Chemical Modification of the Nicotinic Cholinergic Receptor of PC-12 Nerve Cell†

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ABSTRACT: The identity of the protein that mediates the nicotinic acetylcholine sensitivity in neuronal cells has been investigated by chemical modification and affinity labeling. When an ion flux assay is used, it is possible to measure specifically the activity of the ionophore associated with the nicotinic acetylcholine receptor in cultured nerve cells (PC-12 pheochromocytoma). This activity is modulated by modification of the redox state of at least one disulfide bridge located at the vicinity of the agonist binding site. The oxidizing agent 5,5′-dithiobis(nitrobenzoic acid) restores the complete receptor response which had been inhibited by reduction with dithiothreitol. *N*-Ethylmaleimide and the nicotinic affinity labels [4-(*N*-maleimido)benzyl]-α-trimethylammonium iodide and bromoacetylcholine react also with the reduced receptor and irreversibly block the agonist-dependent response of the ionophore. The two affinity labels show strong affinities for the receptor, and apparent IC_{50} values of 20 and 560 nM can be respectively evaluated. Bromoacetylcholine, being an acetylcholine analogue, blocks the receptor function by desensitization, a process in which the constant interaction with the activator causes a shift into an inactive form of the receptor. Bromoacetylcholine can also be shown to activate untreated as well as reduced cells. In this case, the bound label induces a lasting response which is terminated by the irreversible desensitization of the modified receptor. These experiments thus show that the PC-12 nicotinic ionophore shares functional and structural similarities with peripheral receptors. They suggest that nicotinic affinity labels developed for the muscle receptor can also be used as specific markers of the nicotinic neuronal ionophore. They also show that the site labeled by these reagents is a functional site that can be both activated and desensitized.

Nerve signal integration is partly based on the ability of postsynaptic neurotransmitter receptors to modulate the cellular response to a rather featureless signal. The molecular properties of the receptor protein that allow such behavior are beginning to be elucidated in the case of the nicotinic acetylcholine receptor (AChR)[1] of muscle and fish electric organs [for a review see Changeux (1980)].

The kinetics of the modulation of ionic permeabilities by this receptor have been characterized in vivo (Hamill & Sakmann, 1981) as well as in vitro with native receptor in its membrane environment (Hess et al., 1975). In these studies, the receptor activation and inactivation parameters have been well documented (Cash et al., 1980), and a molecular mechanism that includes informations gained by electrophysiology (Katz & Thesleff, 1957) as well as data obtained by the study of the ionic fluxes in receptor-containing membrane vesicles has been presented (Cash & Hess, 1980).

Much information about the structure and mechanism of the acetylcholine receptor has been obtained by its chemical modification. Affinity labeling of the receptor by [4-(*N*-maleimido)benzyl]-α-trimethylammonium iodide (MBTA) or bromoacetylcholine (BAC) (Karlin et al., 1975) after reduction by dithiothreitol (DTT) has been of particular interest. These two affinity compounds can be made radioactive and have been useful as specific labels of the acetylcholine binding sites. Ion flux studies (Cash & Hess, 1980) as well as binding experiments (Neubig & Cohen, 1979; Delegeane & McNamee, 1980) had shown that reversible ligands such as acetylcholine or its analogues bind to two sites per receptor and compete for these sites with α-neurotoxin. BAC (an agonist of the receptor) and MBTA (an antagonist) react with only one of the two available neurotoxin binding sites of the receptor (Damle & Karlin, 1978), while BAC can label both sites in a slightly different experimental system (Wolosin et al., 1980).

Binding sites for nicotinic cholinergic agonists and antagonists in the central nervous system have also been shown to be sensitive to chemical modification (Lukas & Bennett, 1980a,b). Although they display pharmacological characteristics similar to those of the muscle AChR, these CNS cholinergic binding sites need still to be identified as true CNS AChR. So far, except for a few cases (Betz, 1981; Duggan et al., 1976; Miledi & Szczepaniak, 1975), their ability to control ion fluxes has not been demonstrated, and α-bungarotoxin, a specific inhibitor and irreversible marker of the muscle receptor, failed to block central (Duggan et al., 1976; Miledi & Szczepaniak, 1975) and ganglionic cholinergic responses [Bursztajn & Gershon, 1977; but see Chiappinelli & Zigmond (1978)]. As nicotinic characteristics, it is worth noting that the CNS neurotoxin binding site located on brain membrane preparations displays an agonist-induced time-dependent change in its affinity toward cholinergic ligands, as demonstrated by the competition between activating ligands and radiolabeled neurotoxin for the binding sites on the membrane (Lukas & Bennett, 1979). This type of agonist-induced affinity alteration of the receptor is characteristic of the peripheral AChR (Weber et al., 1975; Colquhoun & Rang, 1976; Weiland et al., 1976; Quast et al., 1978; Bulger et al., 1977) and has been identified as a consequence of the receptor conformational changes associated with desensitization. However, affinity labeling of these brain membrane preparations, as monitored by toxin binding, by MBTA or BAC

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[1] Abbreviations: AChR, acetylcholine receptor; MBTA, [4-(*N*-maleimido)benzyl]-α-trimethylammonium iodide; BAC, (bromoacetyl)-choline; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CNS, central nervous system.

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failed to show the half-of-the-site reactivity observed with the fish electric organ membranes (Lukas & Bennett, 1980a). Reversibly associating agonists and antagonists affected the rate of affinity reagent interaction with neurotoxin receptors but only at very high concentrations. Thus, it may be premature to conclude from chemical modifications experiments that the CNS toxin receptor is a true AChR.

One approach that may help in the understanding of this problem is the use of clonal nerve cells in culture. With this material, the functional properties and toxin binding characteristics of the receptor can be assessed at the same time by using a single cell type (Sine & Taylor, 1980). A considerable advance has been made in the isolation and characterization of nerve cell from the central and peripheral nervous system (Bullock et al., 1977). Of particular interest is the clonal pheochromocytoma PC-12 (Greene & Tischler, 1976) that displays nicotinic cholinergic properties (Dichter et al., 1977; Patrick & Stallcup, 1977b; P. Leprince et al., unpublished results). This cell line shows an apparent separation between α-bungarotoxin binding sites and receptors controlling the cholinergic response (Patrick & Stallcup, 1977a,b), a characteristic also present in sympathetic neurons (Carbonetto et al., 1978) and ganglionic cells (Brown & Fumagalli, 1977). This segregation of the two cholinergic functions is a strong support of the opinion that the occurrence of α-neurotoxin binding sites in the brain does not reflect the presence of functional cholinergic receptors in this tissue.

To compare the receptor mediating the ion-translocation function with the toxin binding entities and with the peripheral AChR, we have undertaken the comparison of their behavior after chemical modification. This report deals with the effect of affinity labeling on the ion fluxes mediating receptor; a second paper will be published that involves the toxin receptor of the PC-12 cells.

Materials and Methods

Cell Culture. PC-12 Cells. PC-12 cells are grown in 100-mm plastic dishes (NUNC) and maintained at 37 °C in an atmosphere of 5% CO_2 in air. The culture medium is Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum and 10% heat-inactivated horse serum. The cells are propagated every 4–5 days after reaching confluence.

For experiments, the cells are plated on polylysine-coated 35-mm dishes and used the same day. Three to four 35-mm dishes are made from one confluent 100-mm dish.

Chemical Modification. MBTA was synthesized according to Karlin (1977) and stored in acetonitrile in liquid nitrogen. The observed melting point of the final product was 204 °C (theoretical mp 204–205 °C). The structure of the product was verified by ^{1}H NMR spectroscopy, and its purity was checked by UV spectroscopy (Karlin, 1977).

Before each experiment, an aliquot of MBTA was dried under gentle N_2 stream and redissolved at 100-fold its final concentration in 10^{-4} M HCl. Dilution of this solution into assay buffer to the concentration needed for the experiment was made immediately before use to prevent the self-hydrolysis of the maleimide group.

BAC was synthesized by the method of Damle et al. (1978). Its purity was verified by paper chromatography (Wolosin et al., 1980) and by elemental analysis. BAC was stored as dry crystals at −20 °C and dissolved in 10^{-4} M HCl before each experiment. The same procedure for dilution in assay buffer was followed for BAC as it was for MBTA.

Generally, the cells were first rinsed with assay buffer and then reduced with 1 mM DTT in assay buffer for 20 min. After three rinses with buffer, the label reagent was added for 15 min at a concentration ranging from 0.1 mM to 0.1 nM for MBTA and from 0.1 mM to 10 nM for BAC. This incubation was followed by a 15-min incubation with 0.1 mM DTNB in assay buffer after which the cells were tested for agonist-mediated ion fluxes. Indications are given in the figure legends when this experimental procedure was modified.

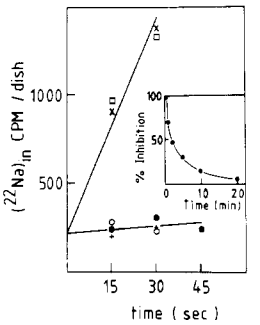

FIGURE 1: Effect of acetylcholine receptor disulfide modification on carbamoylcholine-induced sodium influx in PC-12 cells. The cells were reduced with 1 mM DTT in buffer for 20 min and then incubated with 0.1 mM DTNB for 15 min (□), or with DTT and then with 1 mM *N*-ethylmaleimide for 15 min, followed by 0.1 mM DTNB for 15 min (○), or with DTT and then with buffer only for 15 min (+). Ion flux buffer containing 5 mM ouabain, 1.3 μCi of ^{22}Na/mL, and 2 mM carbamoylcholine was then added for the time shown, and the influx of sodium was measured as described. Control uptake of sodium is determined with cells that were treated with buffer only and that received radiotracer solution without carbamoylcholine (●), while full response of the cells is given by similarly unmodified cells that were tested with 2 mM carbamoylcholine for the time shown (×). Inset: Time course of AChR inhibition by 1 mM DTT. The cells were treated with DTT for the time shown after which their response to 2 mM carbamoylcholine was measured as described.

^{22}Na Fluxes. The ^{22}Na flux assay was as described by Stallcup (1979) with one dish representing one time point. Ion flux buffer was assay buffer (140 mM NaCl, 5,4 mM KCl, 2 mM $CaCl_2$, 5 mM glucose, and 25 mM Hepes-Na adjusted to pH 7.4 with HCl) to which ouabain (5 mM) and ^{22}Na (1.3 μCi/mL) were added. Immediately after the DTNB solution was removed, 0.75 mL of ion flux buffer containing or not 2 mM carbamoylcholine was added to the dish. The Na^+ uptake was terminated by three rinses with assay buffer followed by 30 s of draining. The cells were collected by using two 1-mL aliquots of 0.1 N NaOH and counted in a γ counter.

Results

An important characteristic of peripheral AChR is the presence of several disulfide bridges located between receptor subunits and in the vicinity of the ligand binding sites. The redox state of these disulfide groups appears to play a crucial role in the control of the cholinergic response. After reduction by DTT, a decrease in the efficiency of acetylcholine analogues to induce ion fluxes in electric organ membrane preparations is apparent (Walker et al., 1981a). Similarly, upon reduction by 1 mM DTT, the cholinergic response of PC-12 cells is markedly altered (Figure 1). The response to saturating concentrations of carbamoylcholine is totally abolished, while passive sodium influx, measured in the absence of agonist, is not affected by prior reduction.

The time course of reduction by DTT is shown in the inset to Figure 1. Complete reduction of the PC-12 receptor was reached after 20 min, and in further chemical modification experiments, the incubation with DTT was routinely performed for the same time. This inhibition by DTT could be totally reversed by treatment with the oxidizing agent dithiobis(nitrobenzoic acid) (DTNB) or be irreversibly maintained by treatment with *N*-ethylmaleimide, an alkylating agent (Figure

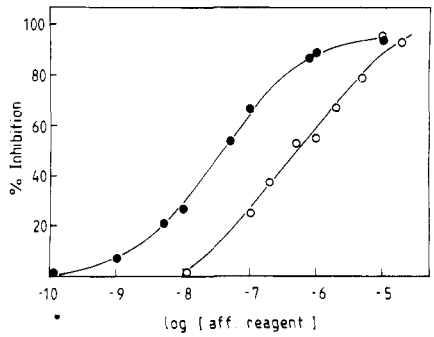

FIGURE 2: Inhibition of AChR-mediated sodium influx by MBTA and BAC. The cells were reduced for 20 min with 1 mM DTT, then incubated with MBTA (●) or BAC (○) for 15 min at the concentrations shown, and then treated with 0.1 mM DTNB for 15 min. Their sensibility to 2 mM carbamoylcholine is then measured as in Figure 1. Control values are given by cells treated with DTT and then with DTNB.

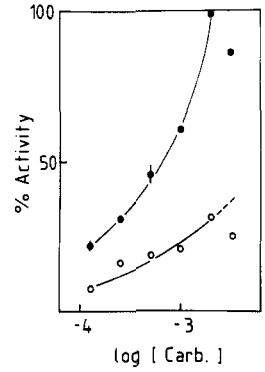

FIGURE 3: Effect of carbamoylcholine concentration on AChR-mediated sodium influx in native and MBTA-treated PC-12 cells. Cells were either reduced with DTT and then reoxidized with DTNB (●) or reduced with DTT, alkylated with 10 μM MBTA, and then reoxidized with DTNB (○) as described in Figure 2. Their response to various concentrations of carbamoylcholine was then measured; 100% activity is the response of native cells to 2 mM agonist. Bars across points show the data dispersion resulting from two independent experiments.

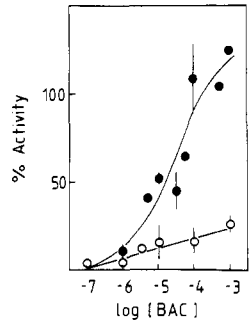

FIGURE 4: Effect of AChR reduction on bromoacetylcholine-induced sodium influx in PC-12 cells. The sodium influx in native cells (●) or cells that had been treated with 1 mM DTT for 30 min (○) induced by the concentrations of bromoacetylcholine shown on the graph was measured as described in Figure 1; 100% activity is the response of untreated cells to 2 mM carbamoylcholine. The figure was compilated from two independent experiments. The bars represent the data dispersion for BAC concentrations that were used in both experiments.

1). As a consequence of this inhibition by DTT, it was necessary after covalent binding of affinity labels to the reduced receptor to reoxidize the unreacted SH groups of the cell membrane with DTNB if the remaining functional response was to be measured. A 15-min treatment of the reduced PC-12 AChR with 0.1 mM DTNB was shown to be sufficient to allow the recovery of a full response to carbamoylcholine (Figure 1).

Affinity labeling experiments using MBTA or BAC may be somehow limited by the spontaneous hydrolysis of both reagents in neutral solutions. For this reason, the labels were allowed to react with the reduced receptor for a maximum of 15 min after which time the unreacted reagent was removed and the cells were treated with DTNB to reoxidize the remaining SH groups.

As shown in Figure 2, the covalent attachment of MBTA to the reduced AChR causes an inhibition of agonist-mediated ^{22}Na influx into the cells. This inhibition is dose dependent as can be expected if one assumes that the covalent binding of the affinity label is the rate-limiting step of the interaction of MBTA with the reduced receptor, and thus, the concentration of MBTA that causes a 50% decrease of the response to 2 mM carbamoylcholine can be calculated to be 20 nmol/L. In these experiments, the concentration of label that causes a 50% decrease of the carbamoylcholine response is likely to be overestimated. Although the rate constant for specific alkylation of electric organ AChR is much larger than the rate constant for nonspecific reactions of the affinity labels with nonreceptor SH groups (Karlin, 1977), the concentration of these nonreceptor SH groups is certainly several orders of magnitude larger than the concentration of AChR, especially when the PC-12 receptor is considered. The data represent thus the concentration of affinity reagent necessary to block a given fraction of the active response of the AChR before the reagent is consummated by nonspecific interactions.

Increasing the concentration of agonist results in a larger response of the PC-12 AChR labeled with an excess concentration of MBTA (Figure 3). However, with neuronal cells, at high agonist concentration desensitization will happen well before the first time point is taken. Thus, no exact measurements of agonist-induced ion flux above 2 mM carbamoylcholine can be made, and it is not possible to show if very large agonist concentrations can effectively suppress the MBTA inhibition.

The covalent binding of bromoacetylcholine to the receptor realized in the presence of eserine, an inhibitor of acetylcholinesterases, also lead to the inhibition of the sodium flux mediated by PC-12 receptors. The alterations of the functional response due to BAC are nevertheless more complex since, as an acetylcholine analogue, BAC behaves in some instances as a receptor agonist. This effect is readily seen upon addition of BAC to untreated cells together with radioactive tracer ion (Figure 4). The concentration of free BAC that elicits a ^{22}Na flux equal to 50% of the carbamoylcholine-induced response can be evaluated to be about 10 μmol/L, a figure not different from the value obtained with acetylcholine. After reduction of the cells by DTT, however, the same concentrations of BAC could elicit only strongly diminished sodium influxes (Figure 4). As with carbamoylcholine, thus, the reduction of a receptor disulfide prevents the subsequent activation by an agonist even if, like BAC, this agonist is capable of alkylating the receptor SH group. After binding of BAC to the reduced receptor and subsequent reoxidation of the unreacted sulfhydryl groups, the affinity label would block 50% of the agonist-induced response at a concentration of about 560 nmol/L (Figure 2).

In contrast with the MBTA effects, this inhibition by bound BAC cannot be reversed, even partially, by increasing the concentration of carbamoylcholine present in the sodium uptake medium. This difference can be explained if ones assumes that the inhibition by BAC results from desensitization of the receptor by bound BAC, making thus the addition of larger concentrations of agonist totally ineffective.

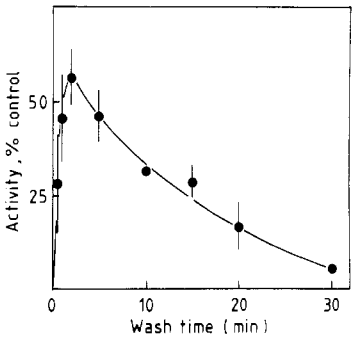

FIGURE 5: PC-12 acetylcholine receptor activation by bound BAC. After reduction of the cells with 1 mM DTT for 30 min, BAC (10 μM) was added for 5 min. The cells were then extensively washed in a large buffer bath. During that wash, the ability to incorporate ^{22}Na into the cells was measured at the time shown without addition of agonists; 100% activity is the response of untreated or DTT–DTNB-treated cells to 2 mM carbamoylcholine. Cells that were not reduced first, or that were not incubated with BAC, showed activity equal to control (0% activity). The figure was compilated from three independent experiments. The bars represent the data dispersion for wash time that was realized in all three experiments.

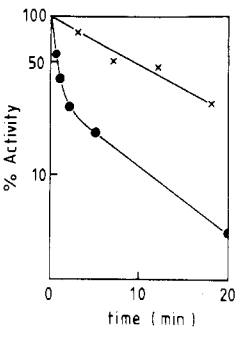

FIGURE 6: Desensitization of PC-12 AChR by free and bound BAC. (●) Free BAC: the cells were incubated with 10 μM BAC without preliminary reduction, and at the time shown their response to 2 mM carbamoylcholine was measured. (×) Bound BAC: the cells were reduced with DTT, alkylated with 10 μM BAC, and washed in buffer, and their activation by bound BAC was measured at the time shown during the washing step as described in Figure 5. For that experiment, 100% activity is the maximum activation by bound BAC observed during the washing procedure (after about a 2-min washing; see Figure 5).

This conclusion implies that shortening the duration of the interaction between bound BAC and reduced receptors would provoke a lesser inhibition of agonist-induced flux. Due to the necessity of going through a reoxidation step with DTNB, this particular experiment is, however, impossible to perform. Desensitization by bound BAC can, however, be demonstrated in experiments where the affinity label is acting as a permanent receptor agonist.

Indeed, BAC can be made to react for a short time with the reduced receptor after which the unreacted label can be completely washed away. Figure 5 shows that in these conditions, the receptor becomes activated although no free agonist is present in the assay medium. The capability of mediating an increased sodium flux first augments rapidly during the washing procedure that follows the labeling step. After about 2 min, the amplitude of the sodium influx levels off and decreases to reach control values after about 30 min. Parallel runs made without prior reduction of the PC-12 cells with dithiothreitol or without addition of BAC after reduction give similar levels of influx not different from the passive sodium uptake of unaltered cells. This time-dependent rise in sodium flux seen with labeled preparation corresponds probably to the recovery from the desensitization by free BAC that occurred during the labeling phase. The slower lowering in activity that follows would thus be the result of the desensitization of these receptors that are subjected to activation by bound BAC.

To support this conclusion, we have compared the rate of desensitization by bound and free BAC. As can be seen from Figure 6, the desensitization by free BAC, measured by evaluating the residual response to 2 mM carbamoylcholine of unreduced cells after various lengths of incubation with 10 μM BAC, is a biphasic process. Interestingly, the slope of the slow phase of that process is almost parallel to the slope describing the apparent desensitization by bound BAC (half-time of 7.5 and 9.5 min, respectively). The lack of a rapid phase in the desensitization by bound BAC can be explained if one recalls that during the labeling reaction the interaction with free BAC has lasted for 5 min and that in Figure 6, the 100% activity value for the bound BAC curve is set to be that amplitude of sodium flux observed after a 2-min washing (2 min during which desensitization by bound BAC has occurred). Thus, although we cannot rule out the existence of a fast phase in the process of the desensitization by bound BAC, the experimental procedure used here precludes its determination.

Discussion

The aim of these experiments was to determine whether affinity labels could be used in the study of a peripheral nervous system nicotinic receptor. These reagents have been shown to react specifically with the receptor of muscle origin. Their interaction with a neurotoxin binding site of the central nervous system has also been documented (Lukas & Bennett, 1980a). In this case, however, only the inhibitory effect of the labels on the binding of a specific snake neurotoxin to its receptor could be studied. Neuronal cells in culture used here are a particularly useful material since both the binding and the functional effects of a ligand can be assessed in similar experimental conditions.

The results suggest that the state of reduction of a disulfide bridge located at the vicinity of the acetylcholine binding site affects the response of the PC-12 receptor to its ligand. Such effects of thio-group modification on ligand-induced ion flux and ligand-binding affinity have been reported (Lukas & Bennett, 1980b; Walker et al., 1981a). With PC-12 cells, reduction by dithiothreitol causes a strong inhibition of carbamoylcholine-induced sodium flux. In contrast, similar experiments carried out with *Torpedo* membrane preparation showed only a shift in the dose–response curve for the carbamoylcholine-induced increase in $^{22}Na^+$ permeability to higher ligand concentrations. In parallel, a decrease in the binding affinity of the AChR for carbamoylcholine was also observed, suggesting that the decreased response was a direct result of the decreased binding affinity (Walker et al., 1981a).

Since our data show that treatment of reduced PC-12 receptors with DTNB reverses completely all DTT effects, we may conclude that native and DTT–DTNB-treated PC-12 receptors are in a similar resting state. Such a result is consistent with similar observations made on *Torpedo* acetylcholine receptor (Walker et al., 1981a) and reinforces the hypothesis that functional nicotinic receptors of all origin possess a disulfide group that can be reversibly reduced by DTT into a sulfhydryl group with a concomitant decrease in the response to agonist. Affinity labels did readily react with the reduced PC-12 receptor. Studies with brain membrane receptor have shown that affinity labels react with the receptor-associated SH group(s) several orders of magnitude faster than with the nonreceptor SH groups. Thus, even if receptor SH groups constitute only a small fraction of all sulfhydryl groups, the specific labeling is completed in a few seconds (Lukas & Bennett, 1980a). Accordingly, in our ex-

Table I: Effect of Chemical Modification on AChR Function and AChR Agonist Affinity in PC-12 Cells and *Torpedo* Electric Organ Preparation[a]

	PC-12 cells	*Torpedo* membrane[b]	
treatment	flux response	flux response	agonist binding[c]
native	no effect	no effect	no effect
DTT	inhibition	decrease in agonist efficiency	decrease in agonist affinity
DTT + DTNB	no effect	no effect	no effect
DTT + NEM	irreversible inhibition	irreversible inhibition	large decrease in agonist affinity
DTT + MBTA	decrease in agonist efficiency	decrease in agonist efficiency	slight increase in agonist affinity
DTT + BAC	activation/inhibition	decrease in agonist efficiency	
BAC	activation		

[a] Chemical modification and flux assay were carried as described under Materials and Methods. Flux response for PC12 cells is usually the ^{22}Na influx elicited by 2 mM carbamoylcholine. Flux response for *Torpedo* membrane is the ^{22}Na efflux elicited by 1 mM carbamoylcholine. [b] Data from Walker et al. (1981a) and Delegeane & McNamee (1980). [c] Measurements of carbamoylcholine dissociation constants by [^{125}I]-α-bungarotoxin competition assay.

periments, the inhibition by the labels appears to be the same whether the alkylation reaction is allowed to proceed for 15 min or is stopped after 5 min of reaction.

Covalently bound MBTA showed only an inhibitory effect on the PC-12 receptor function and had no effect on $^{22}Na^+$ flux in the absence of added agonist. MBTA inhibition resulted in an apparent shift of the carbamoylcholine response to higher concentration although this effect could not be completely documented with the PC-12 receptor. An aberrant behavior is indeed observed when the agonist concentration is raised past 2 mmol/L: the ability to induce an increased sodium influx diminishes as the agonist concentration is raised. The fact that a similar decrease of the active response occurs with both untreated and treated cells suggests that this phenomenon is due to a faster desensitization process at high agonist concentration. Changes in agonist affinity after covalent binding of MBTA have been also observed with *Torpedo* AChR. In this case, however, it was shown that MBTA labels only one of two identical ACh binding sites per receptor and that the remaining site, which can be activated, presents a decreased affinity for the agonist resulting form the DTT reduction step (Delegeane & McNamee, 1980).

All experiments involving BAC presented in this work tend to demonstrate that this affinity reagent labels a receptor site that can be both activated and desensitized. Free BAC induces a functional response of the PC-12 receptor exactly as acetylcholine does. Addition of BAC to the reduced receptor, however, gives only rises to a strongly inhibited ion flux response. Thus, alkylation by the bromoacetyl group of the agonist of the SH group located close to the ACh binding site does not seem to be sufficient to suppress the receptor inactivation initially caused by disulfide reduction. Two mechanisms could equally well explain this fact. The first one would be that receptor reduction causes a decrease in affinity for the agonist so that an effective interaction of BAC with reduced receptor would be less likely to occur. Prolonged incubations (>1 min) with the affinity label would, however, lead to a higher occupation of the active site since the binding of BAC to reduced receptor is irreversible and a significant but transient receptor activation would be apparent before desensitization could occur. Alternatively one could think that more than one disulfide/SH group is involved in the control of the receptor activity and that occupation of the receptor active site by bound BAC would be able to trigger a response only if one or more other SH groups were oxidized or even alkylated by a BAC molecule. If these alternative SH groups show a poor affinity for nicotinic agonists, again a long incubation with BAC would be able to shift the receptor into an active form, but instantaneous addition of affinity label would remain ineffective. These two mechanisms seem to be as good in explaining the results of the experiments described in Figures 4 and 5. In favor of the latter model, it is worth noting that the existence of multiple binding sites for agonists on *Torpedo californica* acetylcholine receptor has recently been documented (Dunn & Raftery, 1982). Desensitization, a functional characteristic of AChR, occurs upon prolonged incubation of the neuronal receptor with free or bound BAC. This phenomenon is complex and not well understood. It consists of at least two independent processes (Walker et al., 1981b; Magleby & Pallotta, 1981; El-Fakahany et al., 1982), and our results with PC-12 cell suggest that desensitization of the neuronal AChR is also biphasic (Figure 6).

A direct determination of the rate of desensitization by bound BAC cannot be made since the addition of BAC to the reduced receptor would merely allow desensitization by free BAC to occur. Rather, covalent binding of BAC followed by removal of unreacted label must be performed before any measurement of the inactivation by bound BAC can be made. Thus, any early event in this process is likely to be missed. The late step of desensitization, however, can be studied and appears to be a slow phase for the effect of both bound and free BAC, with similar inactivation rates in both cases. This result thus shows that reduced PC-12 receptors can be desensitized and that reduction by dithiothreitol and covalent binding of the activating ligand do not alter noticeably at least part of the desensitization process.

Taken together, these results provide a fairly complete picture of how affinity labels react with PC-12 neuronal ACh receptors. The most striking implication emerging from this work is the very close similarity between the properties of peripheral receptor (specially from *Torpedo* electric organ) and of the neuronal receptor from cultivated PC-12 cells (Table I). Differences in behavior between the two proteins as far as chemical modification is concerned appear to be minor ones and can be fairly well accounted for by the known functional disparities between the two receptors. Indeed, the higher affinity for agonists and sensitivity to desensitization characterizing *Torpedo* receptors being established, it is not surprising to observe that after being bound, BAC can still activate the neuronal receptor but not the *Torpedo* AChR and that inhibition by both MBTA and BAC can be fully reversed by increasing the competing agonist concentration with *Torpedo* AChR but not with the neuronal receptor.

Much more profound are the differences between nicotinic ionophore as studied here and neuronal α-neurotoxin binding sites since the apparent affinity of the neurotoxin site for the affinity labels is about 2 orders of magnitude lower for the brain neurotoxin receptor (Lukas & Bennett, 1980a) and the PC-12 neurotoxin binding site (P. Leprince, unpublished observations) than for the PC-12 ionophore acetylcholine binding

site. Also, changes in binding site affinity for agonists are observed after treatment of brain membrane neurotoxin sites with DTT followed by DTNB (Lukas & Bennett, 1980b) while no such effects are seen with *Torpedo* (Walker et al., 1981a) or PC-12 AChR.

The nicotinic nature of toxin binding sites in the central and peripheral nervous system is well supported by pharmacological arguments (Patrick & Stallcup, 1977b). Furthermore, the chemical reactivity and mechanistic characteristics of the brain toxin receptor as studied by Lukas and Bennett suggest a very clear similarity with the toxin site on AChR from *Torpedo*. Like the electric organ protein, the brain receptor possesses disulfide bridges that can be modified with concomitant changes in affinity for ligands; it binds strongly snake α-neurotoxin and displays alterations of affinity for agonists that recall the effect of desensitization (Lukas & Bennett, 1979; Lukas et al., 1979). These observations have, unfortunately, not yet been confirmed by the establishment of a functional role for this nicotinic entity (Miledi & Szczepaniak, 1975; Brown & Fumagalli, 1977; Patrick & Stallcup, 1977b), and the problem of knowing whether α-neurotoxin receptors in the nervous system represent anything more than a binding site remains unsolved.

Our observation that nicotinic affinity reagents label preferentially the ionophore of sympathetic neurones is not likely to resolve this paradox since neurotoxin binding experiments reveal no such affinity label sites with such a low apparent dissociation constant in the brain (Lukas & Bennett, 1980a). Thus, the very existence of a nicotinic ionophore in the brain could be further discussed. Still, our results suggest that, at least for the sympathetic neurones, the exact nature of nicotinic binding entities is open to direct investigation, specific labels being now available for both the α-neurotoxin site and the functional ionophore.

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