A Hydrophobic Inhibitor of the Nicotinic Acetylcholine Receptor Acts on the Resting State†

Ge Wu, Douglas E. Raines, and Keith W. Miller*

Department of Anesthesia, Massachusetts General Hospital, and Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02114

Received July 20, 1994; Revised Manuscript Received September 9, 1994⊗

ABSTRACT: 3-(Trifluoromethyl)-3-(*m*-iodophenyl)diazirine (TID) has recently been found to be a noncompetitive inhibitor of the nicotinic acetylcholine receptor (nAcChoR) by both photolabeling and flux assays (White et al., 1991). However, these experiments were done when TID was in equilibrium with the nAcChoR, and thus only its interactions with the resting and the desensitized states of the nAcChoR were studied. In this work we characterized the interaction between TID and nAcChoR in the open and resting states using a flux assay. When TID and acetylcholine were simultaneously mixed with the nAcChoR in native *Torpedo* vesicles, TID did not inhibit agonist-induced $^{86}Rb^+$ flux. However, following prolonged preincubation (4 min) of TID with nAcChoR, complete inhibition was observed with a half-inhibition constant of 0.4 μM TID and a Hill coefficient of 0.9. This suggested that TID might act either on the resting or the desensitized state in preference to the open state of the nAcChoR. Preincubation of nAcChoR with TID, followed by a 7 ms agonist-induced flux assay, showed that the flux response declined exponentially with preincubation time. Assuming a pseudo-first-order process, analysis revealed the rate constant for the onset of inhibition of nAcChoR in the resting state to be in the range of $(1.2-3.4) \times 10^6$ M^{-1} s^{-1}. To test if fast desensitization was enhanced by TID under these conditions, we used a fluorescent analog of acetylcholine. Stopped-flow fluorimetry showed that the fraction of nAcChoR in the pre-desensitized state did not increase during preincubation with TID. Thus, the inhibition of nAcChoR by TID occurs primarily by an interaction with the resting state of the nAcChoR.

3-(Trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]-TID)[1] is a hydrophobic photoactive reagent which has been widely used to label the membrane-spanning region of transmembrane proteins in a nonspecific way (Brunner & Semenza, 1981; Frielle & Curthoys, 1983; Kahan & Moscarello, 1986; Peitsch et al., 1990; Steffens et al., 1988). However, [^{125}I]TID was recently shown to label a specific component of the nicotinic acetylcholine receptor (nAcChoR) at a site distinct from both the lipid−protein interface and the agonist binding site. Furthermore, nonradioactive TID was shown to inhibit agonist-induced flux (White & Cohen, 1988; White et al., 1991). Thus, TID belongs to the chemically diverse group of noncompetitive inhibitors, which include local anesthetics, long chain alcohols, barbiturates, chlorpromazine, phencyclidine, histrionicotoxin, and triphenylmethylphosphonium. [^{125}I]TID labels all four subunits of the nAcChoR on two distinct components: (1) a specific component which is sensitive to agonist, some noncompetitive inhibitors, and nonradioactive TID; (2) and a nonspecific component at the lipid−protein interface which is insensitive to the above reagents (Blanton & Cohen, 1992, 1994; White & Cohen, 1988; White et al., 1991). The specifically labeled component on the β and δ subunits was identified as a novel binding site in the M2 region that is linked allosterically to the binding sites of both agonists and other noncompetitive antagonists (histrionicotoxin and phencyclidine) (White & Cohen, 1992). It has been suggested that this labeled component is in the lumen of the channel. When agonist was present, the total photoincorporation of [^{125}I]TID in nAcChoR was strongly inhibited (more than 75%), the labeling of the residues in the M2 region was reduced by more than 90%, and the distribution of labeled residues was broadened (White et al., 1991; White & Cohen, 1992).

Under equilibrium conditions, noncompetitive inhibitors can label the nAcChoR in the resting state (in the absence of agonist) and the desensitized state (sustained application of agonist), but rapid labeling techniques must be used to label the transient open state before desensitization (Cox et al., 1984; Heidmann & Changeux, 1984; Muhn et al., 1984). Though the results obtained from these rapid labeling experiments were controversial because of the complication of receptor desensitization (Cox et al., 1985; Heidmann & Changeux, 1984, 1986), there is strong evidence that the transient open state is labeled by [^{3}H]chlorpromazine and [^{3}H]quinacrine azide (DiPaola et al., 1990; Heidmann & Changeux, 1986). To date, photolabeling with [^{125}I]TID has only been done under equilibrium conditions. Thus, only information from the resting state and the desensitized state has been obtained. Since photolabeling of the nAcChoR with [^{125}I]TID has several unique features under equilibrium conditions, it may offer some insight into the interaction between noncompetitive inhibitors and the activated nAcChoR on the millisecond time scale.

† This research was supported by the National Institute of General Medical Science to Harvard Anesthesia Center (GM-15904) and by the Department of Anesthesia, Massachusetts General Hospital.

* Address correspondence to this author at the Department of Anesthesia, Massachusetts General Hospital, Boston, MA 02114.

⊗ Abstract published in *Advance ACS Abstracts,* November 15, 1994.

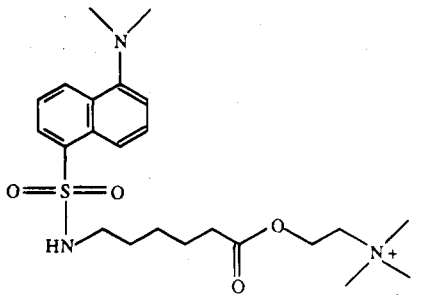
[1] Abbreviations: TID, 3-(trifluoromethyl)-3-(*m*-iodophenyl)diazirine; nAcChoR, nicotinic acetylcholine receptor; DFP, diisopropyl phosphorofluoridate; α-BTX, α-bungarotoxin; TPS, *Torpedo* physiological saline.

0006-2960/94/0433-15375$04.50/0

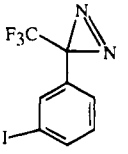
To understand the interaction between TID and nAcChoR in a time-resolved manner, we have studied the inhibition of agonist-induced flux by TID with a rapid quenched-flow assay and also with a stopped-flow fluorescent energy transfer assay. We found that TID does not have detectable inhibitory effects on the nAcChoR in the open state, but it strongly inhibits the nAcChoR in the resting state. The inhibition of the nAcChoR by TID was reversible. The onset rate constant for inhibition of the nAcChoR in the resting state was estimated to be in the range of $(1.2-3.4) \times 10^6$ M^{-1} s^{-1}.

MATERIALS AND METHODS

Diisopropyl phosphorofluoridate (DFP) was from Aldrich Chemical Co. (Milwaukee, WI). α-Bungarotoxin (α-BTX) was from the Miami Serpentarium (Miami, FL) and was purified by CM-cellulose chromatography (Lee et al., 1972). Acetylcholine chloride, procaine hydrochloride, buffer reagents. and Dowex resin were from Sigma Chemical Co. (St. Louis, MO). ^{86}RbCl was from New England Nuclear (Boston, MA). TID was made according to Brunner and Semenza (1981) and stored in methanol at −80 °C. Dns-C_6-Cho was made according to Waksman et al. (1980).



Dns-C_6-Cho



TID

Vesicle Preparation. Postsynaptic membranes from freshly dissected electroplaques of *Torpedo nobiliana* (Biofish Associates, Georgetown, MA) were prepared using sucrose density gradient centrifugation at 4 °C essentially as described previously (Braswell et al., 1984). Membrane suspensions were kept frozen at −80 °C in *Torpedo* physiological saline (TPS: 250 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 5 mM PO_4^{2-}, 0.02%Na_3N; pH 7.0) and thawed within 48 h of use. The thawed nAcChoR was then incubated with $^{86}Rb^+$ for 5 h or longer to allow $^{86}Rb^+$ to permeate into the vesicles and achieve equilibrium. The $^{86}Rb^+$ outside the vesicles was removed by a 30 × 0.5 cm Sephadex G50 column. The vesicles were incubated for 20 min with 0.1 mM DFP at 4 °C to inhibit acetylcholinesterase activity. Flux responses were not altered by this treatment (Forman et al., 1989). Prior to flux assays, $^{86}Rb^+$ that had leaked out of the vesicles during the 20 min incubation was removed by a 2 cm × 1 cm Dowex cation exchange column. When necessary, the number of active receptor−channel complexes was reduced by blocking with the irreversible inhibitor, α-BTX, to prevent full equilibration of $^{86}Rb^+$ within the experimental assay time (Miller et al., 1987).

Quenched-Flow Assay. Channel function was assayed by measuring agonist-induced $^{86}Rb^+$ efflux from spontaneously sealed native *Torpedo* electroplaque vesicles. A rapid quenched-flow technique, as described in detail previously (Forman et al., 1987), was used for time-resolved studies. For concentration−response curve measurements, vesicles containing $^{86}Rb^+$ and an equal volume of agonist solution were pushed through a mixer into a delay tube, which was usually set to give a 7 ms incubation. The combined solution was then mixed with an equal volume of 100 mM procaine at a second mixer to quench the flux.

To determine the kinetics of TID inhibition, equal volumes of $^{86}Rb^+$ loaded vesicles and TID solution were pushed through the first mixer and flowed through a delay tube. The combined solution was then mixed with acetylcholine to give a final concentration of 1 mM at the second mixer. After a 7 ms delay the efflux of $^{86}Rb^+$ was quenched with procaine to give a final concentration of 50 mM at the third mixer.

The 1-octanol inhibition experiments were done by first mixing 1-octanol and acetylcholine in one solution and then mixing it with $^{86}Rb^+$ loaded vesicles (in the presence or absence of TID), and the flux was quenched as before after 7 ms.

In all the above procedures, following quenching, the reaction mixture was *immediately* filtered through a glass microfiber filter (Whatman GF/F). The radioactivity in the filtrate was assayed with a Packard 1900CA liquid scintillation analyzer using Packard Ultima Gold XR (Meriden, CT) scintillation solution.

Manual 10 s Assay. $^{86}Rb^+$ loaded vesicles, preincubated with or without TID, were mixed with acetylcholine to give a final concentration of 1 mM. After 10 s, the reaction mixture was filtered as above and the filtrate assayed as in the quenched-flow assay.

Reversal of TID Inhibition. $^{86}Rb^+$ loaded vesicles were divided into two aliquots. The first was incubated with 10^{-5} M TID for 4 min and then quickly diluted 100 times into TPS. The $^{86}Rb^+$ efflux was then measured by exposing the vesicles to 1 mM acetylcholine for 10 s. The other vesicles were diluted 100 times into TPS, and two control experiments were done: (1) to measure the flux activity of the vesicles in the absence of TID to obtain the maximum value of the flux in the above dilution experiment, and (2) to measure the flux activity of diluted vesicles that were incubated with 10^{-5} M TID for 4 min, which gives the minimum value of the flux in the above dilution experiment. Both of these control experiments were done under the same conditions as the dilution experiment.

Data Analysis. The $^{86}Rb^+$ efflux from the vesicles is described by a parameter, F_c (Wu et al., 1994), which takes into account $^{86}Rb^+$ leak from vesicles and also is corrected for the limited internal volume of the vesicles. TID does not induce leakage of the vesicles in our experimental conditions. The flux of vesicles was analyzed with the logistic equations for the acetylcholine activation curve (eq 1) and the inhibitory curves of noncompetitive inhibitors (eq 2),

$$F_c = F_c^{max} \frac{[A]^n}{[A]^n + K_A^n} \quad (1)$$

$$F_c = F_c^{max} \frac{[I]^n}{[I]^n + IC_{50}^n} \quad (2)$$

where F_c is the $^{86}Rb^+$ efflux from the vesicles at a given acetylcholine concentration, n is the Hill coefficient (which

has a positive value for activation and a negative one for inhibition), [A] is the acetylcholine concentration, [I] is the concentration of noncompetitive inhibitor, and K_A and IC_{50} are the concentrations of acetylcholine or inhibitor that produce half-maximal activation or inhibition, respectively. For convenience, in each experiment values of F_c were normalized to an F_c^{max} of 1.

When the rate of inhibition was measured, the concentration of TID was much higher than nAcChoR, and the data were fitted to a pseudo-first-order process as shown:

$$F_c = F_c(\infty) + [F_c(0) - F_c(\infty)] \exp(-kt) \qquad (3)$$

$F_c(0)$ is the initial value of F_c in the absence of TID and is taken as 1. $F_c(\infty)$ was obtained from the value in Figure 1 when different concentrations of TID and nAcChoR are at equilibrium. k is the pseudo-first-order rate constant which is equal to $[TID]k_{on} + k_{off}$, where k_{on} is the onset rate constant for TID association with the nAcChoR and k_{off} is the rate constant for the dissociation of the complex of TID and the nAcChoR.

Stopped-Flow Fluorescent Energy Transfer Assay. The stopped-flow fluorescent energy transfer experiments were done with a BioSX-17 MV spectrofluorimeter with a 150 W lamp (Applied Photophysics, England). Equal volumes of Dns-C_6-Cho (4 μM) and vesicles (1 μM) were rapidly mixed. Tryptophan residues in proteins were excited at 290 nm, and the binding of Dns-C_6-Cho was recorded (9.5 nm slit with a 530 nm bandpass filter). The resulting increase in fluorescence was followed for 250 s after mixing, and the data were collected on a log time base. In each experiment, three traces (2000 points in each trace) were collected and averaged.

RESULTS

TID Inhibition of Initial Flux Rate. The $^{86}Rb^+$ that was trapped inside the vesicles can only flux out of the vesicles through the activated (opened) nAcChoR channels. Thus, the flux rate is proportional to the number of the opened channels. Most of the activatable nAchoR channels are opened immediately (less than 100 μs) after exposure to 1 mM of the full agonist acetylcholine (Colquhoun & Ogden, 1988), and the maximum $^{86}Rb^+$ flux rate is observed. The flux rate in the present experiment was controlled by irreversibly blocking some nAcChoR with α-BTX so that the integrated flux was linear and measurable over the 7 ms assay time (Wu et al., 1994). Thus, the fraction of nAcChoR inhibited by TID can be assayed by measuring the decrease in flux, F_c, at fixed time. Figure 1 shows the effect of various concentrations of TID on F_c. No inhibition of $^{86}Rb^+$ efflux was observed when acetylcholine and TID (up to 30 μM) were simultaneously mixed with nAcChoR and flux was allowed to proceed for 7 ms. Under such conditions, all nAcChoR should be in the open state throughout the assay. In contrast, a complete inhibition curve was observed when TID was preincubated with nAcChoR for more than 4 min followed by exposure to 1 mM acetylcholine for 7 ms. The curve in Figure 1 was generated by a nonlinear least squares fit to eq 2, which yields a Hill coefficient of $n = -0.9 \pm 0.11$ and a half-maximum inhibition concentration or $IC_{50} = 0.40 \pm 0.074$ μM. When nAcChoR was preincubated with TID for 132 ms before it was exposed to 1 mM acetylcholine, complete inhibition of the nAcChoR by TID was also attained, but TID appeared less potent. A nonlinear least squares fit to eq 2 yielded $n = -1.08 \pm 0.090$ and $IC_{50} = 3.4 \pm 0.29$ μM. This apparent reduction of TID potency following a short preincubation hints that TID inhibition of nAcChoR is a slow process.

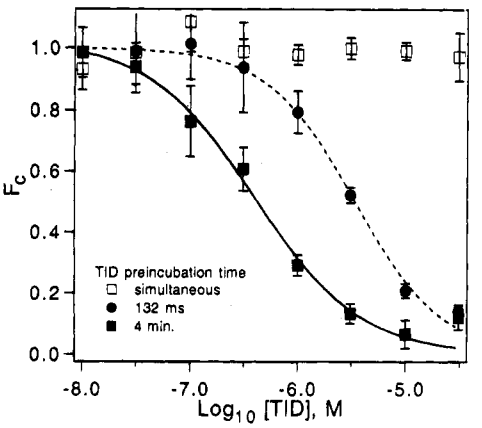


FIGURE 1: Inhibition of acetylcholine-induced $^{86}Rb^+$ flux by TID at 4 °C with 7 ms assay. No inhibition of $^{86}Rb^+$ flux was observed when TID and acetylcholine were mixed with the nAcChoR simultaneously (□). Strong inhibition was observed when TID was preincubated with the nAcChoR for more than 4 min before $^{86}Rb^+$ flux was induced by 1 mM acetylcholine (■). The parameters fitted to eq 2 for the inhibition curve were obtained as $n = -0.9 \pm 0.11$ and $IC_{50} = 0.4 \pm 0.11$ μM. The inhibition by TID appeared less potent if TID and nAcChoR were preincubated for 132 ms before exposure to acetylcholine (●). The parameters for the inhibition curve were obtained as $n = -1.07 \pm 0.090$ and $IC_{50} = 3.4 \pm 0.29$ μM. The data were obtained in quadruplicate and averaged.

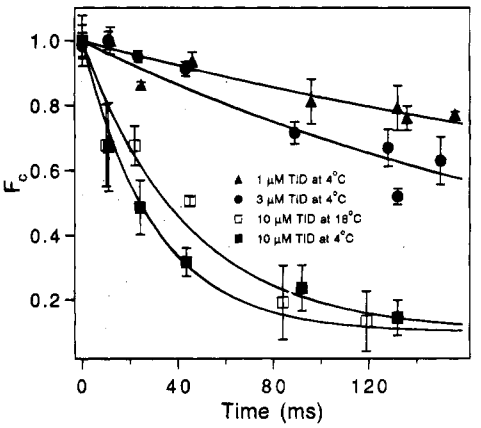


FIGURE 2: Rate of inhibition of 1 mM acetylcholine-induced flux by TID. TID was incubated with nAcChoR for various times and then exposed to acetylcholine (final concentration of 1 mM). (▲) 1 μM TID at 4 °C, (●) 3 μM TID at 4 °C, (■) 10 μM TID at 4 °C, (□) 10 μM TID at 18 °C. The data were fitted to a first-order exponential process (eq 3). The extent of the inhibition at equilibrium was obtained from Figure 1. The resulting inhibition rates are summarized in Table 1.

Determination of TID Inhibition Rate. The slow inhibition of nAcChoR by TID was further investigated by preincubating several concentrations of TID with $^{86}Rb^+$ loaded nAcChoR vesicles for various times and measuring the decrease in the 1 mM acetylcholine-induced $^{86}Rb^+$ flux relative to a control in which nAcChoR was preincubated with TPS instead of TID. Figure 2 shows the percent inhibition of flux by TID at 1, 3, and 10 μM at 4 °C and 10 μM at 18 °C as a function of preincubation time. The data were fit by nonlinear least squares analysis to a pseudo-first-order kinetic equation (eq 3), with maximal inhibition fixed at the values obtained from Figure 1 for 4 min preincubation.

Table 1: Rate Constants for the Inhibition of the Resting State nAcChoR by TID[a]

TID	pseudo-first-order rate constant (s^{-1})	onset rate constant (M^{-1} s^{-1}) (estimate a)	onset rate constant (M^{-1} s^{-1}) (estimate b)
1 μM at 4 °C	2.8 ± 0.31	(2.8 ± 0.31) × 10^6	(2.0 ± 0.22) × 10^6
3 μM at 4 °C	4.2 ± 0.41	(1.4 ± 0.14) × 10^6	(1.2 ± 0.12) × 10^6
10 μM at 4 °C	34 ± 3.3	(3.4 ± 0.33) × 10^6	(3.3 ± 0.32) × 10^6
10 μM at 18 °C	23 ± 3.2	(2.3 ± 0.32) × 10^6	(2.2 ± 0.31) × 10^6

[a] The pseudo-first-order rate constant was obtained by fitting the data in Figure 2 to a pseudo-first-order process (eq 3). The onset rate constant was estimated using two assumptions: (a) the off-rate is significantly slower than the on-rate and is therefore negligible; (b) the dissociation constant of the TID•nAcChoR complex is equal to the IC_{50} as determined in Figure 1.

The pseudo-first-order inhibition rate constants obtained from the fitting are summarized in Table 1. Since there is no information regarding the off-rate of TID dissociation from nAcChoR nor the value of the dissociation constant of the nAcChoR•TID complex, we can only estimate the onset rate constant of inhibition with the assumptions that either (a) the off-rate is very slow compared with the onset rate, i.e., $[TID]k_{on} \gg k_{off}$; or (b) the dissociation constant of the nAcChoR•TID complex is equal to the IC_{50} determined in Figure 1. The onset rate constants for TID thus estimated are summarized in Table 1. Though the data were obtained in quadruplicates and averaged, we estimated that the absolute error was 5 times larger than the goodness of fit errors given in Table 1.

TID Inhibition of Flux over 10 s. The above experiments demonstrated that the rate of TID inhibition of nAcChoR at the resting state is much slower than the flux rate. It is also possible that TID inhibits nAcChoR in the open state with the same slow rate, and if so, we would not be able to observe this in the above simultaneous mixing experiment in 7 ms (see Discussion section). To maximize the chance of observing the inhibition of nAcChoR in the open state, we used a 10 s flux assay with acetylcholine and TID simultaneously mixed with the nAcChoR. In this assay, the flux rate is linear for 10−20 ms and then decays slowly because of desensitization (Wu & Miller, 1994). The $^{86}Rb^+$ inside the vesicles will reach equilibrium with the outside and the flux stops (at least 30−40 ms after mixing). The 10 s assay, therefore, offers the maximum flux assay time while nAcChoR is in the open state, and any inhibition of the nAcChoR by TID faster than the assay time (at least 30−40 ms) should be observed. Figure 3 shows that, as in the 7 ms assay, no inhibition was observed when TID and acetylcholine were simultaneously mixed with nAcChoR while strong inhibition was observed when TID and nAcChoR were preincubated (see Discussion section).

Reversal of TID Inhibition. TID inhibition of nAcChoR was reversed when the TID concentration was reduced by dilution with large amount of TPS. In a 10 s flux assay, the vesicles in the absence of TID released 58 ± 4% of $^{86}Rb^+$ in the presence of 1 mM acetylcholine. When the vesicles were incubated with 10 μM TID for 4 min and then exposed to 1 mM acetylcholine, negligible flux was observed. When the vesicles that had been equilibrated with 10 μM TID were rapidly diluted 100 times with TPS to a final concentration of 0.1 μM TID, 52 ± 3% of the $^{86}Rb^+$ was released by 1 mM acetylcholine. The recovery of the nAcChoR activity was complete within 30 s after rapid dilution.

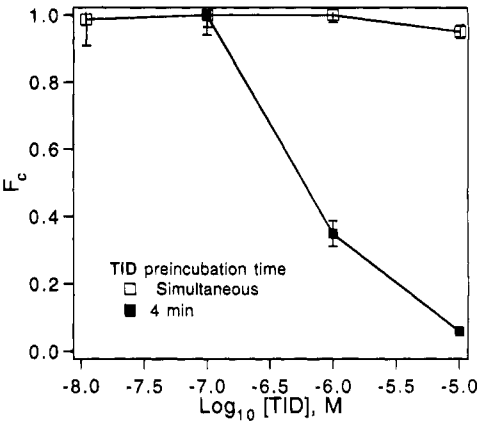


FIGURE 3: Inhibition of acetylcholine-induced $^{86}Rb^+$ flux by TID at 4 °C with 10 s assay. No inhibition of $^{86}Rb^+$ flux was observed when TID and acetylcholine were mixed with nAcChoR simultaneously (□). Strong inhibition was observed when TID was preincubated with nAcChoR for more than 4 min and then the $^{86}Rb^+$ flux was induced by 1 mM acetylcholine (■).

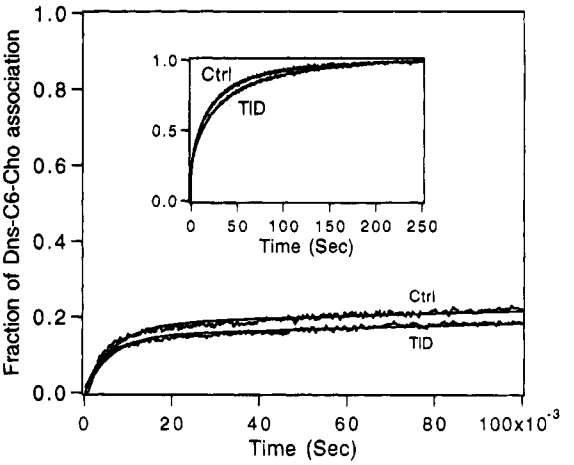


FIGURE 4: Time course of association of Dns-C_6-Cho with nAcChoR with or without preincubation with 10 μM TID. The traces are the average of 3 determinations in each case. Equilibrium was reached 250 s after mixing equal volumes of 4 μM Dns-C_6-Cho and 1 μM nAcChoR at 20 °C (insert). The association of Dns-C_6-Cho with the nAcChoR in the pre-desensitized state occurs in the first 100 ms. A slight decrease in the fraction of the pre-desensitized state is observed after preincubation with TID (from 17.3 ± 0.28% to 15.3 ± 0.25%).

TID's Effects on the Equilibrium between Resting and Desensitized States. In the absence of agonist, nAcChoR is present in an equilibrium between the resting and the desensitized states. The desensitized state has a high affinity for agonist and accounts for about 20% of the total receptor population. Here we used a well-established stopped-flow fluorescent energy transfer assay to study the effect of TID on the fraction of receptors pre-existing in the desensitized state [for a review see Changeux (1990)]. Figure 4 shows the time course of the association of Dns-C_6-Cho, a fluorescent analog of acetylcholine, with nAcChoR which had been preincubated with 10 μM TID for more than 4 min. A control experiment in the absence of TID was also done under the same conditions. When the concentration of Dns-C_6-Cho is low, it binds rapidly and solely to the pre-existing desensitized states within the first 100 ms (Heidmann & Changeux, 1980). Figure 4 shows that there was a small

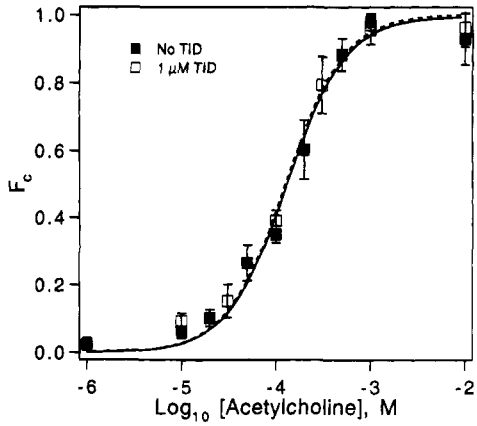


FIGURE 5: Preincubating nAcChoR with TID does not affect the acetylcholine concentration–response curve. The acetylcholine concentration–response curves for the flux in the presence and absence of 1 μM TID (preincubated for 4 min) at 4 °C are shown. The data were fitted to the Hill equation (eq 1), and in the absence of TID (■), $n = 1.4 \pm 0.2$ and $K_A = 130 \pm 12$ μM, and in the presence of 1 μM TID (□), $n = 1.4 \pm 0.2$ and $K_A = 126 \pm 10$ μM.

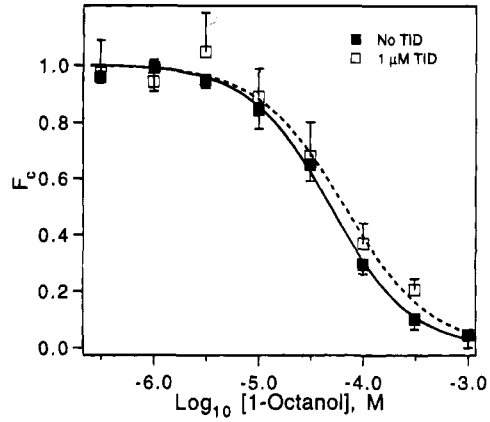


FIGURE 6: Preincubating nAcChoR with TID does not affect the 1-octanol inhibition curve. The 1-octanol inhibition curves of the 1 mM acetylcholine-induced flux with and without preincubation with 1 μM TID at 4 °C are shown. Acetylcholine and 1-octanol were premixed before mixing with nAcChoR. The data were fitted to eq 2, and the results are listed below. In the absence of TID (■), $n = -1.1 \pm 0.1$ and $IC_{50} = 48 \pm 4.4$ μM. In the presence of 1 μM TID (□), $n = -1.1 \pm 0.2$ and $IC_{50} = 66 \pm 12$ μM.

decrease in the fraction of the desensitized state after preincubating the nAcChoR with TID (from 17.3 ± 0.28% to 15.3 ± 0.25%). The complete traces of the Dns-C_6-Cho binding fluorescence were also shown in Figure 4 (insert).

TID Does Not Affect the Acetylcholine Concentration–Response Curve. Figure 5 shows that preincubation of 1 μM TID with nAcChoR did not change the acetylcholine concentration–response curve. Since 1 μM TID dramatically decreased the number of receptors that could respond to acetylcholine, the assay could only be performed when the nAcChoR was not modified by α-BTX. A control acetylcholine concentration–response curve was also performed in the absence of TID with a fraction of the nAcChoR blocked by α-BTX. The maximum flux in both experiments were normalized to 1. All points were obtained in quadruplicate and averaged. The curves were obtained by nonlinear least squares fitting to eq 1. The two curves were superimposed after normalization to its maximum flux, with Hill coefficient $n = 1.4 \pm 0.16$ and $K_A = 130 \pm 12$ μM in the absence of TID, and $n = 1.4 \pm 0.19$ and $K_A = 126 \pm 10$ μM in the presence of TID.

TID Does Not Affect the 1-Octanol Inhibition Curve. Figure 6 shows the inhibitory action of 1-octanol on the 1 mM acetylcholine induced $^{86}Rb^+$ efflux in the presence (preincubated for more than 4 min) and absence of 1 μM TID. In both cases, acetylcholine and 1-octanol were mixed with nAcChoR simultaneously. Again, the vesicles used in the presence of 1 μM TID were not modified by α-BTX. In both experiments, the maximum flux was normalized to 1. The data were obtained in quadruplicate and averaged, and the curves were obtained by nonlinear least squares fitting to eq 2. In the absence of TID, 1-octanol's inhibitory curve had a Hill coefficient $n = -1.1 \pm 0.1$ and an $IC_{50} = 48 \pm 4.4$ μM. In the presence of 1 μM TID, 1-octanol's inhibitory curve had $n = -1.1 \pm 0.2$ and $IC_{50} = 66 \pm 12$ μM. Furthermore, in a similar experiment where 1-octanol was preincubated with the nAcChoR, 1-octanol's IC_{50} was the same whether or not 1 μM TID was included in the preincubation mixture.

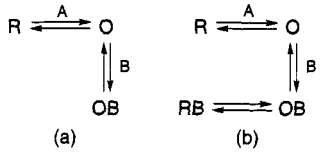
DISCUSSION

The present experiments show that the nAcChoR was inhibited by TID only after preincubation and the rate constant of the inhibition was in the order of 10^6 M^{-1} s^{-1}. When TID (up to 30 μM) and acetylcholine (1 mM) were simultaneously mixed with nAcChoR, no detectable inhibition by TID was observed. Because the concentration of acetylcholine was much higher than that of TID in this experiment, most channels were opened before nAcChoR had time to interact with TID. The inhibition of the nAcChoR was reversible, and the rate of activated flux was fully recovered within 30 s after diluting TID.

From the above observations, several models for TID inhibition of the nAcChoR are possible. Model 1: TID desensitized the nAcChoR in the absence of agonist after preincubation. Thus, most nAcChoR was in the desensitized state after preincubation with TID, and channels did not open when exposed to acetylcholine. Model 2: TID inhibited nAcChoR in the open state, but the inhibition of this state is very slow compared with the agonist-induced flux rate. Thus, when acetylcholine and TID are simultaneously mixed with nAcChoR, the channel opens rapidly and flux occurs, while binding by TID was so slow that inhibition did not occur until after all $^{86}Rb^+$ fluxed out of the vesicles. Model 3: TID inhibited nAcChoR in the resting state but not the open state. Thus, strong inhibition was observed when TID was preincubated with nAcChoR in the resting state (in the absence of agonist), and no inhibition was observed when nAcChoR was in the open state in the experiments with simultaneous mixing. We will discuss each of these possibilities in the following paragraphs.

Model 1. It is known that nAcChoR exists in equilibrium between the desensitized state and the resting state in the absence of agonist. The nAcChoR in the desensitized state normally accounts for about 20% of the total receptors. After sustained application of agonist, the equilibrium is changed and most nAcChoR is converted to the desensitized state. Furthermore, some noncompetitive inhibitors are capable of changing the nAcChoR from the resting state to the desensitized state even in the absence of agonist (Boyd & Cohen, 1984; Heidmann & Changeux, 1979; Heidmann et

Scheme 1: Open Channel Blocking Models by NCIs (Model 2)[a]



[a] R = resting state; O = open state; A = agonist; B = inhibitor; OB = blocked open state; RB = blocked resting state.

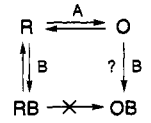
al., 1983; Oswald et al., 1983). Since both the desensitized state and the inhibited state are characterized by their inability to allow ions to pass through the channel, it is impossible to distinguish between the two states by flux studies alone. Stopped-flow fluorescent energy transfer assay [for a review see Changeux (1990)] of nAcChoR with an agonist analog, Dns-C_6-Cho, demonstrated that the desensitized receptors have the highest affinity and highest association rate for Dns-C_6-Cho, and the reaction was complete within the first 100 ms after mixing the nAcChoR and Dns-C_6-Cho. Our results (Figure 4) were consistent with their observations and showed that TID hardly had any effect on the equilibrium between the resting state and the pre-desensitized state of nAcChoR. Further evidence for the insensitivity of the desensitized state to TID is that photoincorporation of [^{3}H]-TID to nAcChoR was significantly inhibited by preincubation with agonist (White & Cohen, 1988). Thus, the possibility that TID desensitized nAcChoR during preincubation can be ruled out.

Model 2. Electrophysiological observations based on "noise" analysis, relaxation kinetics after voltage jump, or single channel recordings have led to the proposal that many noncompetitive inhibitors enter the pore of the opened channel and sterically inhibit ion transport (Scheme 1a) [for a review see Adams (1981)]. This model has been further modified to allow the blocked open channel state to be in equilibrium with a blocked resting state (Scheme 1b) (Dilger & Brett, 1991; Neely & Lingle, 1986). Most noncompetitive inhibitors exert little action unless the ion channel has been opened by an agonist (Adams, 1976; Changeux, 1990; Neely & Lingle, 1986; Neher & Steinbach, 1978; Ogden et al., 1981). Time-resolved photolabeling offered further evidence for the open state inhibition model with photoactive [^{3}H]-chlorpromazine and [^{3}H]quinacrine azide as the inhibitor (Heidmann & Changeux, 1984; Heidmann & Changeux, 1986; DiPaola et al., 1990).

Similar to the action of most other noncompetitive inhibitors, Model 2 suggests that TID inhibits nAcChoR in the open state. The open channel may be inhibited either by physically plugging the channel with noncompetitive inhibitors or by a structural transition triggered by their binding to nAcChoR. Thus, the slow action of TID on the open channel may be because either (a) the diffusion of TID into the channel is sterically hindered ("wiggling in") or (b) its diffusion to its site is not hindered, but the structural transition to the inhibited state is very slow. We examine these two hypotheses below:

(a) Steric hindrance at the channel is unlikely to account for the results of the experiment where TID and acetylcholine were simultaneously mixed with the nAcChoR because (1) 1-octanol, whose size is not much smaller than TID, and which has been proposed to inhibit the open channel, inhibits the nAcChoR under similar experimental conditions (Figure 6); and (2) time-resolved photolabeling by [^{3}H]chlorpromazine, which is much bulkier than TID, revealed that the onset rate constant for photolabeling is 1.1×10^7 M^{-1} s^{-1} (Heidmann & Changeux, 1984) after rapid mixing, about 10 times faster than TID.

Scheme 2: Proposed Model for TID Inhibition (Model 3)



(b) It is unlikely that TID inhibits the open state of nAcChoR by triggering a slow structural transition to the inhibited state after its rapid binding to the open state. First, after preincubating TID for 4 min with the nAcChoR, TID was able to inhibit most of the nAcChoR within 7 ms. This fast action was not compatible with the assumption that TID bound to nAcChoR at the open state and then triggered a slow structural transition unless it is assumed that TID also acted on the resting state and inhibited most of nAcChoR before it was exposed to acetylcholine. Second, in 10 s flux experiments with simultaneously mixed TID, acetylcholine, and nAcChoR, which offered the longest time for TID to inhibit the transient open state (it takes at least 30 ms for the inside−outside $^{86}Rb^+$ concentration gradient to reach equilibrium), no inhibition was observed (Figure 3). If we assume that TID inhibits the resting state and open state with the same kinetics, we can simulate the time course of the inhibition of the open state using the parameters obtained from the resting state. The simulation shows that almost half of the open nAcChoRs ought be inhibited 30 ms after simultaneously mixing with 10 μM TID and 1 mM acetylcholine. Such inhibition should be observed in the 10 s assay. Therefore, this hypothesis is unlikely, but if true, any inhibitory conformation change must be much slower than we could measure.

Model 3. Inhibition of the resting state of nAcChoR by TID is most compatible with our experiments demonstrating that preincubation of TID completely inhibited nAcChoR but that there was no inhibition when TID, acetylcholine, and nAcChoR were simultaneously mixed. Since the inhibition of ion flux was dependent on pre-equilibration of TID with the resting state, TID inhibits the resting state of the nAcChoR directly (Scheme 2) in contrast to the open state inhibition shown in Scheme 1. Although we cannot exclusively rule out inhibition of the open state, it could only contribute to a small degree compared to the inhibition of the resting state. The inhibited resting state cannot be converted to the inhibited open state (or it may happen but extremely slowly) in the presence of agonist because negligible flux was observed when the inhibited resting state receptors were incubated with 1 mM acetylcholine for 10 s.

Preincubation of TID with the nAcChoR had no apparent effect on the acetylcholine concentration−response curve (Figure 5). This is compatible with the assumption that TID primarily acts at the resting state. TID's effect is to eliminate some of the nAcChoR that would otherwise be open in the presence of agonist and the remaining nAcChoR is open in the normal fashion when exposed to acetylcholine. This experiment also showed that the reverse rate for the inhibition of TID must be very slow compared with channel opening and the flux rate. Otherwise, a rightward shift in the

acetylcholine concentration−response curve in the presence of TID should be observed as more nAcChoR move from the RB state to the R state and then to the O state at high acetylcholine concentrations (see Scheme 2). We have shown that the inhibition of nAcChoR by TID can be fully reversed by TID within 30 s. Assuming the dissociation constant of the nAcChoR·TID complex equals the IC_{50} (0.4 μM) and $k_{on} = 2 \times 10^6$ M^{-1} s^{-1} (see Table 1), the reverse rate, k_{off}, can be estimated to be 0.8 s^{-1}, which is beyond the resolution of our manual assay.

Preincubation with TID had no significant effect on the inhibition curve of 1-octanol (Figure 6), because TID inhibits nAcChoR at the resting receptor (proposed Model 3 show in Scheme 2) while 1-octanol inhibits the nAcChoR in the open state (Scheme 1) (Dilger & Brett, 1991; Murrell & Haydon, 1991). If equilibrium between the R and RB states could be established extremely rapidly, a right shift in the 1-octanol inhibition curve should be observed after preincubation with TID. However, as argued before, the rate of transition from RB to R is slow compared with the assay time, and thus only the nAcChoR not preinhibited by TID participated in the 1-octanol inhibition equilibrium (Scheme 2). Thus, preincubating TID with the nAcChoR only reduced the amplitude of the maximum response of the nAcChoR to 1-octanol but did not alter its inhibition curve.

Because the inhibition which we observed occurs on the resting state, the photolabeling studies already performed indicate that two classes of TID binding site on nAcChoR might be responsible. The studies of nAcChoR photolabeling by [^{125}I]TID (Blanton & Cohen, 1992, 1994; White & Cohen, 1988; White et al., 1991) and several other radioactive noncompetitive inhibitors [for a review see Lena and Changeux (1993)] have shown that they bind to nAcChoR at either the channel lumen (high affinity binding) or the lipid−protein interface (low affinity or nonspecific binding). White et al. (1991) have argued that the former class of sites is most likely to be the inhibitory site of TID, but were unable to unequivocally rule out the second class of sites. Our data are consistent with binding to either a single site or many sites of equal affinity and therefore do not provide a more definitive resolution of the dichotomy that they discussed. This problem could be resolved by comparing the time constant for development of inhibition to that which could be obtained for the two components by time-resolved photolabeling experiments.

ACKNOWLEDGMENT

We would like to thank Dr. Shaukat Husain for synthesizing TID and Dns-C_6-Cho.

REFERENCES

Adams, P. R. (1976) *J. Physiol. (London) 260*, 531−552.
Adams, P. R. (1981) *J. Membr. Biol. 58*, 161−174.
Blanton, M. P., & Cohen, J. B. (1992) *Biochemistry 31*, 3738−3750.
Blanton, M. P., & Cohen, J. B. (1994) *Biochemistry 33*, 2859−2872.
Boyd, N. D., & Cohen, J. B. (1984) *Biochemistry 23*, 4023−4033.
Brunner, J. , & Semenza, G. (1981) *Biochemistry 20*, 7174−7182.
Changeux, J.-P. (1990) *Fidia Res. Found. Neurosci. Award Lect. 4*, 21−168.
Colquhoun, D., & Ogden, D. C. (1988) *J. Physiol. (London) 395*, 131−159.
Cox, R. N., Kaldany, R. R., Brandt, P. W., Ferren, B., Hudson, R. A., & Karlin, A. (1984) *Anal. Biochem. 136*, 476−486.
Cox, R. N., Kaldany, R. R., DiPaola, M., & Karlin, A. (1985) *J. Biol. Chem. 260*, 7186−7193.
Dilger, J. P., & Brett, R. S. (1991) *Ann. N.Y. Acad. Sci. 625*, 616−627.
DiPaola, M., Kao, P. N., & Karlin, A. (1990) *J. Biol. Chem. 265*, 11017−11029.
Frielle, T., & Curthoys, N. P. (1983) *Ciba Found. Symp. 95*, 73−91.
Heidmann, T., & Changeux, J. P. (1979) *Eur. J. Biochem. 94*, 281−296.
Heidmann, T., & Changeux, J. P. (1980) *Biochem. Biophys. Res. Commun. 97*, 889−896.
Heidmann, T., & Changeux, J. P. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1897−1901.
Heidmann, T., & Changeux, J. P. (1986) *Biochemistry 25*, 6109−6113.
Heidmann, T., Oswald, R. E., & Changeux, J. P. (1983) *Biochemistry 22*, 3112−3127.
Kahan, I., & Moscarello, M. A. (1986) *Biochim. Biophys. Acta 862*, 223−226.
Lena, C., & Changeux, J. P. (1993) *Trends Neurosci. 16*, 181−186.
Muhn, P., Fahr, A., & Hucho, F. (1984) *Biochemistry 23*, 2725−2730.
Murrell, R. D. , & Haydon, D. A. (1991) *Ann. N.Y. Acad. Sci. 625*, 365−374.
Neely, A., & Lingle, C. J. (1986) *Biophys. J. 50*, 981−986.
Neher, E., & Steinbach, J. H. (1978) *J. Physiol. 277*, 153−176.
Ogden, D. C., Siegelbaum, S. A., & Colquhoun, D. (1981) *Nature 289*, 596−598.
Oswald, R. E., Heidmann, T., & Changeux, J. P. (1983) *Biochemistry 22*, 3128−3136.
Peitsch, M. C., Amiguet, P., Guy, R., Brunner, J., Maizel, J. V., Jr., & Tschopp, J. (1990) *Mol. Immunol. 27*, 589−602.
Steffens, K., Hoppe, J., & Altendorf, K. (1988) *Eur. J. Biochem. 170*, 627−630.
Waksman, G., Changeux, J. P., & Roques, B. P. (1980) *Mol. Pharmacol. 18*, 20−27.
White, B. H., & Cohen, J. B. (1988) *Biochemistry 27*, 8741−8751.
White, B. H., & Cohen, J. B. (1992) *J. Biol. Chem. 267*, 15770−15783.
White, B. H., Howard, S., Cohen, S. G., & Cohen, J. B. (1991) *J. Biol. Chem. 266*, 21595−21607.
Wu, G., & Miller, K. W. (1994) *Biochemistry 33*, 9085−9091.
Wu, G., Tonner, P. H., & Miller, K. W. (1994) *Mol. Pharmacol. 45*, 102−108.