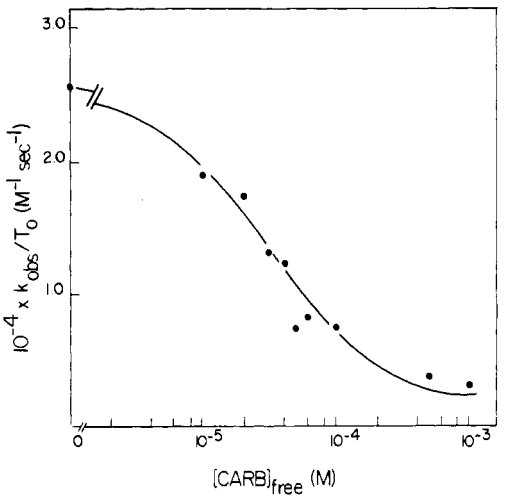


FIGURE 4: K_d of Carb for AcChR modified with PCMB (as in Figure 3). PCMB-treated AcChR [(6–8) × 10^{-8} M] was preincubated with various concentrations of Carb for at least 30 min. [^{125}I]-α-BuTx was added to a concentration of 5 × 10^{-7} M, and the initial rate of toxin–receptor complex formation was followed by DEAE disc assay. The data were fitted to the equation (Quast et al., 1978) $k_{obsd} = kT_0/(1 + [L]/K_{app})$, where T_0 is the total toxin concentration ($T_0 >> [AcChR]$), k is the bimolecular rate constant for toxin binding to AcChR, [L] is the free Carb concentration, and K_{app} is the apparent dissociation constant for Carb. The predicted curve for K_d = 30 μM from the fit is indicated as a solid line.

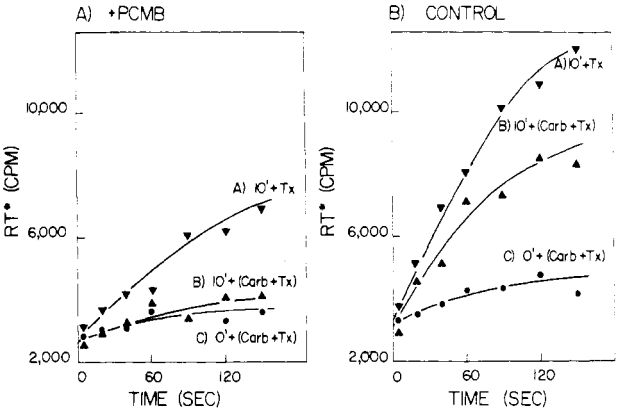


FIGURE 5: Effect of PCMB on the recovery of AcChR-containing membrane fragments to low affinity from Carb-induced high ligand affinity. AcChR-containing membranes (1.8 × 10^{-7} M in *Torpedo* Ringer's solution) were incubated with 3 μM Carb for 25 min at room temperature. (A) PCMB (1 mM) (in 200 mM Tris, pH 8.5) was added, and the reaction was allowed to proceed for 25 min. The reaction was then diluted 40-fold into *Torpedo* Ringer's solution; 0 (●) and 10 min (▲) after dilution, [^{125}I]-α-BuTx (5 × 10^{-7} M) and Carb (1 μM) were added simultaneously, and formation of the toxin–receptor complex was followed by disc assay. (▼) is identical with (▲), except that only toxin was added 10 min after dilution. (B) Control, same as in (A) except that PCMB was replaced by Tris buffer. Carb concentration after dilution was 75 nM.

AcChR was stabilized in a low-affinity form(s) after PCMB modification (K_d = 30 μM; Figure 4).

Effect of PCMB on Recovery of AcChR-Enriched Membrane Fragments to Low Affinity from Carb-Induced High Ligand Affinity. Membrane-bound AcChR (1.4 μM in α-BuTx sites) was first converted to the high-affinity state by incubation with 3 μM Carb. After 50 min at room temperature, the mixture was diluted 40-fold into *Torpedo* Ringer's solution, and the reversion of the AcChR to the low-affinity form was followed by the toxin binding assay method. Figure 5B illustrates the results. At different times after dilution, 1 μM Carb and 4 μg/mL of α-BuTx were added simultaneously, and the initial toxin binding rates were

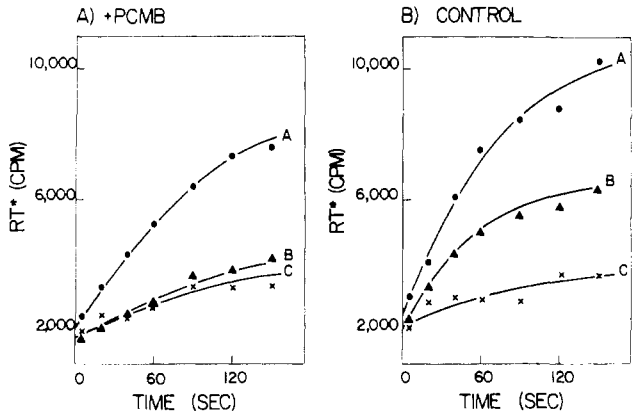


FIGURE 6: Effect of PCMB on the recovery of AcChR-containing membrane fragments to low affinity from Carb-induced high ligand affinity. Membrane fragments, modified with PCMB as described in the legend to Figure 5, were washed extensively with *Torpedo* Ringer's solution to remove Carb and then assayed by the method described in the Figure 1 for the state of receptor in affinity toward Carb. (A) PCMB-treated membrane fragments. (B) Control.

monitored by DEAE disc assay. As has been shown previously (Lee et al., 1977), recovery of low ligand affinity was essentially complete 10 min after dilution. However, if after 25 min of incubation with Carb, the AcChR was allowed to react with 1 mM PCMB for 25 min, the dilution experiment gave a completely different result (Figure 5A); there was essentially no reversion of the AcChR to the low-affinity form, as shown by the nearly superimposable curves (curves B and C in Figure 5A).

In another experiment, the PCMB-modified membrane fragments described above were washed extensively to remove the added Carb (this amounted to 75 nM after 40-fold dilution from 3 μM used to the high-affinity state). The membrane preparation was then tested for its state of affinity for Carb (Figure 6). The PCMB-treated membrane fragments showed only high affinity for Carb, whereas the control membrane fragments exhibited over 80% recovery of the low-affinity form. Thus, PCMB treatment of the Carb-converted AcChR seemed to stabilize the receptor in a high-affinity form(s).

The toxin binding time course of PCMB-treated AcChR 10 min after dilution (Figure 5A, curve A) was 30% of the control value. After complete removal of Carb, this rate (Figure 6A, curve A) was measured to be 50–65% of the control value; the total number of α-BuTx sites, however, remained unaffected.

Discussion

After reduction by DTT, the AcChR-containing membrane preparation from *T. californica* can no longer be converted from a low-affinity form(s) to one(s) of higher affinity by 1 μM Carb. Removal of excess reducing reagents gradually restored the ability to undergo the conversion, presumably by allowing reoxidation of a DTT reduced -S-S- bond(s). Alkylation of the reduced receptor with iodoacetamide or iodoacetic acid, however, irreversibly modified the reduced disulfide bridges, and no recovery of interconversion of states of ligand affinity was observed after removal of excess reagents. Since iodoacetamide and iodoacetic acid alone at similar concentrations did not have any significant effect on the Carb-induced conversion, it is concluded *from this result alone* that disulfide bond(s), rather than free sulfhydryl groups, in the native receptor play an important role in the effects described above.

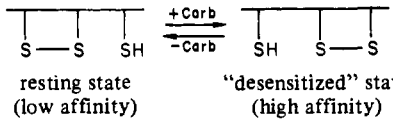
The apparent K_d for Carb of a reduced, iodoacetate-alkylated membrane preparation as determined by the inhibition of the initial α-BuTx binding rate was approximately 30 μM, a value 250-fold higher than that of an untreated preparation ($K_d \sim 120$ nM; Quast et al., 1978). The results indicate that one or more disulfide bonds are involved in the ligand-induced conversion between states of differing affinity. Reduction and alkylation of such bonds inhibited this process and irreversibly stabilized the AcChR in a low-affinity state(s), i.e., prevented conversion of the system to a state(s) of high affinity as in the case of unmodified receptor. Further study is required to determine if these bonds are (1) the "reactive" disulfide(s) in the preparation (Karlin & Bartels, 1966; Ben-Haim et al., 1973) that resulted in altered electrophysiological responses following DTT treatment and that was labeled by the antagonist MBTA (Weill et al., 1974) or the agonist bromoacetylcholine (Moore & Raftery, 1979) or (2) those involved in AcChR dimer formation through the 65 000–67 000-dalton subunits (Suarez-Isla & Hucho, 1977; Chang & Bock, 1977; Hamilton et al., 1977; Witzemann & Raftery, 1978) or (3) still other disulfides.

Treatment of the low-affinity form of the AcChR with PCMB, unlike iodoacetic acid or iodoacetamide, stabilized the receptor in a low-affinity state(s). This indicates the possibility that a sulfhydryl group(s), as well as a disulfide bridge(s), is important for the interconversion of affinity states in vitro. Observations consistent with this notion have been reported by Karlin & Bartels (1966), who demonstrated that treatment of intact electroplaques with PCMB affected the AcCh-dependent excitability, whereas NEM had no effect. The lack of effect of iodoacetic acid and iodoacetamide, like NEM, may be due to differing reactivities with freely accessible or difficultly accessible sulfhydryl groups in the AcChR. This suggestion is based on the relative ease with which PCMB is known to form mercaptides relative to reacting with other amino acid side chains (Means & Feeney, 1971), but reaction with other residues cannot be totally excluded. In addition, PCMB is known to react with buried sulfhydryl residues in proteins such as hemoglobin which are unreactive toward iodoacetic acid or iodoacetamide (Kilmartin & Wooton, 1970). It was possible to show that in addition to preventing the change to a high affinity form(s) by Carb the PCMB treated material had an apparent dissociation constant for Carb similar to the unmodified low-affinity form (Quast et al., 1978). It is not yet known, however, whether this stabilized low ligand affinity form(s) can be transformed by cholinergic agonists into an activated state capable of translocation of cations. In the future it should be useful for mechanistic studies irrespective of whether or not such is the case.

PCMB modification of the AcChR-enriched membranes which were first converted to the high-affinity state by preincubation with Carb prevented reversion from this state(s) to the resting state of low ligand affinity when the Carb concentration was reduced. Such treatment caused a slower α-BuTx binding time course as depicted in Figure 5A. This result would be expected if the AcChR were stabilized in a high-affinity form(s), since the residual Carb concentration after dilution was still large (75 nM) compared to the K_d of Carb for the high-affinity form (50 nM) (Quast et al., 1978). However, after complete removal of residual Carb, the toxin binding rate of the treated AcChR was not totally restored to the control value (Figure 6A). The precise reason for this observation remains to be elucidated; it could, for example, be caused by steric effects of the added PMB residues, resulting in a slowing of the kinetics of α-toxin binding.

There are two possible explanations for the effects of PCMB. First, PCMB may interact with the AcChR in such a way that

it sterically blocks conformational transitions, thus stabilizing a low-affinity form(s) when allowed to react with the resting state of the AcChR and stabilizing a high-affinity form(s) when allowed to react with this form following induction by Carb. Alternatively, the results could be explained in terms of a disulfide interchange model, depicted as



In this case, modfication of the SH group of either state would prevent conversion to the other state. Preliminary observations favor the notion that disulfide interchange processes can affect the transitions between states of low and high affinity: preparations found to be in a state of high ligand affinity upon isolation (i.e., isolated in a "desensitized" form) were reduced and dialyzed extensively to remove the reductant and allow reoxidation to take place. Such preparations were found to be in a state of low ligand affinity following this treatment and were capable of ligand-induced conversion to a state of high affinity. One simple interpretation of these results would be that the receptor, as isolated in these cases, had undergone sulfhydryl–disulfide interchange reactions that prevented normal function. Further supporting evidence, however, is needed to establish the validity of this hypothetical model of the interconversion process.

In addition, it is possible that PCMB treatment labilizes some component(s) of the membrane, resulting in either its inactivation or removal from the system. In this respect we have not yet conducted rigorous analyses of the constituent polypeptides remaining after the chemical modifications have been carried out.

In summary, these studies indicate that free sulfhydryl groups and disulfide bonds are both present in AcChR-enriched membrane preparations and their modification inhibits interconversion of the affinity states of the AcChR. The significance of these results is that they suggest a possible chemical base for the ligand-induced affinity change of membrane-bound AcChR.

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