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The effects of drugs on the incorporation of a conformationally-sensitive, hydrophobic probe into the ion channel of the nicotinic acetylcholine receptor

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Abstract

The pattern of incorporation of the hydrophobic photolabel 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine([^{125}I]TID) into the nicotinic acetylcholine receptor (AChR) is a sensitive measure of AChR conformation (resting state or desensitized). We determined the ability of tetracaine, dibucaine, procaine, lidocaine, chlorpromazine or phencyclidine to inhibit [^{125}I]TID photolabeling of the AChR as a function of drug concentration, both as a measure of the ability of these drugs to desensitize the AChR, and to characterize the [^{125}I]TID binding site. To localize the site(s) of drug action, experiments were performed in the absence and presence of saturating concentrations of α-bungarotoxin (BgTx), to block drug binding to the agonist binding site. On the basis of the concentration dependence of their effects, which was not altered by the presence of BgTx, tetracaine and dibucaine appeared to block [^{125}I]TID incorporation competitively, suggesting that the high-affinity [^{125}I]TID binding site is the non-competitive blocker binding site presumed to exist in the interior of the AChR ion channel. Procaine, chlorpromazine, lidocaine and phencyclidine blocked [^{125}I]TID incorporation at lower concentrations in the absence of BgTx than in its presence, suggesting that these drugs block incorporation by inducing desensitization when bound to their high-affinity non-competitive blocker binding sites and that BgTx countered the drug effect by allosterically stabilizing the resting state.

Key words: Acetylcholine receptor; Local anesthetic; Noncompetitive blocker; Photolabeling; Desensitization

1. Introduction

The nicotinic acetylcholine receptor (AChR) is the primary signal transducer at the neuromuscular junction. The AChR is a multi-subunit, intrinsic membrane protein (reviews of the structural and biochemical aspects of AChR function include [1–4]). The AChR assumes at least four conformations; the resting, open, intermediate desensitized, and equilibrium desensitized states. Agonist binding to resting state AChR induces transient channel openings, rapidly depolarizing the post-synaptic membrane and initiating muscle contraction. Exposure to μmolar agonist for durations greater than 0.2 s causes the AChR to isomerize to the desensitized conformation(s). There are at least two kinetically differentiable forms of desensitization, a fast onset intermediate form and a slow onset equilibrium form [5]. Equilibrium-desensitized AChR binds agonist about a thousand-fold more tightly than the initial, resting state conformation [6], but in desensitized AChR the binding of agonist does not result in channel opening.

Drugs which modify the behavior of the AChR can be roughly separated into two classes; desensitizing drugs, and non-competitive (or open channel) blockers (NCBs), on the basis of their mode of action when bound to their high-affinity binding sites. Ideally, NCBs should bind within the ion channel of open state AChR

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Abbreviations: AChR, acetylcholine receptor; BgTx, α-bungarotoxin; [^{125}I]TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine; NCB, non-competitive blocker; Mops, 3-(*N*-morpholino)propanesulfonic acid; EGTA, [ethylenebis(oxoethylenenitrilo)]tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HTX, *d,l*-decahydro(pentenyl) histrionicotoxin.

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and block ion conduction sterically. However, in practice, most drugs classified as NCBs also appear to bind to resting state and desensitized AChR, although typically to the same high-affinity site to which they bind open state AChR. In addition, many NCBs induce some degree of AChR desensitization, and some desensitizing drugs act as NCBs, as a function of their concentration. These overlapping modes of action are well described by the model of Heidmann, Oswald and Changeux [7], wherein NCBs have a single high-affinity AChR binding site, within the ion channel region, while desensitizing drugs have multiple binding sites, both at the agonist binding site, and numerous, non-specific binding sites presumed to be at the lipid/protein interface. At higher concentrations, NCBs can also bind at the agonist binding sites, and desensitizing drugs can bind within the AChR ion channel.

In this report, we used the covalent-labeling reagent 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine-([^{125}I]TID) as a probe to characterize the effects of a number of drugs on the conformation of the AChR. [^{125}I]TID is a hydrophobic probe which, upon photoactivation by long-wavelength ultraviolet light, incorporates non-specifically into the alkyl side chains of amino acids, both at the lipid/protein interface and in hydrophobic pockets in the interior of proteins [8,9]. In previous studies we have shown that agonist-induced equilibrium desensitization of both partially-purified AChR in native lipids and affinity-purified AChR reconstituted into supportive lipids was characterized by a dramatic reduction in the levels of incorporation of [^{125}I]TID into all four subunits of the AChR [10,11]. In addition, reconstitution of the AChR into lipids which stabilize the desensitized conformation, or solubilization in some (but not all) non-ionic detergents, was shown to have the same effect on the pattern of [^{125}I]TID incorporation as agonist-induced desensitization [11]. We interpreted these results to suggest that agonists, lipids, and detergents, although potentially quite different in their modes of action, all induced the same final, desensitized conformation of the AChR. We compare here the effects of tetracaine, dibucaine, procaine, lidocaine, chlorpromazine and phencyclidine on the patterns of [^{125}I]TID incorporation into the AChR, both as a measure of their effects on the conformation of the AChR and to gain insight into the location of the conformationally-sensitive [^{125}I]TID-labeling site. While this work was in progress, studies recently published by Cohen and co-workers have demonstrated that [^{125}I]TID itself acts as an NCB [12], and have localized the conformationally-sensitive [^{125}I]TID binding site to the region of the AChR thought to line the ion channel [13]. Our results are in agreement with the conclusion of Cohen and co-workers that desensitization involves a change in the local conformation of the AChR ion channel, and suggest that one of the drugs we tested, tetracaine, may act to stabilize the resting state.

2. Materials and methods

Materials. [^{125}I]TID (8–10 Ci/mmol) and [^{125}I]iodo-α-bungarotoxin (^{125}I-BgTx) (3000 Ci/mmol) were purchased from Amersham (Arlington Heights, IL). Egg lecithin (phosphatidylcholine, predominantly dioleoyl) and dioleoylphosphatidic acid were purchased from Avanti Polar Lipids (Pelham, AL). Cholesterol, carbamylcholine, dibucaine, lidocaine, tetracaine, procaine, phencyclidine and chlorpromazine were purchased from Sigma Chemical (St. Louis, MO). Live *Torpedines californicae* were purchased from Marinus (Long Beach, CA).

Acetylcholine receptor purification and reconstitution. The preparation of *Torpedo californica* electric organs, and affinity purification of the AChR was performed as described [11]. AChR purified and reconstituted in this manner has been shown to be functional by a number of criteria [11,14]. Basically, *Torpedo* electric organs were excised and stored in liquid N_2 until use. 600 g of the frozen electric organ fragments were homogenized, and after a low speed spin to remove large debris, material sedimented by a higher speed spin (25 000 $\times g$ for 4 h) was solubilized in 1% sodium cholate. The cholate-solubilized *Torpedo* electroplaque membranes, which contain the AChR, were applied to a Bio-Rad Affi-gel 10 column derivatized with bromoacetylcholine. The column was washed extensively with buffer containing the lipids egg lecithin, dioleoylphosphatidic acid, and cholesterol (in a molar ratio of 3 : 1 : 1), both to remove contaminating proteins and to exchange native electroplaque lipids for defined lipids. AChR was eluted from the column with buffer containing 10 mM carbamylcholine, and fractions containing AChR were pooled and extensively dialyzed against 10 mM Mops, 100 mM NaCl, 0.1 mM EDTA, 0.2% NaN_3 (pH 7.5) (DB) to remove the carbamylcholine and cholate and reconstitute the AChR into the defined lipids. The dialyzed, reconstituted AChR was typically stored in small aliquots ($\sim$ 300 μl) in liquid N_2 at a protein concentration 2–5 mg/ml.

Five separate preparations of affinity-purified AChR were used in these experiments. The AChR was 100% pure on the basis of SDS-PAGE analysis and 91% pure on the basis of ^{125}I-BgTx binding (averaging 6.8 $\pm$ 0.8 nM BgTx bound/mg protein) [15]. The amount of phospholipid present was determined by phosphate assay [16], and in the different preparations averaged 205 $\pm$ 79 moles phospholipid/mole protein, which has been shown in the past to support functional AChR [17]. The K_d for [^{3}H]phencyclidine binding to the affinity-purified AChR was 6.4 + 0.4 μM, which is very

similar to values determined in earlier studies using different preparations of AChR [7,36].

[^{125}I]TID Labeling. All of the [^{125}I]TID photolabeling experiments were performed in the same manner. In a final volume of 45 μl, AChR (0.4 μM) in DB was equilibrated for 30 min at room temperature in the absence or presence of 300 μM carbamylcholine, or drugs at various concentrations (see text). In some experiments, the AChR was pre-equilibrated with 10 μM BgTx (13-fold excess over binding sites) for 30 min at room temperature before drugs were added. [^{125}I]TID (1 μCi; final ratio of 18 pmol AChR/80–100 pmol [^{125}I]TID) was then added, mixed by repeated pipeting, and equilibrated for 10 min. Photolabeling of the AChR with [^{125}I]TID was performed by exposing each reaction mixture to long-wavelength UV light (λ_{max} = 366 nm) at a distance of 3 cm for 10 min. In trial experiments, under these conditions [^{125}I]TID incorporation into the AChR peaked within 8–10 min of exposure to UV light. Photolabeling was terminated by removing the reaction mixes from the UV light. 6 μl of 2 mM dithiothreitol was then added to each reaction mixture, to reduce disulfides, followed by the addition of 15 μl of IEF sample buffer [18]. The reaction mixtures were then equilibrated for 30 min at room temperature prior to SDS-PAGE analysis. 10–12-μl aliquots of each reduced reaction mix were applied to each lane of the gel. SDS-PAGE separation of the [^{125}I]TID-labeled AChR subunits was performed as described previously [11]. Autoradiograms were prepared using Kodak X-Omat RP film at −70°C, typically in the presence of an intensifying screen.

[^{125}I]TID incorporation into the subunits of the AChR was quantitated by cutting out the bands of interest and determining the amount of ^{125}I present in each in a Packard gamma counter. In Figs. 2 and 3, the data points were determined according to the equation:

$$\% \text{ control} = (CPM_{[drug]} - CPM_{ago}) / (CPM_{max} - CPM_{ago}) \times 100$$

where $CPM_{[drug]}$ is the amount of [^{125}I]TID incorporated at various drug concentrations, CPM_{ago} is the amount of [^{125}I]TID incorporated in the presence of carbamylcholine, and CPM_{max} is the maximal amount of [^{125}I]TID incorporated in the absence of added ligands. Subtracting the CPM incorporated in the presence of agonist from the data points allowed us to concentrate upon only conformationally-sensitive [^{125}I]TID incorporation. CPM_{ago} and CPM_{max} were determined individually for each experiment; basically, each gel, as in Fig. 1, contained reference lanes of AChR labeled in the absence or presence of agonist. The concentrations at which drugs diminished [^{125}I]TID incorporation by 50% of their maximal effect (IC_{50}) was determined by fitting the dose–response data to the four parameter logistic equation. Curve fitting was performed by non-linear least-squares analysis using the graphical curve-fitting program INPLOT, which applies the Marquardt algorithm [19]. As is true for all parameters calculated by non-linear least-squares methods, the standard errors reported for the calculated IC_{50} values are approximate. In all cases, the data could be adequately fit assuming a single class of drug binding sites.

3. Results

In these studies, we wished to determine if AChR desensitization, induced by the binding of desensitizing drugs, had the same effect on [^{125}I]TID labeling as desensitization induced by other mechanisms. In earlier studies performed upon partially purified AChR, the NCB phencyclidine at 2 μM had no effect on [^{125}I]TID incorporation [10], and at higher concentrations (10–50 μM) only reduced [^{125}I]TID incorporation 20–30% [20]. We re-examined the effect of different concentrations of phencyclidine upon [^{125}I]TID incorporation into affinity-purified AChR reconstituted into defined lipids, and also studied the effects of other tertiary amines such as the local anesthetics lidocaine, procaine, tetracaine and dibucaine and the phenothiazine antipsychotic chlorpromazine.

The experimental design was straightforward. When mixtures of resting-state AChR and [^{125}I]TID were exposed to long-wavelength ultraviolet light, all four subunits of the AChR were photolabeled, with enhanced labeling of the γ subunit (approximately twice as much as the other subunits; Fig. 1, lane 1). The band beneath the β subunit is most likely a proteolytic product of the γ subunit which is sometimes observed [20]; however, as we did not explicitly determine the identity of this band in each preparation, we did not consider [^{125}I]TID associated with this band as part of the total [^{125}I]TID incorporation into the γ subunit. Pre-equilibration with desensitizing concentrations of agonist reduced the levels of [^{125}I]TID incorporation into all four subunits of the AChR, most dramatically for the γ subunit (incorporation was decreased 75–80%; Fig. 1, lane 2). This effect has been shown to be due to agonist-induced desensitization, and not merely competition between agonist and [^{125}I]TID for the same binding site, as competitive antagonists such as BgTx did not diminish [^{125}I]TID incorporation [10,20]. Pre-equilibration of the AChR with drugs also reduced the levels of [^{125}I]TID incorporation, as a function of the drug concentration. For example, prior equilibration with tetracaine at concentrations greater than 10 μM reduced the levels of [^{125}I]TID incorporation into

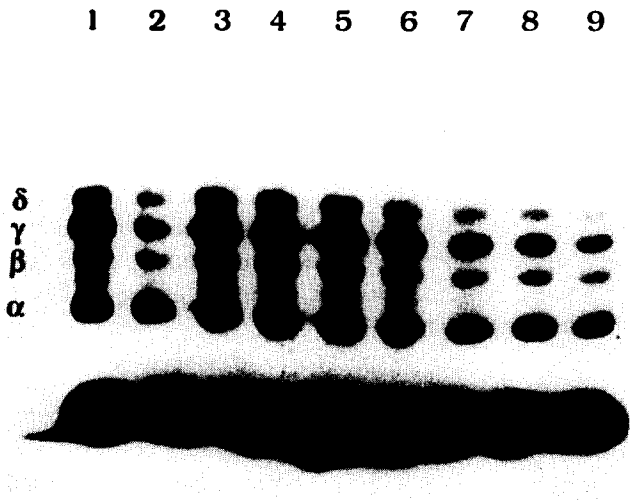

Fig. 1. Effects of carbamylcholine or tetracaine on the incorporation of [^{125}I]TID into the AChR. Shown is an autoradiogram of [^{125}I]TID-labeled AChR subunits. AChR was pre-equilibrated for 30 min in the absence (lane 1) or presence (lane 2) of 300 μM carbamylcholine or in the presence of 10 nM, 100 nM, 500 nM, 1 μM, 5 μM, 10 μM, or 50 μM tetracaine (Lanes 3–9) prior to equilibration with [^{125}I]TID for 10 min and subsequent photolabeling. Labeled AChR subunits were then separated by SDS-PAGE. The positions of the AChR subunits are indicated.

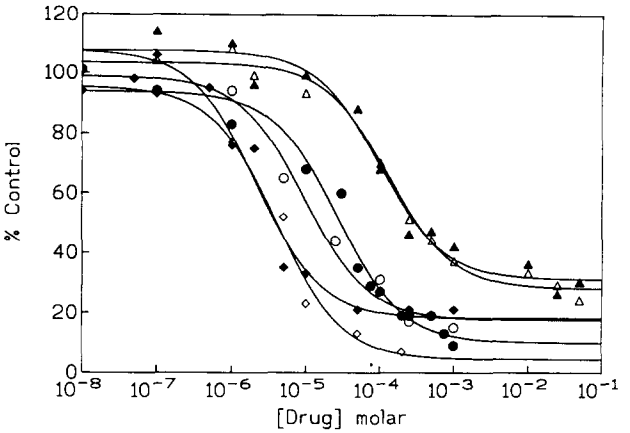

Fig. 2. Effects of different drugs on the incorporation of [^{125}I]TID into the γ subunit of the AChR. AChR was pre-equilibrated for 30 min in the presence of different concentrations of drug. After equilibration with [^{125}I]TID for an additional 10 min, the reaction mixtures were photolabeled for 10 min at 22°C. Labeled AChR subunits were then separated by SDS-PAGE. The individual subunit bands were cut out of the gel, and the amount of [^{125}I]TID remaining associated with each subunit was determined in a gamma counter. The data points were calculated as described in Materials and methods and are the average of at least three separation determinations ($n = 3$–6). The IC_{50} values calculated from these curves are shown in Table 1. Tetracaine (♦), chlorpromazine (◇), phencyclidine (○), dibucaine (●), procaine (▲), lidocaine (△).

all four AChR subunits to the same extent as desensitizing amounts of agonist (Fig. 1, lanes 3–9).

We determined the concentration dependence of the reduction in [^{125}I]TID incorporation induced by drugs by cutting the relevant bands out of the dried polyacrylamide gel, and determining the amount of [^{125}I] present in each band in a gamma counter. The amount of [^{125}I]TID remaining associated with each subunit band relative to the amount found in control AChR (i.e., AChR in the absence of carbamylcholine or drug) was then plotted as a function of drug concentration. The concentration dependence of drug inhibition of [^{125}I]TID labeling was essentially the same for all four subunits for all the drugs tested (data not shown). With tetracaine, for example, the calculated IC_{50} values (the concentration of drug required to achieve 50% of maximal reduction in [^{125}I]TID incorporation) for the α, β, γ, and δ subunits equalled 5.6, 2.8, 2.2, and 2.3 μM, respectively. This is the expected pattern if the drugs were acting to inhibit [^{125}I]TID binding to a single site within the AChR.

Drug-induced reduction of [^{125}I]TID incorporation could occur by two mechanisms. First, the drugs could directly compete with [^{125}I]TID for binding to a common site, or second, the drugs could act like agonists and other agents, and reduce [^{125}I]TID incorporation by inducing desensitization. To differentiate between these two possibilities, we determined the concentration dependence of the effects of drugs on [^{125}I]TID incorporation into the γ subunit of the AChR. The concentrations at which these drugs act either as NCBs, or desensitizing agents, have been well characterized. The concentration dependence of the effects of drugs on [^{125}I]TID incorporation into the γ subunit of the AChR is shown in Fig. 2. The IC_{50} values of these drugs are shown in Table 1. Tetracaine and chlorpromazine were the most potent, followed by phen-

Table 1
Calculated IC_{50} values for drug blockage of [^{125}I]TID incorporation into the γ subunit

	Tetracaine	CPZ	PCP	Dibucaine	Procaine	Lidocaine
− BgTx	2.2 ± 0.5 (9)	4.1 ± 1 (4)	10 ± 4 (6)	27 ± 5 (13)	101 ± 22 (10)	131 ± 19 (9)
+ BgTx	2.8 ± 1 (3)	15 ± 3 (6)	748 ± 74 (9)	42 ± 12 (3)	273 ± 82 (6)	1 890 ± 510 (6)

The μM IC_{50} values (concentrations at which drugs diminished [^{125}I]TID incorporation into the γ subunit of the AChR by 50% of their maximal effect) and standard errors were determined by non-linear least-squares analysis. CPZ stands for chlorpromazine and PCP stands for phencyclidine. [^{125}I]TID labeling was performed as described in Materials and methods, either with or without pre-equilibration with 10 mM BgTx for 30 min prior to drug addition and subsequent photolabeling. The number of experiments performed to determine each IC_{50} is shown in parentheses.

cyclidine and dibucaine, while procaine and lidocaine required concentrations in excess of 100 μM to achieve dramatic reduction in label incorporation. The concentration dependence of phencyclidine reduction of [^{125}I]TID incorporation is comparable to that determined earlier for partially purified AChR in native lipids (7 μM) [20]. However, in the earlier studies, phencyclidine only reduced [^{125}I]TID incorporation into the γ subunit by about 30%, while in our experiments phencyclidine reduced [^{125}I]TID incorporation into the γ subunit greater than 80%, perhaps as a result of the different purity and lipid environments of the two preparations of AChR.

In an effort to separate the effects on [^{125}I]TID incorporation due to drug binding at the NCB binding site from those due to drug binding to the agonist binding site, we determined the effects of drugs on [^{125}I]TID incorporation in the presence of excess BgTx, which will block drug binding to the agonist binding site. For tetracaine and dibucaine, BgTx had only a slight influence on the calculated IC_{50} (Table 1), suggesting that the effects of these drugs on [^{125}I]TID binding is mediated solely through the NCB binding site. The calculated IC_{50} for procaine or chlorpromazine was increased roughly 2.5-fold and 4-fold by saturating BgTx. However, the presence of BgTx had a much more pronounced effect upon the concentration-dependent inhibition of [^{125}I]TID incorporation by lidocaine and phencyclidine, and increased the IC_{50} 14-fold and 75-fold, respectively (Table 1; Fig. 3). This suggests either that some or all of the reduction in [^{125}I]TID incorporation induced by low concentrations of drugs such as phencyclidine and lidocaine is mediated by desensitization, presumably caused by drug binding to the agonist binding site, or more likely, that BgTx is stabilizing the resting state to some extent, and that it merely requires more of these drug to overcome the effects of BgTx.

There is evidence that some drugs, such as dibucaine and chlorpromazine, may act on the AChR somewhat non-specifically at the protein/lipid interface, particularly at higher concentrations [7]. Some of the residual [^{125}I]TID labeling observed following agonist-induced desensitization is on amino acids presumed to be at the protein/lipid interface [13,21]. It is possible, therefore, that at high concentration drugs might also interfere with [^{125}I]TID attachment to amino acids at the protein/lipid interface. However, even at very high concentrations (50–500-times greater than the IC_{50} values listed in Table 1), none of the drugs studied here reduced [^{125}I]TID incorporation to a significantly greater extent than agonist alone (data not shown). Also, the order of drug and [^{125}I]TID addition did not alter the final extent of [^{125}I]TID incorporation. The level of [^{125}I]TID incorporation was the same whether the AChR was equilibrated with tetracaine (10 μM) or lidocaine (750 μM) for 5–20 min before or after equilibration with [^{125}I]TID for 5–10 min. This suggests that under our experimental conditions true equilibrium had been reached and that there was no

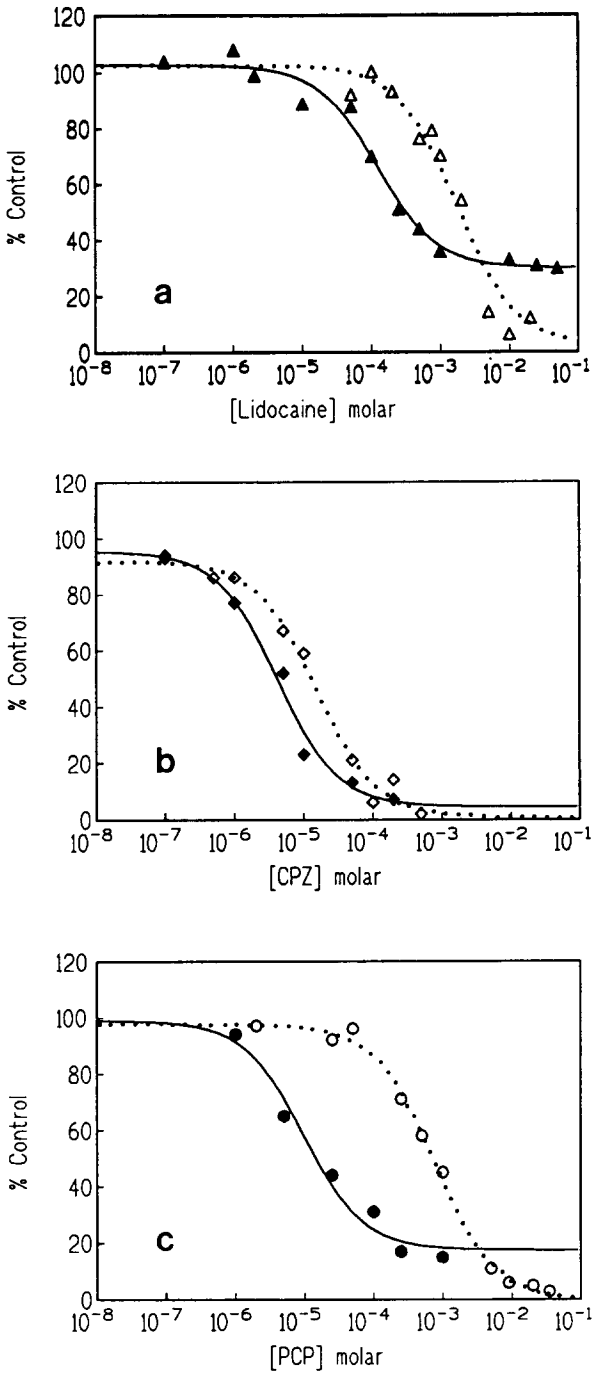

Fig. 3. Effects of BgTx on the reduction by drugs of [^{125}I]TID incorporation into the γ subunit. Prior to addition of drugs, AChR was pre-equilibrated for 30 min in the presence of saturating BgTx. The rest of the experimental conditions are described in the legend to Fig. 2. The IC_{50} values calculated from these curves are shown in Table 1. Plus BgTx, open symbols and dotted lines, minus BgTx, filled symbols and solid lines. (a) Lidocaine, (b) chlorpromazine, (c) phencyclidine.

hysteresis. Finally, as there is evidence that other channel blockers can become 'trapped' in the the closed ion channel [22], we tested whether [^{125}I]TID can become trapped in its specific binding site following addition of agonist. However, agonist reduced the level of [^{125}I]TID incorporation into all four subunits to the same extent regardless of whether the agonist was added before or after equilibration with [^{125}I]TID, followed by photolabeling, suggesting that in desensitized AChR [^{125}I]TID can escape its high-affinity binding site.

4. Discussion

In these experiments, we examined the effects of different drugs on the incorporation of [^{125}I]TID into the AChR. Interestingly, the simplest interpretation is that in one particular case (tetracaine), drug reduced [^{125}I]TID incorporation by competing for the same or overlapping binding sites, and not by inducing desensitization, as is the case for agonists, lipids and some detergents [10,11,20]. However, the interpretation of the effects of other drugs is confused both by their ability to desensitize the AChR and by the effects of BgTx. Therefore, for the other drugs, it is not possible to distinguish absolutely between direct competition between drug and [^{125}I]TID for the same binding site, or allosteric reduction in [^{125}I]TID incorporation due to desensitization. The effects of the individual drugs are discussed more fully below.

The effects of tetracaine on [^{125}I]TID incorporation appear to be due entirely to competition, a conclusion further supported by the recent work of Cohen and co-workers (summarized below). Tetracaine reduced [^{125}I]TID incorporation with an IC_{50} of 2.2 μM (Table 1). Similarly, in earlier studies, tetracaine blocked the binding of the NCBs phencyclidine and *d,l*-decahydro (pentenyl)histrionicotoxin (HTX) to *Torpedo* AChR with K_i values of 1.8 and 1.5 μM, respectively [23,24]. Unlike most other NCBs, tetracaine binds preferentially to resting state AChR [25]. As tetracaine does not desensitize the AChR at micromolar concentrations (in fact, it appears to stabilize the resting conformation), the reduction in [^{125}I]TID incorporation caused by micromolar tetracaine is almost certainly due to competition. The inability of saturating BgTx to block the effect of tetracaine on [^{125}I]TID incorporation is consistent with this conclusion.

The mechanisms by which the other drugs reduced [^{125}I]TID incorporation are not as straightforward. Like tetracaine, the effect of dibucaine upon [^{125}I]TID incorporation (IC_{50} of 27 μM; Table 1) is probably due to competition for the same binding site, as is the case for tetracaine. In earlier studies, dibucaine blocked phencyclidine and HTX binding to *Torpedo* AChR with K_i values of 45–57 and 80 μM respectively [23,24,26]. However, unlike tetracaine, dibucaine also was also observed to induce desensitization in this concentration range, probably by binding to the agonist binding site [23]. As BgTx had no significant effect on the ability of dibucaine to block [^{125}I]TID incorporation, it is clear that dibucaine did not reduce [^{125}I]TID incorporation allosterically, by binding to the agonist binding site and inducing desensitization. However, it is possible that dibucaine induced desensitization by binding to a site other than the agonist binding site.

In the absence of BgTx, phencyclidine and chlorpromazine blocked [^{125}I]TID incorporation at the same concentrations where they have been observed to bind to the NCB site in earlier studies [7,24,26–28]. This suggests that phencyclidine and chlorpromazine, like tetracaine and dibucaine, could be competing with [^{125}I]TID for the NCB binding site. However, unlike tetracaine and dibucaine, the addition of BgTx shifted the concentrations at which phencyclidine and chlorpromazine effectively blocked [^{125}I]TID incorporation. The simplest explanation, but one we feel is unlikely, is that the reduction in [^{125}I]TID incorporation observed at lower concentrations of phencyclidine and chlorpromazine could be due to desensitization induced by drug binding to the agonist binding site, while the reduction in [^{125}I]TID incorporation seen at higher concentrations of drug in the presence of BgTx could be caused by direct competition with [^{125}I]TID for binding to the NCB binding site. For example, phencyclidine has been observed to bind to the agonist binding site in some studies, although at much higher concentrations [7,24]. We postulate, however, that the most probable explanation is that BgTx is stabilizing the resting state, and thereby reducing the affinity for phencyclidine and chlorpromazine, which are known to bind preferentially to agonist-desensitized AChR [7,27–29]. BgTx has been observed to stabilize the resting state in some [30,31], (but not all [32,33]) earlier studies. In addition, with the affinity-purified AChR used in these studies, we have observed that BgTx increased the extent of [^{125}I]TID incorporation, suggesting that BgTx increased the amount of AChR in the resting state (Moore and McCarthy, unpublished observations). Therefore, in the presence of BgTx, it may simply have required a higher concentration of phencyclidine (or chlorpromazine) to bind to the NCB site, and reduce [^{125}I]TID incorporation competitively. This effect may be more prominent in our studies, as phencyclidine reduced [^{125}I]TID incorporation to a much greater extent in our affinity preparations than it did for AChR in native lipids [20].

Interpretation of the effects of lidocaine and procaine on [^{125}I]TID incorporation is confounded by the fact that these drugs appear to act both as NCBs, and to bind to the agonist binding site and induce desensitization, at about the same concentrations. In our stud-

ies, lidocaine and procaine blocked [^{125}I]TID incorporation with IC_{50} values of 131 μM and 100 μM, respectively. Addition of BgTx shifted the calculated IC_{50} values of lidocaine and procaine to higher concentrations (Table 1). In earlier experiments, lidocaine appeared to bind to both the NCB site and the agonist binding site of *Torpedo* AChR at mM or greater concentrations [23,25,34,35]. Similarly, procaine was reported to act at the NCB site of *Torpedo* AChR only at relatively high (mM) concentrations [23,34,35]. However, in other studies, procaine was observed to inhibit phencyclidine binding with a K_i of 60 μM [36], and to block *Torpedo* AChR ion flux at 50–300 μM by acting at the voltage-dependent (NCB) binding site [37–39]. In the studies reported here performed in the absence of BgTx, we can not distinguish whether lidocaine and procaine reduced [^{125}I]TID incorporation by binding to the agonist binding site and inducing desensitization, or by directly competing with [^{125}I]TID binding to the NCB site. However, in the presence of BgTx, both lidocaine and procaine most likely reduced [^{125}I]TID incorporation by binding to the NCB binding site at roughly mM concentrations, in agreement with earlier studies [23,35].

[^{125}I]TID has proven to be a surprisingly useful reagent for the study of the AChR. The initial studies demonstrated that [^{125}I]TID incorporation was sensitive to the conformation of the AChR, as label incorporation was dramatically reduced upon agonist-induced desensitization [10,20]. We have taken advantage of this fact, and used the patterns of [^{125}I]TID incorporation to identify lipids and detergents which desensitize the AChR [11]. However, HTX, a non-desensitizing NCB, was also shown to block [^{125}I]TID incorporation, providing the first evidence that there was a specific [^{125}I]TID-binding site on the AChR which partially or completely overlapped the NCB binding site [20]. Further studies by Cohen and co-workers suggested that the [^{125}I]TID binding site was complex. It was shown that TID could act as an NCB, blocking $^{22}Na^+$ flux with an IC_{50} of about 1 μM. However, while TID reduced HTX binding both in the presence and absence of agonist, it only reduced phencyclidine binding in the presence of agonist. Finally, TID inhibited phencyclidine and HTX binding in the presence of 100 μM carbamylcholine, conditions under which no TID binding to the NCB site would be predicted, on the basis [^{125}I]TID photolabeling experiments [12]. Recently, Cohen and co-workers have identified the conformationally-sensitive [^{125}I]TID binding sites on the α, β, and δ subunits [13,21]. In agreement with the ability of TID to act as an NCB, [^{125}I]TID labeled homologous amino acids on all three subunits in the M2 region of the AChR. On the basis of several labeling and mutagenesis studies, the M2 regions of the subunits are thought to form α-helices (but see [40]) which combine to form the lining of the AChR ion channel (reviewed in [2,3]). Interestingly, upon agonist-induced desensitization, the pattern of [^{125}I]TID labeling changes, and reduced label incorporation was observed further down the putative M2 α-helices, and in one case on the other side of the putative M2 α-helix of the δ subunit [13].

On the basis of their results, White and Cohen [13] suggested a model of AChR gating, which is supported by our results. They speculated that the ring of hydrophobic amino acids labeled by [^{125}I]TID may form the gate which opens upon agonist binding. Upon desensitization, this region is predicted to remain opened up, making it a poor binding site for [^{125}I]TID. This type of conformational change could easily be accomplished by changing the tilt angle, or cross-over position, of a ring of aligned α-helices. As suggested by White and Cohen [13], [^{125}I]TID binding might stabilize the resting state conformation of the AChR. As tetracaine also appears to bind preferentially to resting state AChR, and was the most potent inhibitor of [^{125}I]TID incorporation in our studies, an obvious extension of this model is that tetracaine and [^{125}I]TID bind to the same portion of the NCB binding region. Whether or not the [^{125}I]TID binding site in resting state AChR forms the ion channel gate, the fact that [^{125}I]TID labeling patterns of resting state and desensitized AChR are different indicates that desensitization is not simply a small local change (as suggested by earlier studies of global conformation [41]) at the agonist binding site which uncouples agonist binding from channel opening. Rather, desensitization clearly involves a change in the channel region of the AChR which probably involves 'closure' of a region of the channel which is normally open in the resting state.

5. References

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