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Snake venom toxins, unlike smaller antagonists, appear to stabilize a resting state conformation of the nicotinic acetylcholine receptor

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Abstract

Previous studies have shown that the pattern and degree of 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID) photoincorporation into the nicotinic acetylcholine receptor (nAChR) can be used as a sensitive measure of nAChR conformation. Upon desensitization by prolonged exposure to agonists, certain drugs and detergents, or reconstitution into desensitizing lipids, the levels of [^{125}I]TID incorporation into the subunits of the nAChR are dramatically reduced. In this study, we characterized the effects of the snake venom proteins α-bungarotoxin and α-cobrotoxin, as well as the smaller antagonists tubocurarine and gallamine, on [^{125}I]TID incorporation into the subunits of both partially-purified nAChR in native lipids, or affinity-purified nAChR reconstituted into different combinations of lipids. Unlike all other compounds previously tested, α-bungarotoxin and α-cobrotoxin reproducibly increased the level of [^{125}I]TID incorporation into all four subunits of nAChR reconstituted into dioleoylphosphatidylcholine, dioleoylphosphatidic acid and cholesterol. Gallamine had little or no effect on [^{125}I]TID incorporation at any concentration tested (0.1 μM–5 mM). Tubocurarine had no effect on [^{125}I]TID incorporation at low concentrations, but at higher concentrations reduced the level of [^{125}I]TID labeling. The snake venom proteins may shift the population of nAChR, which exists as a mixture of resting state and desensitized conformations, entirely to the resting state. However, the binding of the snake venom toxins does not appear sufficient to induce the resting state conformation in nAChR which have been desensitized by other means, such as solubilization in desensitizing detergents or reconstitution in desensitizing lipids.

Keywords: Acetylcholine receptor; α-Bungarotoxin; Toxin; Photolabeling; Conformation; Antagonist

1. Introduction

The nicotinic acetylcholine receptor (nAChR) is the primary signal transducer at the neuromuscular junction. The nAChR is a multi-subunit, intrinsic membrane protein (reviews of the structural and biochemical aspects of nAChR function include [1–3]). Operationally, the nAChR can be thought to assume at least four conformational states; the resting, open, intermediate desensitized, and equilibrium desensitized states. Binding of the agonist acetylcholine causes the nAChR ion channel to open, allowing the depolarizing influx of cations through the muscle cell membrane. A number of agents block the action of agonists at the nAChR, both competitively and non-competitively. The competitive blockers can be arbitrarily separated into two groups; snake venom toxins ($\approx$ 8000 Da proteins), and lower molecular weight compounds. The snake venom toxins, such as α-bungarotoxin and α-cobrotoxin, bind with high affinity (K_d less than nM) [4–7] to neuromuscular nAChR and competitively block agonist binding. The smaller antagonists, such as the plant alkaloid d-tubocurarine (the active ingredient of curare) or gallamine bind with weaker affinity to the nAChR [8–10]. Non-competitive blockers reduce agonist activity by inducing desensitization and/or blocking ion flow through the protein, and not surprisingly their action appears to be quite sensitive to the functional conformation of the nAChR [11]. However, in some (but not all) earlier

Abbreviations: nAChR, nicotinic acetylcholine receptor; BgTx, α-bungarotoxin; [^{125}I]TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)-diazirine; octylglucoside, n-octyl-β-D-glucopyranoside; DOPC, dioleoylphosphatidylcholine; DOPA, dioleoylphosphatidic acid; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis.

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studies performed upon partially-purified nAChR, the snake venom toxins appeared relatively insensitive to the conformational state of the nAChR.

We re-determined the effects of competitive antagonists on the conformation of the nAChR using the hydrophobic photoprobe, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)-diazirine ([^{125}I]TID). The pattern of [^{125}I]TID incorporation into the nAChR is dependent upon the functional conformation of the nAChR. All four subunits of resting state nAChR are labeled by [^{125}I]TID, with enhanced labeling of the γ-subunit (ratio of label incorporation into the γ-subunit relative to the α-subunit > 2 [12]). Upon desensitization by prolonged exposure to agonist, label incorporation into all four subunits is reduced [13,14], and the ratio of label incorporated into the γ-subunit relative to the α-subunit is approximately equal [12]. We have also shown that reconstitution into certain lipids, or solubilization in some (but not all) detergents, had the same effect on nAChR [^{125}I]TID-labeling as prolonged incubation with agonist, suggesting that agonists, and particular lipids and detergents, all induced the same final desensitized nAChR conformation [12]. Recently, Cohen and co-workers demonstrated that the conformationally-sensitive [^{125}I]TID-incorporation site is within the portion of the nAChR thought to form the lining of the ion channel [15,16], in agreement with other studies which demonstrate that [^{125}I]TID binding and labeling is competitively blocked by certain non-competitive blockers [13,17,18].

In the studies reported here, we observed that, unlike all other reagents tested to date, the snake venom toxins α-bungarotoxin (BgTx; from *Bungarus multicinctus*) and α-cobrotoxin (from *Naja naja kaouthia*) increased the levels of [^{125}I]TID incorporation (largely into the γ-subunit) of both affinity-purified nAChR reconstituted into defined lipids and enriched nAChR-containing membranes in native lipids. However, the smaller antagonists d-tubocurarine and gallamine had no effect on [^{125}I]TID incorporation at low concentrations, while at higher concentrations d-tubocurarine reduced the levels of [^{125}I]TID labeling. Our results suggest that the snake venom toxins act to stabilize the resting state conformation of the nAChR, leading to increased [^{125}I]TID incorporation, while at higher concentrations d-tubocurarine may reduce [^{125}I]TID incorporation by inducing desensitization, or by competing with [^{125}I]TID binding to the ion channel region of the nAChR directly.

2. Experimental procedures

2.1. Materials

[^{125}I]TID (8–10 Ci/mmol) and [^{125}I]-α-bungarotoxin ([^{125}I]BgTx) (3000 Ci/mmol) were purchased from Amersham (Arlington Heights, IL). Egg lecithin (dioleoylphosphatidylcholine; DOPC) and dioleoylphosphatidic acid (DOPA) were purchased from Avanti Polar Lipids (Pelham, AL). Cholesterol was purchased from Boehringer Mannheim (Indianapolis, IN). Carbamylcholine, gallamine triethiodide, d-tubocurarine chloride, α-bungarotoxin (from *Bungarus multicinctus*) and α-cobrotoxin (from *Naja naja kaouthia*) were purchased from Sigma (St. Louis, MO). Live *Torpedo californica* were purchased from Marinus (Long Beach, CA).

2.2. Acetylcholine receptor purification and reconstitution

Torpedo californica electric organs were collected, and both enriched nAChR membranes in native lipids [14,19], or affinity-purified nAChR reconstituted into either DOPC alone, or a mixture of DOPC/DOPA/cholesterol (molar ratio between the lipids of 3:1:1) [12,18], were prepared as described previously. nAChR reconstituted in DOPC/DOPA/cholesterol appears functional by a number of criteria, while nAChR reconstituted in DOPC appears to be essentially locked in the desensitized conformation [12,20,21]. Small aliquots (≈ 300 μl) of the purified nAChR were stored in liquid N_2 at a protein concentration of 1.5 mg/ml until use, typically within 3 weeks of preparation. In these studies, five separate preparations of enriched nAChR membranes and four preparations of affinity-purified nAChR (three reconstituted into DOPC/DOPA/cholesterol and one reconstituted into DOPC alone) were tested. On the basis of [^{125}I]BgTx binding [22], the enriched nAChR membranes averaged 70% pure (5.23 ± 0.38 nmol [^{125}I]BgTx bound/mg protein) while the affinity-purified nAChR were 100% pure (7.47 ± 0.34 nmol [^{125}I]BgTx bound/mg protein).

2.3. [^{125}I]TID labelling

The [^{125}I]TID photolabeling experiments were performed largely as described in earlier studies [12,18]. Basically, in a final volume of 50 μl, nAChR (0.4 μM) in 10 mM Mops, 100 mM NaCl, 0.1 mM EDTA, 0.2% NaN_3, pH 7.5 was equilibrated for 30 min at room temperature in the absence or presence of 100–300 μM carbamylcholine, α-bungarotoxin or α-cobrotoxin (2–10 $\times$ molar excess over nAChR binding sites), or gallamine triethiodide or d-tubocurarine (0.1 μM 5 mM) (see text for drug concentrations used in individual experiments). [^{125}I]TID (1 μCi) was then mixed with the nAChR/ligand solution by repeated pipeting (final ratio of 18 pmol AChR/80–100 pmol [^{125}I]TID), and equilibrated for 10 min. Photolabeling of the nAChR with [^{125}I]TID was performed by exposing each reaction mixture to long-wavelength UV light ($\lambda_{max} = 366$ nm) at a distance of 3 cm for 10 min (conditions which yield maximal label incorporation). In experiments where the effects of detergent on [^{125}I]TID incorporation in the presence of toxins was tested, the nAChR was solubilized in octylglucoside (1%, w/v) for 30 min at room temperature prior to

addition of toxin and photolabel. Photolabeling was terminated by removal of the reaction mixtures from the UV light, after which each mixture was reduced by the addition of 6 μl of 2 mM dithiothreitol. 15 μl aliquots of each reaction mixture were then mixed with 15 μl of IEF sample buffer [23] and then equilibrated for 30 min at room temperature prior to SDS/PAGE analysis. 20 μl aliquots of each reduced reaction mix were applied per gel lane. SDS/PAGE separation of the [^{125}I]TID-labeled nAChR subunits was performed as described previously [12]. Autoradiograms were prepared using Kodak X-Omat RP film at $-70°$ C in the presence of a Cronex Lightning Plus intensifying screen.

The extent of [^{125}I]TID incorporation into the subunits of the nAChR was quantitated by cutting out the bands of interest and measuring the amount of [^{125}I] present in each band using a Packard Cobra γ counter. The concentration at which d-tubocurarine diminished [^{125}I]TID incorporation by 50% of its maximal effect (IC_{50}) was determined by fitting the dose-response data to the four parameter logistic equation as described [18].

3. Results

These studies were prompted by earlier nAChR [^{125}I]TID-labeling experiments in which we used BgTx to block drug binding to the agonist binding site [18]. Unexpectedly, in some instances, the amount of [^{125}I]TID incorporated into affinity-purified nAChR was significantly increased in the presence of BgTx. This observation was unexpected because in earlier studies performed both by ourselves [14] and others [13], BgTx had virtually no effect on [^{125}I]TID incorporation into enriched nAChR membranes. We therefore decided to re-determine the effects of antagonists on the patterns of [^{125}I]TID into the subunits of the nAChR, to test whether antagonist binding altered nAChR conformation.

First, we determined whether snake venom toxins such as BgTx enhanced [^{125}I]TID incorporation into both functionally-reconstituted affinity-purified nAChR and enriched nAChR membranes. As shown in Fig. 1, BgTx binding caused increased [^{125}I]TID labeling into both types of nAChR preparation, although the effect was more pronounced for functionally-reconstituted affinity-purified nAChR (Fig. 1b) than for enriched nAChR membranes (Fig. 1a). The extent to which BgTx enhanced [^{125}I]TID into affinity-purified nAChR varied somewhat (between 30–80%) with different affinity preparations; in the preparation shown in Fig. 1b, BgTx increased [^{125}I]TID incorporation into the γ-subunit by $40 \pm 11\%$ (average and standard deviation of six determinations). However, while enhanced [^{125}I]TID incorporation in the presence of snake venom toxins was always observed for affinity-purified nAChR reconstituted into DOPC/DOPA/cholesterol (three separate preparations tested), the effect was more

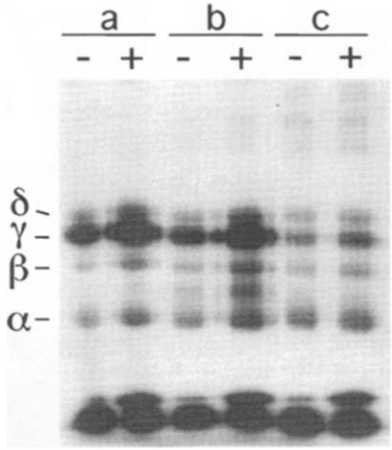


Fig. 1. Effects of antagonists on [^{125}I]TID-labeling of different nAChR preparations. nAChR was pre-equilibrated for 30 min in the absence (−) or presence (+) of saturating BgTx (5× molar excess over binding sites) prior to equilibration with [^{125}I]TID for 10 min and subsequent photolabeling. Labeled nAChR subunits were then separated by SDS-PAGE. Shown is an autoradiogram of a, enriched nAChR in native lipids, b, affinity-purified nAChR reconstituted into DOPC/DOPA/cholesterol, and c, affinity-purified nAChR reconstituted into DOPC, labeled with [^{125}I]TID in the presence or absence of BgTx. The positions of the nAChR subunits are indicated. The band beneath the β subunit is most likely a proteolytic product of the γ subunit which is sometimes observed [13].

variable for enriched nAChR membranes. Two enriched nAChR membranes preparations showed no change in [^{125}I]TID incorporation in the presence of BgTx, two showed moderate but significant enhancement ($\approx$ 15–25%) and in one preparation, BgTx binding increased [^{125}I]TID incorporation by $\approx$ 50%. As described more fully in Section 4, we believe the variable effects of BgTx on nAChR conformation observed with different preparations of nAChR, as detected by [^{125}I]TID labeling, is determined by the pre-existing resting state/desensitized state ratio of each nAChR preparation. However, BgTx did not alter the level of [^{125}I]TID incorporation into nAChR which was no longer able to shift between the resting and desensitized states. As shown Fig. 1c, affinity-purified nAChR reconstituted into DOPC alone was labeled to about the same extent by [^{125}I]TID in both the presence and absence of BgTx (the slight enhancement of [^{125}I]TID incorporation into nAChR reconstituted into DOPC by BgTx apparent in Fig. 1 was neither significant or reproducibly observed, averaged over six determinations). In earlier studies, both ourselves and others have shown that nAChR reconstituted solely into DOPC is not able to undergo the change in conformation between the resting and desensitized states, and in fact appears to be entirely in the desensitized conformation [12,21].

The observation that BgTx increased the extent of [^{125}I]TID incorporation into functional nAChR suggested to us that snake toxin venoms might be acting to shift a portion of the nAChR population from a desensitized state to the resting state conformation. However, the inability of

BgTx to enhance [^{125}I]TID labeling of nAChR reconstituted into DOPC implied that snake venom toxins could not shift 'profoundly' desensitized nAChR to the resting state. To test this notion further, we determined the effect of BgTx on [^{125}I]TID labeling of detergent-solubilized nAChR using the non-denaturing detergent octylglucoside, which appears on the basis of its effects on [^{125}I]TID labeling to desensitize the nAChR [12]. As shown in Fig. 2, solubilization in octylglucoside reduced [^{125}I]TID incorporation into enriched nAChR membranes to the same extent as incubation with desensitizing concentrations of the agonist carbamylcholine; compare the leftmost lane (−additives) with samples labeled in the presence of carbamylcholine (c) or octylglucoside (OG), while BgTx increased [^{125}I]TID incorporation into membrane-bound nAChR; compare the sample labeled in the absence of additives (−) with that labeled in the presence of BgTx (TX). However, for octylglucoside-solubilized nAChR, BgTx did not induce the pattern of [^{125}I]TID labeling characteristic of resting state nAChR (increased labeling of all four subunits, with enhanced labeling of the γ-subunit), as shown in the rightmost lane (TX/OG). The inability of BgTx to induce the resting state pattern of [^{125}I]TID labeling for nAChR reconstituted in DOPC or solubilized in octylglucoside suggests that snake venom toxins can not shift nAChR from the equilibrium desensitized state back to the resting state.

We also tested whether other antagonists influenced labeling of the nAChR by [^{125}I]TID. As shown in Fig. 3, like BgTx, α-cobrotoxin (3 × molar excess over binding sites) increased the extent of [^{125}I]TID incorporation into enriched nAChR membranes; compare the left most lane (−additives) with the rightmost lane (TX). However, the non-proteinaceous antagonists d-tubocurarine and gallamine (at 10 μM) had essentially no effect on [^{125}I]TID incorporation; compare the sample labeled in the absence of additives (−) with that labeled in the presence of d-tubocurarine (Tu) or gallamine (Ga). This suggests that the relatively large (≈ 8000 Da) snake venom toxins can stabilize the resting state conformation of the nAChR whereas the smaller antagonists can not. The concentrations of d-tubocurarine and gallamine used in the experiment depicted in Fig. 3 are well over the dissociation constant for the high affinity d-tubocurarine binding site and slightly above the dissociation constants for lower affinity d-tubocurarine and gallamine binding [8–10,24,25]. However, we tested the effects of d-tubocurarine and gallamine on nAChR [^{125}I]TID labeling over a wide range of concentrations, to determine if these ligands could alter nAChR conformation at higher concentrations, in particular as an earlier publication had shown that 100 μM d-tubocurarine began to reduce [^{125}I]TID incorporation significantly [13]. As shown in Fig. 4a, at relatively high concentrations, d-tubocurarine reduced [^{125}I]TID incorporation into enriched nAChR membranes to the same extent as carbamylcholine. The inhibition of [^{125}I]TID incorporation by high concentrations of d-tubocurarine could be due to d-tubocurarine induced desensitization, or direct competition by d-tubocurarine for the [^{125}I]TID binding site. The calculated IC_{50} for d-tubocurarine inhibition of [^{125}I]TID incorporation into the nAChR γ-subunit, determined from six experiments performed as in Fig. 4a, was 453 μM. However, as shown in Fig. 4b, at concentrations as high as 5 mM, gallamine had little effect on labeling of the nAChR by [^{125}I]TID. This suggests that, even at high concentrations, gallamine did not desensitize enriched nAChR membranes, in agreement with earlier studies

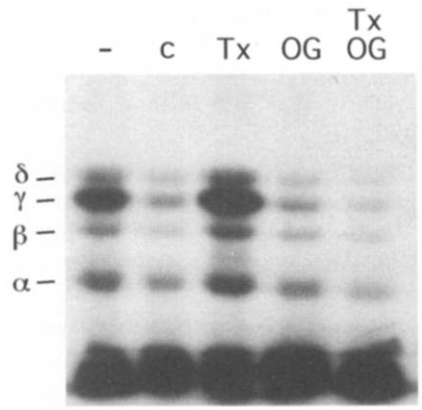


Fig. 2. Effects of detergent and antagonists on nAChR conformation. [^{125}I]TID-labeling was performed as described in the legend to Fig. 1. Detergent solubilization was performed as described in Experimental procedures. Shown is an autoradiogram of enriched nAChR membranes labeled with [^{125}I]TID in the absence (−) or presence of 300 μM carbamylcholine (c), saturating α-bungarotoxin (Tx; 7× molar excess over binding sites), 1% octylglucoside (OG) or both α-bungarotoxin and 1% octylglucoside (Tx/OG). The positions of the nAChR subunits are indicated.

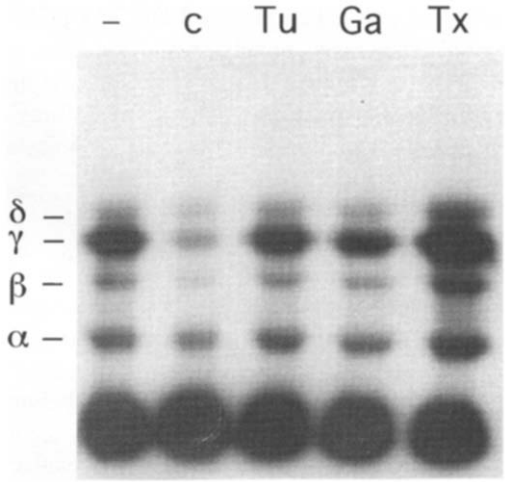
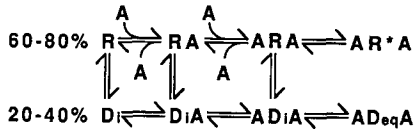


Fig. 3. Effects of antagonists on nAChR [^{125}I]TID-labeling. [^{125}I]TID labeling was performed as described in the legend to Fig. 1. Shown is an autoradiogram of enriched nAChR membranes [^{125}I]TID-labeled in the absence (−) or presence of 300 μM carbamylcholine (c), 10 μM d-tubocurarine (Tu), 10 μM gallamine (Ga), or saturating α-cobrotoxin (Tx; 3× molar excess over binding sites). The positions of the nAChR subunits are indicated.

demonstrating that gallamine functions as a pure, non-desensitizing antagonists. Finally, while the data shown in Fig. 4 were determined using enriched nAChR membranes, the results were the same using affinity-purified nAChR reconstituted into DOPC/DOPA/cholesterol.

4. Discussion

The primary observation of this publication is that snake venom toxin proteins such as BgTx and α-cobrotoxin, but not the smaller antagonists d-tubocurarine and gallamine, increase the degree of [^{125}I]TID labeling of both enriched nAChR membranes and functionally reconstituted affinity-purified nAChR. The simplest mechanism to explain this observation is that the snake venom toxins, but not other nAChR antagonists, shift the population of nAChR entirely to the resting state. As depicted in the reaction scheme below, modified from earlier studies based on radioactive ion flux and other measurements [26–28], the population of nAChR exists in a ratio of resting/desensitized states even in the absence of agonists or other functional modulators.



In this scheme, R is resting state or activatable nAChR, A is agonist, R* is open state nAChR, Di is intermediate desensitized nAChR and Deq is equilibrium desensitized nAChR (although not shown for reasons of clarity, agonist can bind to the un- and mono-liganded Di conformation of the nAChR as well). The numbers to the left of the reaction scheme are the predicted proportions of *Torpedo* nAChR thought to pre-exist in the resting or desensitized states in the absence of ligands. Enriched nAChR membranes apparently are $\approx$ 10–20% in the high-affinity (desensitized) conformation in the absence of agonists [29–31], while functionally-reconstituted affinity-purified nAChR may vary between 20–40% in the pre-existing desensitized state [21]. Binding of snake venom toxins apparently shifts the population of nAChR entirely to the resting state conformation. This would result in increased [^{125}I]TID incorporation into its specific labeling site within the ion channel of the nAChR, which is labeled by [^{125}I]TID predominantly in resting state, but not desensitized nAChR [17]. The magnitude of the enhancement in [^{125}I]TID incorporation in the presence of snake venom toxins agrees with this mechanism, and would explain why the effect was greater with affinity-purified nAChR than with enriched nAChR membranes. The variability of the effect of snake venom toxins on [^{125}I]TID incorporation between different preparations of nAChR also agrees with this mechanism, as different preparations of nAChR have been shown to vary in their pre-existing resting/desensitized state ratio [30,31].

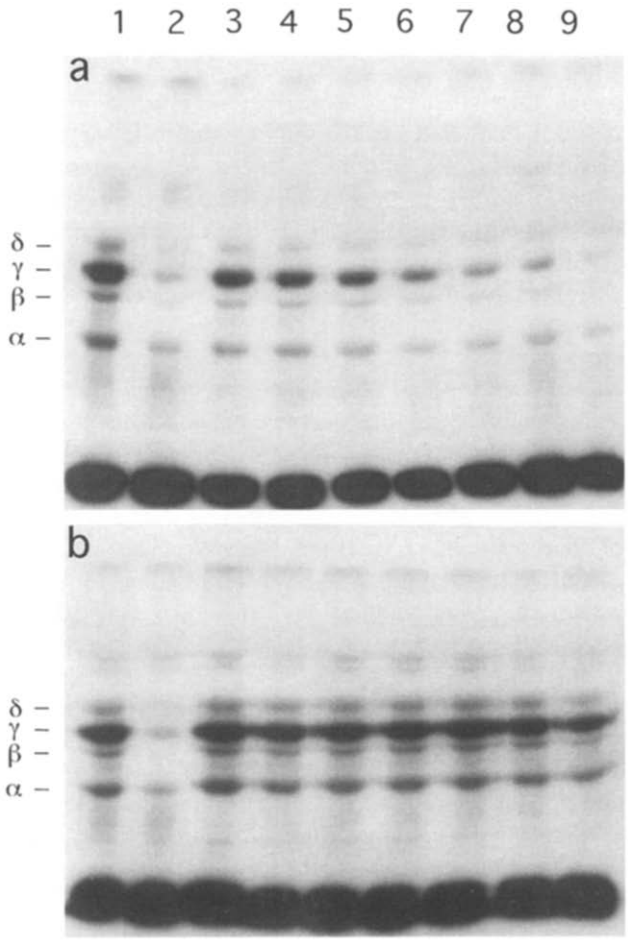


Fig. 4. Effects of d-tubocurarine or gallamine on [^{125}I]TID incorporation into the nAChR. [^{125}I]TID-labeling was performed as described in the legend to Fig. 1. Shown are autoradiograms of enriched nAChR membranes labeled with [^{125}I]TID in the absence (lane 1) or presence of 100 μM carbamylcholine (lane 2) or d-tubocurarine (a) or gallamine (b) at 0.1 μM, 1 μM, 25 μM, 250 μM, 750 μM, 1 M, and 5 mM (lanes 3–9). The positions of the nAChR subunits are indicated.

Earlier workers have split on whether snake venom toxins bind preferentially to one or another functional state, or cause changes in nAChR conformation. While some early studies suggested that snake venom toxins did not stabilize the resting state [32], and that snake venom toxins bound with equal affinity and kinetics to both resting state and desensitized *Torpedo* nAChR [33,34], a number of other lines of research suggest that snake venom toxins do stabilize the resting state. Perhaps the best evidence is provided by the numerous studies which show that snake venom toxins allosterically inhibit the binding of certain non-competitive blockers which are known to bind with higher affinity to desensitized nAChR [11,35–38], while in a more recent study, α-cobrotoxin was seen to block enhanced binding of ethidium to the NCB site by general anesthetics [39]. Further support for

the ability of snake venom toxins to stabilize the resting state conformation is found in studies of the opposing effects of agonists and snake venom toxins on the nAChR. For example, in studies of *T. californica* nAChR, BgTx enhanced quenching of the fluorescent probe pyrene sulfonyl azide while carbamylcholine inhibited quenching [40], BgTx binding caused increased calcium uptake while carbamylcholine induced calcium release [41], BgTx diminished [^{3}H]ethidium labeling while carbamylcholine enhanced label incorporation into all four subunits [42], and in our own earlier studies, BgTx retarded exchange of bound tritium while agonists either slightly accelerated exchange or had no effect [19]. The bulk of the earlier work, and our results reported here, are clearly most consistent with a model in which snake venom toxin binding stabilizes the resting state conformation of the nAChR, and the discrepant results between different studies (including our earlier report of the effects of BgTx on nAChR [^{125}I]TID labeling [14]), are probably due to variation in the ratio between resting and desensitized nAChR pre-existent in different preparations of the protein. However, since the snake venom toxins were not able to stabilize the resting state in nAChR preparations which were profoundly desensitized, by reconstitution into DOPC or solubilization in desensitizing detergent, it appears that the desensitized form of the nAChR which makes up a fraction of the total nAChR population in the absence of ligands is the intermediate desensitized state (Di in the reaction scheme above) and that BgTx will not induce or stabilize the resting state in equilibrium desensitized nAChR.

The effects of d-tubocurarine and gallamine on [^{125}I]TID labeling of the nAChR are consistent with earlier studies. At high (mM) concentrations, d-tubocurarine reduced [^{125}I]TID incorporation to the same extent as agonist-induced desensitization. However, the mechanism by which d-tubocurarine effected this reduction is not clear. In addition to acting as a competitive inhibitor blocker of agonist binding [43], with *Torpedo* nAChR d-tubocurarine has been observed to induce desensitization in some [9,34] (but not all [44]) studies and both to activate or block non-competitively the nAChR ion channel in rat muscle nAChR [45,46] or *Electrophorus electricus* nAChR [47], respectively. In the studies reported here, the calculated IC_{50} for d-tubocurarine reduction of [^{125}I]TID incorporation was 453 μM, which is in general much higher than the concentration of d-tubocurarine required to produce the effects reported in the earlier studies cited above. Therefore, d-tubocurarine reduction of [^{125}I]TID incorporation might be due to induction of desensitization, or perhaps more likely, given the concentration dependence of the effect, by direct competition of d-tubocurarine for the specific [^{125}I]TID binding site in the non-competitive blocker binding region in the nAChR channel. Gallamine, on the other hand, did not reproducibly diminish [^{125}I]TID incorporation even at high gallamine concentrations. In earlier studies, gallamine did not appear to induce nAChR desensitization [44], but did act as a non-competitive blocker of frog muscle nAChR [48]. The failure of gallamine to diminish [^{125}I]TID incorporation is in agreement with the earlier observation that gallamine did not desensitize the nAChR, and if gallamine does bind to the ion channel region of *Torpedo californica* nAChR, this binding apparently does not compete with [^{125}I]TID binding to its high affinity site.

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