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Effects of Oxidizing and Reducing Analogs of Acetylcholine on Neuronal Nicotinic Receptors

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SUMMARY

The synthesis and pharmacological characterization of dithiobisacetylcholine and dithiobis-*N,N*-dimethyl-4-acetylpiperazinium (two oxidizing analogs of acetylcholine), as well as those of their reduced counterparts, are described. Both the oxidizing and reducing analogs stimulate nicotinic receptors in the chick retina and block the binding of ^{125}I-labeled neuronal bungarotoxin to retinal homogenates (IC_{50} values of 2×10^{-6} to 6×10^{-5} M). Both oxidizing compounds reverse the physiological effects of reduction by dithiothreitol on nicotinic function in intact chick retina, when applied for 2 sec (EC_{50} values of about 10^{-5} M). This effect is selective, insofar as neither agent alters the effects of dithiothreitol treatment on receptors for *N*-methyl-D-aspartate. Reoxidation takes place at the disulfide located near the nicotinic receptor agonist binding site, inasmuch as reoxidation by these agents prevents affinity alkylation by bromoacetylcholine, and occupation by the competitive antagonist *d*-tubocurarine prevents reoxidation. Unlike thiocholine, a weak agonist with a free sulfhydryl that, paradoxically, is reported to oxidize nicotinic receptors in electroplax, the reduced forms, mercaptoacetylcholine and *N,N*-dimethylamino-4-mercaptoacetylpiperazinium, have no direct redox effects on retinal receptors, but they do protect the receptors against reduction by dithiothreitol.

One highly conserved property of nicotinic receptors is that reduction by the reducing agent DTT of a disulfide bond near the agonist binding site causes a dramatic decrease in receptor responses to agonists (1) (reviewed in Ref. 2). Amino acid sequences established from cDNA clones of *Torpedo* nicotinic receptor subunits (3), and labeling by affinity alkylating agents (4–6) (reviewed in Ref. 7), determined that this disulfide is formed by two adjacent half-cystine residues located on the α subunit. Site-directed mutagenesis of the adjacent cysteines (Cys^{192} and Cys^{193}) leads to inactive *Torpedo* receptors, when expressed in oocytes (8). Subsequent cloning of neuronal nicotinic receptor subunits suggests that this disulfide is conserved in the α subunits of all known nicotinic receptor subtypes (9) (reviewed in Ref. 10). In addition, this receptor disulfide undergoes a conformational change involved in agonist binding. Thus, agonists, but not antagonists, protect the disulfide in the agonist site against reduction by DTT, in nicotinic receptors found in snail neurons (12) and *Torpedo* electroplax (11).

A disulfide formed between adjacent cysteine residues is expected to be a highly strained ring structure (6), which may explain the greater susceptibility of the agonist binding site disulfide to reduction with DTT (11). In spite of this, Karlin and Bartels (1) demonstrated that, once reduced, the disulfide can be reoxidized by the nonselective disulfide-containing reagent DTNB. However, not all disulfide-containing compounds reoxidize reduced receptors, even if they react at the agonist binding site. Recently, Czajkowski and Karlin (13) demonstrated that *S*-(2-[^{3}H]glycylamidoethyl)dithio-2-pyridine reacts with reduced *Torpedo* receptors to form a mixed disulfide intermediate that is stable for at least hours. Thus, disulfide-containing reagents vary tremendously in oxidizing capacity for reduced nicotinic receptors. Cholinedisulfide, which acts as an antagonist of electroplax receptors and is a disulfide-containing analog of hexamethonium, reoxidizes reduced nicotinic receptors found in *Electrophorus* (14) and snail neurons (12) with a potency 1000-fold higher than that of DTNB. However, the reduced form of cholinedisulfide, thiocholine, is expected to be released during the reaction of cholinedisulfide with reduced receptors. Thiocholine itself is a weak nicotinic agonist that, paradoxically, acts as a potent oxidizer of reduced nicotinic receptors, through a mechanism that is not understood (14). Aside from thiocholine, oxidizing agonists for reduced nicotinic receptors have not been described.

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ABBREVIATIONS: DTT, dithiothreitol; DTNB, dithiobisnitrobenzoic acid; DT-ACh, dithiobisacetylcholine; ACh-SH, mercaptoacetylcholine; DMAP, *N,N*-dimethyl-4-acetylpiperazinium; DT-DMAP, dithiobis-*N,N*-dimethyl-4-acetylpiperazinium; DMAP-SH, *N,N*-dimethyl-4-mercaptoacetylpiperazinium; NBT, neuronal bungarotoxin; BGT, α-bungarotoxin; DMPP, dimethylphenylpiperazinium; KA, kainic acid; NMDA, *N*-methyl-D-aspartic acid; DTC, *d*-tubocurarine; HEX, hexamethonium; BAC, bromoacetylcholine; m.p., melting point.

At present, no selective reducing agent exists that reduces only the agonist binding site disulfide of nicotinic receptors. Such a reagent could be very useful. In addition to the disulfide found on the α subunits near the agonist binding site, every subunit of every known nicotinic receptor subtype has cysteines homologous to residues 128 and 142 of the *Torpedo* α subunit (7). Site-directed mutagenesis studies (8) indicate that a disulfide between residues αCys^{128} and αCys^{142} in *Torpedo* (15) is also essential for proper functioning of nicotinic receptors. At concentrations of DTT commonly used to reduce the disulfide in the agonist binding site (>0.2 mM), the Cys^{128}-Cys^{142} disulfide could also be affected.

A close analog of acetylcholine, DT-ACh, has been mentioned in the literature (16), but neither its synthesis nor its actions as an oxidizing agent for reduced nicotinic receptors have been previously described. We synthesized DT-ACh, as well as an oxidizing analog of the rigid cholinergic agonist DMAP (17, 18), and investigated the pharmacological effects of these compounds, as well as those of their reduced forms, on nicotinic receptors. Preliminary reports of these results have appeared previously (19, 20).

Materials and Methods

Synthesis of DT-ACh and DT-DMAP. DT-ACh (see Fig. 1 for structure) was prepared by acylating choline bromide (386 mg, 2 mmol) with dithiobisglycolyl chloride (252 mg, 1.15 mmol) (21). The crude product was converted to the bisperchlorate, which was crystallized from H_2O/isopropanol to give analytically pure DT-ACh (75 mg; 14% yield; m.p., 137.5–139°).

To obtain DT-DMAP (see Fig. 1 for structure), a solution of *N*-methylpiperazine (4 ml, 36.5 mmol) in 40 ml of ether, stirred at 0°, was treated dropwise with an ethereal solution of dithiobisglycolyl chloride (2.0 g, 9.12 mmol). The crude product was chromatographed on an alumina column to give waxy orange-yellow crystals, which were crystallized from ether/ethanol to give the analytically pure bis-tertiary amine (1.32 g; 42% yield; m.p., 110–112°). The bis-tertiary amine was quaternized to the diiodo salt by treatment of its ethanol solution with an 8–10-fold excess of iodomethane at room temperature. The precipitate was collected by filtration, triturated with hot methanol, and dried *in vacuo* to give analytically pure DT-DMAP [460 mg; 73% yield; m.p., 230–232° (decomposed)].

Synthesis of ACh-SH and DMAP-SH. ACh-SH was made by reduction of DT-ACh with DTT. DTT (20 mM) was added to 10 mM DT-ACh, in a final volume of 1 ml, and was mixed well for 10 min.

To produce small amounts of DMAP-SH, 3 mg of $NaBH_4$ were added to 6.22 mg of DT-DMAP, in 0.73 ml of H_2O solution, and were mixed well for 5 min. Then, 1 N HCl was added to remove excess $NaBH_4$, until bubbles no longer formed after addition of HCl, and the solution was then neutralized with 1 N NaOH. The presence of free sulfhydryls was demonstrated by using DTNB in the Ellman assay (22). No evidence of free sulfhydryls was observed in a control solution that lacked DT-DMAP but contained $NaBH_4$ and was treated with HCl/NaOH.

DMAP-SH was also produced in sufficient quantity for elemental

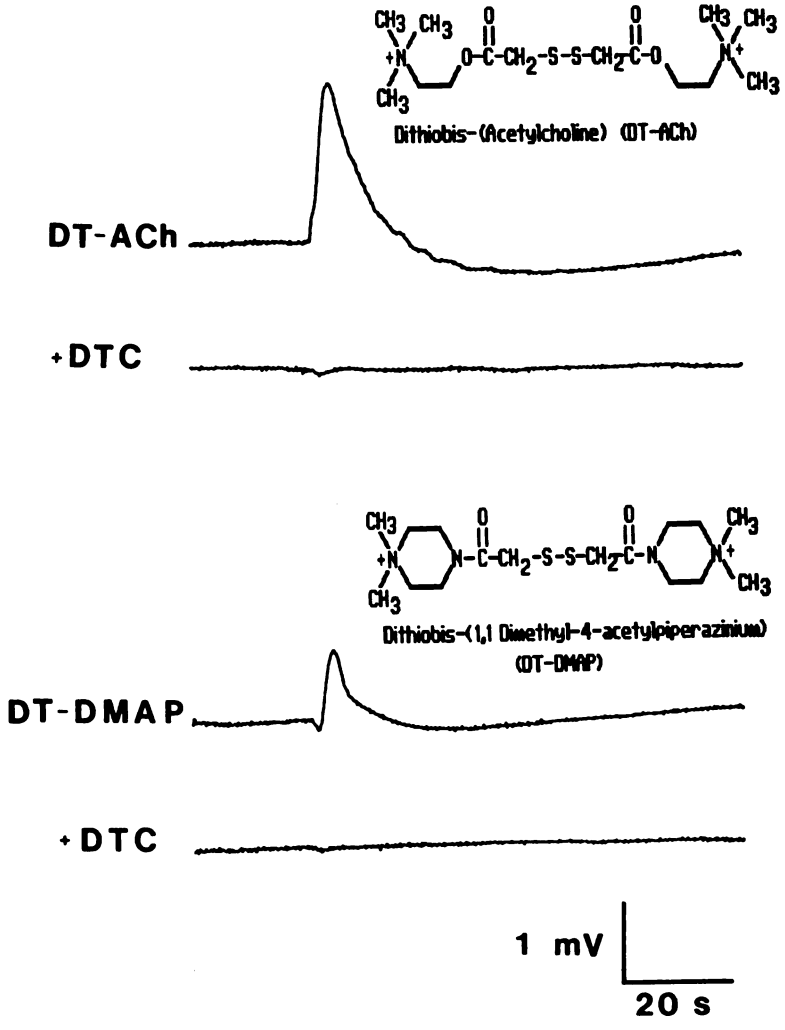

Fig. 1. DT-ACh and DT-DMAP are nicotinic agonists in chick retina. Both DT-ACh (0.5 mM) and DT-DMAP (3 mM) induce depolarizations in chick retina (*top traces*). Chemical structures are also shown. Depolarizations induced by DT-ACh and DT-DMAP are blocked by 100 μM DTC (*bottom traces*).

analysis, by reduction of DT-DMAP (150 mg, 0.24 mmol) with 2 mol equivalents of sodium borohydride in 4 ml of 50% aqueous ethanol. After 20 min, the reaction mixture was acidified with a few drops of HI, diluted with 5 volumes of acetone, and stored in the freezer. After 1 week, the solvent was decanted from a white crytalline precipitate, which was washed with ethanol and dried *in vacuo* [yield, 32 mg (20%); m.p., 243–245° (decomposed)]. The crystalline product gave a positive Ellman reaction.

Electrophysiological measurements. Electrophysiological measurements from the ganglion cell population were made from intact retinas of 1–14-day chicks (23). Briefly, DC potentials were recorded between a suction electrode placed over the cut optic nerve (consisting of axons from the retina ganglion cells) and a second electrode placed within the chick eyecup perfusion medium. Intact retinas were perfused with Tyrode's solution (130 mM NaCl, 3 mM KCl, 20 mM $NaHCO_3$, 17 mM dextrose, 0.01%, w/v, phenol red, bubbled with 95% O_2/5% CO_2) containing 7 mM $MgCl_2$ and 0.1 mM $CaCl_2$, to inhibit synaptic interactions. Agonists were applied via a solenoid valve system located near the eyecup (24). Retinas experienced a maximum agonist concentration for a period not exceeding 2 sec, and 5-min intervals between agonist applications were maintained, to minimize receptor desensitization.

Binding assays. Centrifugation assays for the binding of ^{125}I-labeled NBT, a snake venom neurotoxin that blocks certain subtypes of nicotinic receptors, were performed in quadruplicate (23). Briefly, retinal homogenates were incubated with ^{125}I-NBT (25), with or without competing nicotinic agonists, in a final volume of 100 μl, for 2 hr at room temperature. After centrifugation and washing, the radioactivity bound to the pellet was determined in a γ counter. ^{125}I-NBT binding to a site of unknown function, shared with BGT, was blocked in the presence of 1 μM BGT in all binding assays. Nonspecific binding was determined by incubation with 1 μM NBT before addition of ^{125}I-NBT. IC_{50} values were calculated using GraphPAD InPlot (GraphPAD Software, San Diego, CA). Previous studies with chick retina homogenate (23) indicated that ^{125}I-NBT binding reaches equilibrium by 2 hr at room temperature, with an apparent K_d of 2–3 nM, and that the dissociation of ^{125}I-NBT is complex; 60% of the binding dissocates within 1 hr at room temperature and the remainder dissociates with a $t_{1/2}$ of >10.5 hr. Furthermore, a good correlation was observed between the concentrations of agonists necessary to desensitize 50% of the functional responses in chick retina and the IC_{50} values for agonist blockade of ^{125}I-NBT binding (26). In contrast, the concentrations of agonists necessary to produce 50% of maximum depolarizations were 30-fold higher than the IC_{50} for ^{125}I-NBT binding. These data suggest that the IC_{50} values for ^{125}I-NBT binding correlate most closely with the binding of agonists to desensitized receptors.

For observation of reoxidation effects on ^{125}I-NBT binding to nicotinic receptors, chick retina homogenates were treated with 2 mM DTT for 20 min at room temperature. The homogenate was centrifuged (14,000 × *g*) and washed twice with 1 ml of homogenization buffer. The pellet was resuspended with varying concentrations of DT-ACH or DT-DMAP (5 min). After centrifugation and washing three times, to remove any bound agonist, the resuspended pellet was incubated for 20 min with 100 μM BAC and 2 μM neostigmine. After washing an additional three times, the resuspended and sonicated pellet was assayed for ^{125}I-NBT binding as outlined above. Control experiments determined that the washing conditions used were sufficient to remove noncovalently bound agonists under these conditions (data not shown).

Results

Applications of DT-ACh (0.5 mM) and DT-DMAP (3 mM) both produced depolarizations in chick retina (Fig. 1, *top traces*). These responses were blocked by incubations with nicotinic antagonists, such as DTC (100 μM) (Fig. 1, *bottom traces*) and 100 μM HEX (data not shown). DT-ACh was both more potent and more efficacious than DT-DMAP as an agonist of nicotinic receptors in chick retina (data not shown).

DMAP-SH was approximately 30-fold more potent in producing depolarizations than was the oxidized parent compound, DT-DMAP (data not shown). The mild reducing conditions used to produce ACh-SH were necessary because of the labile ester group. Several studies were performed to confirm the production of ACh-SH from DT-ACh. ACh-SH is similar in structure to acetylcholine and is readily hydrolyzed by acetylcholinesterase (Table 1), whereas DT-ACh is largely resistant to cholinesterases in the retina. However, in the presence of neostigmine (10^{-6} M), ACh-SH was more efficacious than DT-ACh (Table 1; other data not shown). ACh-SH and DMAP-SH induced nicotinic depolarizations that were blocked by DTC (100 μM) (data not shown).

All four compounds displaced ^{125}I-NBT binding to chick retinal homogenates (Fig. 2). The rank order of potency was DT-ACh > DT-DMAP > DMAP-SH > ACh-SH. In several repetitions of these assays, the IC_{50} values were 1.97 ± 0.30 × 10^{-6} M for DT-ACh, 5.71 ± 0.04 × 10^{-5} M for ACh-SH (four experiments each), 5.04 ± 0.07 × 10^{-6} M for DT-DMAP, and 1.58 ± 0.07 × 10^{-5} M for DMAP-SH (three experiments each).

One feature of all known nicotinic receptors is that reduction by DTT of an invariant disulfide bond near the agonist binding site substantially decreases the responsiveness of receptor to

TABLE 1

Neostigmine protects ACh-SH from cholinesterases

Errors represent the standard deviation (at least three recordings).

Agonist	Depolarization	
	Control	With neostigmine (10^{-6} M)
M	*mV*	
KA (1×10^{-4})	1.91 ± 0.11	2.02 ± 0.24
DMPP (3×10^{-4})	1.84 ± 0.16	1.88 ± 0.21
DT-ACh (3×10^{-4})	1.24 ± 0.18	1.20 ± 0.22
ACh-SH (3×10^{-4})[a]	0.37 ± 0.05	1.70 ± 0.24[b]

[a] Concentration assumes quantitative reduction of DT-ACh.
[b] $p < 0.01$ (*t* test), comparing control responses with those with neostigmine.

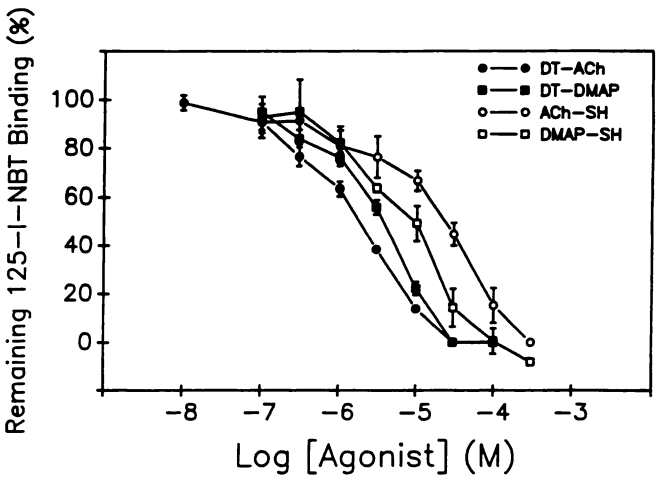

Fig. 2. DT-ACh, DT-DMAP, and their corresponding reduced agonists displace ^{125}I-NBT binding from chick retinal homogenates. Varying concentrations of DT-ACh (●), DT-DMAP (■), ACh-SH (○), and DMAP-SH (□) displace ^{125}I-NBT (5 nM) binding from chick retinal homogenates (values represent the mean ± standard deviation, four experiments). In several repetitions of this experiment, the IC_{50} values were 1.97 ± 0.30 × 10^{-6} M for DT-ACh, 5.71 ± 0.04 × 10^{-5} M for ACh-SH (four experiments each), 5.04 ± 0.07 × 10^{-6} M for DT-DMAP, and 1.58 ± 0.07 × 10^{-5} M for DMAP-SH (three experiments each).

agonists (1, 12, 23, 26). It has also been shown that DTT enhances the response of NMDA receptors (27, 28). A 20-min perfusion with 2 mM DTT in chick retina shifted the NMDA dose-response curve to the left (Fig. 3, *upper*), whereas the same DTT treatment shifted the dose-response curve of the nicotinic agonist DMPP to the right and may also have affected the efficacy of DMPP (Fig. 3, *lower*). Reducing effects of DTT on both DMPP and NMDA responses could be reversed by the nonselective oxidizing agent DTNB (1 mM, 5 min) (data not shown). Thus, changes in the DMPP and NMDA dose-response curves can be used as an index of the selectivity of oxidizing and reducing agents in chick retinas.

DT-ACh and DT-DMAP selectively reoxidized DTT-treated chick retinas (e.g., Fig. 4). KA (100 μM) and NMDA (300 μM) (analogs of excitatory amino acids), as well as 300 μM DMPP, induced depolarizations in intact chick retina under control conditions (Fig. 4, *Control*). The KA responses were a measure of the responsiveness of the retina preparation and were shown previously to be unaffected by redox reagents (23). After 20-min treatment with 2 mM DTT, the KA responses remained unchanged, the DMPP responses were substantially diminished (18 ± 17% of control; 12 experiments), and the NMDA response was enhanced (290 ± 90%; 12 experiments; e.g., Fig. 4, *DTT*). After a single 2-sec application of 3 mM DT-DMAP, the DMPP responses were restored to control levels, whereas NMDA responses were unchanged (Fig. 4, *DT-DMAP*). Treatment with the oxidizing agent DTNB (1 mM, 5 min) returned the NMDA response to control level but did not cause further oxidation of nicotinic receptors (Fig. 4, *DTNB*). Similar results were observed in other experiments with 3 mM DT-DMAP (recovery = 94 ± 8%; three experiments) or after a single 2-sec application of 1 mM DT-ACh to DTT-treated chick retina (recovery = 91 ± 8%; three experiments; data not shown). By varying concentrations of the oxidizing agonists applied for 2 sec and then treating with DTT between applications, to reestablish the basal DMPP response after reduction, the EC_{50} for reoxidation by DT-ACh and DT-DMAP could be determined (data not shown). Both DT-ACh and DT-DMAP were rapid, potent, and selective oxidizing agonists of DTT-treated nicotinic receptors, with EC_{50} values of 30 and 20 μM (when applied for only 2 sec), respectively.

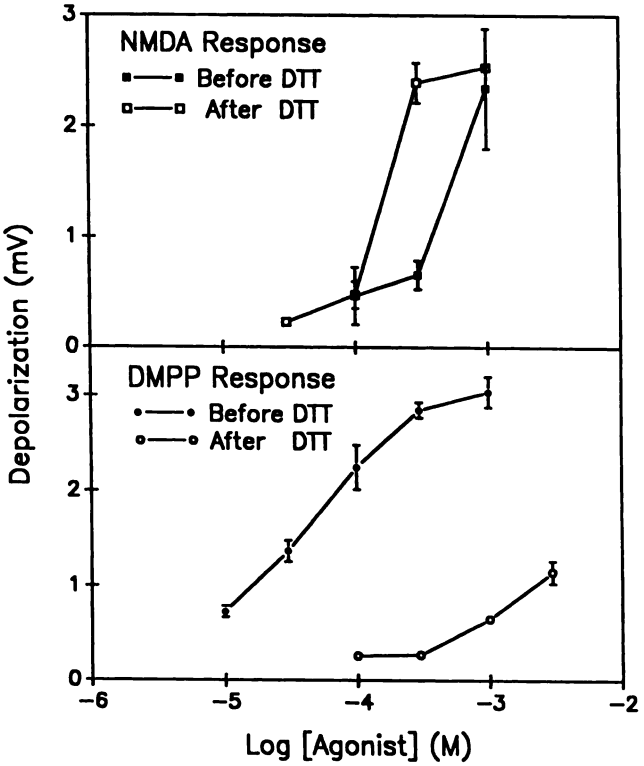

Fig. 3. Effects of DTT on the dose-response curves of NMDA and DMPP in chick retina. *Upper*, DTT (2 mM, 20 min) shifts the dose-response curve of NMDA (before DTT) (■) to the left (after DTT) (□); *lower*, DTT (2 mM, 20 min) shifts the dose-response curve of DMPP (before DTT) (●) to the right (after DTT) (○). DTT may also affect the efficacy of DMPP, but a full dose-response curve after DTT is not technically feasible. The data represent the mean ± standard deviation of three agonist applications.

Because DTT-treated receptors are largely inactivated, the ability of DT-ACh or DT-DMAP to reactivate these receptors may be a useful electrophysiological measure of the occupancy of the reduced agonist binding site by other compounds (Fig. 5). To test this, we compared the ability of DTC and HEX to protect against reoxidation. DTC displaces ^{125}I-NBT binding in chick retina, whereas HEX does not (23). In addition, DTC (data not shown) and dihydro-β-erythroidine displace the DMPP-induced dose-response curves for chick retina depolarizations rightward, in a competitive manner, whereas HEX shifts the dose-response curves downward, in a noncompetitive manner (24). These results suggest that DTC is a competitive inhibitor in chick retina, whereas HEX is a noncompetitive antagonist. Indeed, HEX has been suggested by others to be a nicotinic receptor channel blocker (29, 30). DTT (2 mM, 20 min) substantially diminished DMPP responses, relative to the control values (Fig. 5, control versus DTT groups). A single 2-sec application of 300 μM DT-ACh in the presence of 300 μM DTC did not restore subsequent DMPP responses (Fig. 5, DTC plus DT-ACh group). Control experiments established that a 10-min wash period was sufficient to remove DTC and DT-ACh before testing of recovery of receptor function. However, DT-ACh (300 μM, 2 sec) alone restored the DMPP responses substantially (Fig. 5, DT-ACh group; recovery = 75 ± 10%; six experiments). Treatment with DTT (2 mM, 20 min) again diminished the DMPP responses. In contrast, after application of 300 μM DT-ACh in the presence of 500 μM HEX, the subsequent DMPP responses were restored (Fig. 5, HEX plus DT-ACh group) to the levels seen after reoxidation by DT-ACh alone. The KA responses remained unchanged throughout the experiment. Control studies demonstrated that both DTC (100 μM) and HEX (500 μM) completely abolished DMPP responses (data not shown). Thus, these results indicate that reoxidation by DT-ACh can be prevented by DTC but not by HEX.

BAC irreversibly blocks *Torpedo* nicotinic receptors after DTT treatment. This is believed to occur by alkylation of the reduced disulfide bond found in or near the agonist binding site (4, 11), thus irreversibly inhibiting the binding of ^{125}I-BGT to *Torpedo* membranes. Similarly, it has been shown previously that BAC (with neostigmine) irreversibly blocks nicotinic receptor functions in DTT-treated chick retina and also irreversibly blocks ^{125}I-NBT binding to DTT-treated retinal homogenates (26), with the IC_{50} values for block of physiological function and toxin binding being equal. DTT-treated chick retina homogenate was incubated with 10 nM ^{125}I-NBT (with 1 μM BGT) either without any further treatment (Fig. 6, reduced control), after treatment with 100 μM BAC in the presence of

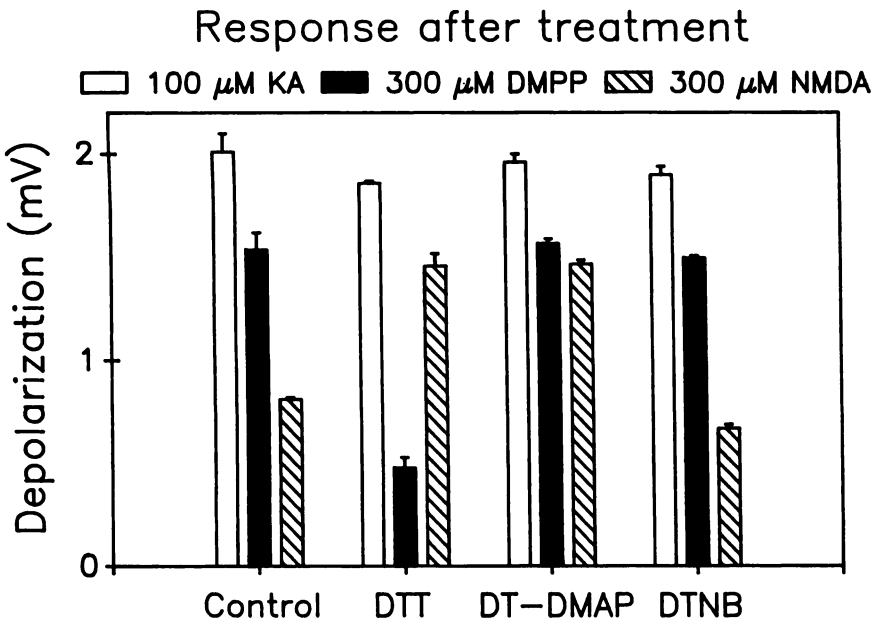

Fig. 4. Reoxidation of DTT-treated chick retina by DT-ACh is selective for nicotinic receptors. Responses (three experiments) were obtained for 2-sec applications of KA (100 μM) (□), DMPP (300 μM) (■), and NMDA (300 μM) (▨) (*Control*). After a 20-min treatment with 2 mM DTT and then washing, the KA responses remain unchanged, the DMPP responses are substantially diminished, and the NMDA responses are enhanced (*DTT*). After a single 2-sec application of DT-DMAP (3 mM), the DMPP responses are restored to the control levels, but the NMDA responses and the KA responses are unaffected (*DT-DMAP*). Treatment with DTNB (1 mM, 5 min), followed by washout, returns the NMDA responses to control values and has no effect on the DMPP responses (*DTNB*).

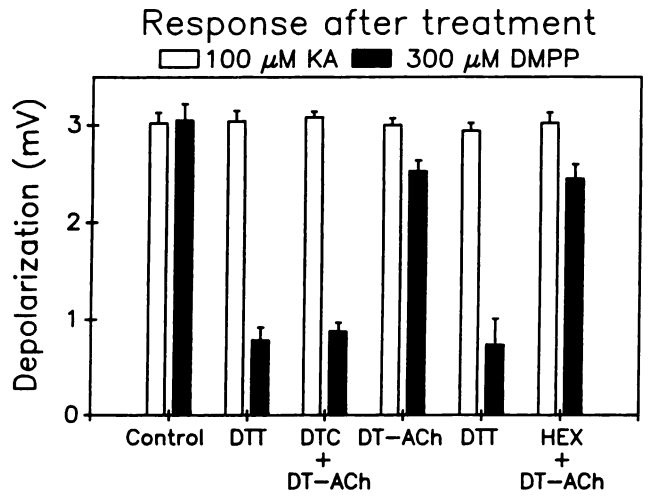

Fig. 5. DTC protects against reoxidation by DT-ACh in DTT-treated chick retina, whereas HEX does not. Responses to 100 μM KA (□) and 300 μM DMPP (■) are shown (*Control*). After the treatment with DTT (2 mM, 20 min), the DMPP responses are substantially decreased (*left DTT*). When 300 μM DT-ACh is given for 2 sec in the presence of 300 μM DTC, followed by washout of both drugs, there is no recovery of DMPP responses (*DTC + DT-ACh*). However, when 300 μM DT-ACh is applied for 2 sec in the absence of DTC, the DMPP responses are substantially recovered (*DT-ACh*). Treatment with DTT (2 mM, 20 min) again decreases the DMPP responses, while leaving the KA responses unchanged (*right DTT*). After application of 300 μM DT-ACh (2 sec) in the presence of 500 μM HEX, followed by washout of the drugs, the DMPP responses are substantially restored (*HEX + DT-ACh*) to the values previously observed after reoxidation by DT-ACh alone (*DT-ACh*).

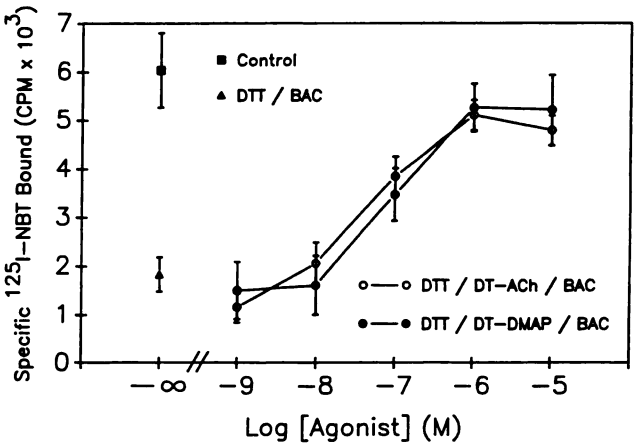

Fig. 6. DT-ACh and DT-DMAP protect against BAC alkylation of DTT-treated chick retinal homogenates. The details of this experiment are given in Materials and Methods. Briefly, homogenates of DTT-treated chick retinas were incubated with ^{125}I-NBT (10 nM) and 1 μM BGT, either in the absence of any further treatment (■), after treatment with 1 mM BAC (▲), or after a 5-min treatment with varying concentrations of either DT-ACh (○) or DT-DMAP (●) before the treatment with BAC.

2 μM neostigmine (Fig. 6, DTT/BAC), or after treatment with varying concentrations of DT-ACh or DT-DMAP (5 min) before treatment with BAC (Fig. 6, DTT/DT-ACh/BAC or DTT/DT-DMAP/BAC). Both DT-ACh and DT-DMAP reoxidized the DTT-reduced receptors and prevented alkylation with BAC, with EC_{50} values (5-min application) of approximately 10^{-7} M.

Having established that dithiobis analogs of acetylcholine are selective reoxidizing agents for reduced receptors in chick retina, we next investigated whether the thiol analogs have any redox effects. Gumulka and Kostkowski (31) reported that ACh-SH inactivated both muscle and neuronal nicotinic receptors, but the description was unclear as to whether this was a redox effect or simple desensitization. In contrast, Bregestovsky *et al.* (12) demonstrated that agonists, but not antagonists, protected the disulfide bond located in the nicotinic agonist binding site of cholinoceptive snail neurons against reduction by DTT. Similar results were reported for *Torpedo* electroplax (11). Therefore, studies were undertaken to determine whether ACh-SH and DMAP-SH either protect retinal receptors against reduction with DTT or reduce the receptors themselves. However, because of notable pharmacological differences between the neuronal nicotinic receptors in chick retina and invertebrate neuronal and vertebrate muscle receptors (e.g., sensitivity to BGT), control studies were carried out to determine whether agonists, but not antagonists, also protect against DTT reduction of neuronal nicotinic receptors in chick retina.

Table 2 indicates the protection afforded against reduction by DTT by desensitizing doses of five agonists and compares that with the protection afforded by five blocking doses of competitive antagonists. A 5–20-min perfusion with the indicated doses of agonists and antagonists blocked the responses to 2-sec test pulses of 300 μM DMPP by 84 ± 11% (22 different drug applications). The concentrations of agonists and antagonists were chosen so that the percentage of nicotinic blockade was comparable (83 ± 5% block for five agonists versus 88 ± 15% block for five antagonists, all before the addition of DTT). DTT (2 mM) was then applied with the blocking drug for 20 min, and then the DTT and the blocking drug were washed out for a period of 30–50 min. At that point, a maximum recovery of the responses to DMPP test pulses was obtained, and the DMPP response remaining was recorded as a percentage of the initial DMPP responses. The lowest level of protection observed with agonist was 77% for 10 μM DMPP, and the highest was 93% for 30 μM acetylcholine (with 1 μM neostigmine). In contrast, no significant protection was observed for antagonists; the remaining DMPP response after DTT alone was 18 ± 17% (12 experiments), whereas responses after applications of DTT plus antagonists ranged from 6% for 300 μM pancuronium to 17% for 100 μM trimethaphan. Thus, the phenomenon reported by Bregestovsky *et al.* (12) also holds for chick retina receptors. Similarly, ACh-SH (30 μM, with 1 μM neostigmine) and DMAP-SH (300 μM) protected nicotinically mediated DMPP responses from the reduction by DTT (Table 2), and we have no evidence that these agents have any reducing effect on chick neuronal receptors.

TABLE 2

Reducing and nonreducing nicotinic agonists protect against DTT reduction, unlike antagonists

After determination of control responses to test DMPP applications (2 sec, 300 μM), agonists or antagonists were applied at the indicated concentrations, for 5–20 min, before testing for blockade of test DMPP responses (before DTT). After determination of the maximum percentage blockade in the presence of an agonist or antagonist, 2 mM DTT was applied with the drug for 20 min, and then both were washed out for a period of at least 30 min, when the maximum response to test DMPP applications was recorded (percentage of remaining DMPP response after DTT). Errors represent the standard deviation for $n \geq 3$ or the range for $n = 2$. n, number of experiments.

Treatment	Concentration	Remaining DMPP response (after DTT)
	M	%
DTT + agonist[a]		
Nicotine	1×10^{-5}	83 ± 1 ($n = 2$)
Cytisine	1×10^{-5}	81 ± 2 ($n = 3$)
DMPP	1×10^{-5}	77 ± 6 ($n = 3$)
Acetylcholine[b]	3×10^{-5}	93 ± 6 ($n = 2$)
Carbamylcholine	1×10^{-4}	80 ± 8 ($n = 3$)
DTT + antagonist[c]		
Dihydro-β-erythroidine	1×10^{-4}	7 ± 3 ($n = 3$)
Trimethaphan	1×10^{-4}	17 ± 4 ($n = 2$)
DTC	1×10^{-4}	8 ± 8 ($n = 3$)
Pancuronium	3×10^{-4}	6 ± 1 ($n = 2$)
Benzoquinonium	1×10^{-3}	7 ± 0 ($n = 2$)
DTT alone	2×10^{-3}	18 ± 17 ($n = 12$)
DTT + reducing agonist		
ACh-SH[b, d]	3×10^{-5}	88 ± 8 ($n = 3$)
DMAP-SH	3×10^{-4}	83 ± 12 ($n = 3$)

[a] The average blockade by the five agonists before DTT application was 83 ± 5%.
[b] Determined in the presence of 10^{-6} M neostigmine.
[c] The average blockade by the five antagonists before DTT application was 88 ± 15%.
[d] Concentration assumes quantitative reduction of DT-ACh.

Bartels *et al.* (14) reported that some sulfhydryl-containing reagents, such as cysteine or thiocholine ($EC_{50} = 10^{-6}$ M at 5 min), reoxidize DTT-treated nicotinic receptors from *Electrophorus* electroplax, through an unknown mechanism. Therefore, the ability of ACh-SH and DMAP-SH to reoxidize DTT-reduced receptors in chick retina was determined. Because ACh-SH and DMAP-SH can protect against reduction with DTT (see above), 2 mM DTT was included with the reducing agonists, to prevent autooxidation into the correspondingly more potent oxidizing agonists. When 2×10^{-4} M ACh-SH or 3×10^{-4} M DMAP-SH was applied (5 min), in the presence of DTT, to DTT-treated chick retinas, no evidence for significant restoration of the nicotinic responses was observed (e.g., Fig. 7; three experiments). Thus, we have no evidence for reoxidation by ACh-SH or DMAP-SH of reduced chick neuronal receptors, under these conditions.

Discussion

Both DT-ACh and DT-DMAP act to oxidize DTT-reduced nicotinic receptors in chick retina. The oxidizing potency of the two compounds is approximately equal (Fig. 6), in spite of DT-ACh being a more potent and efficacious agonist than DT-DMAP (e.g., Fig. 1). These data suggest that reoxidation by

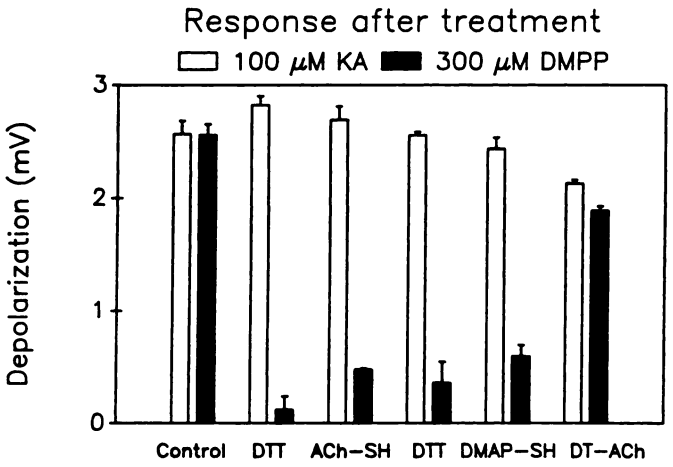

Fig. 7. ACh-SH and DMAP-SH do not act as oxidizing agents for reduced nicotinic receptors in chick retina. Under control conditions, 2-sec applications of either 10^{-4} M KA (□) or 3×10^{-4} M DMPP (■) give similar depolarizing responses (*Control*). After 20-min treatment with 2 mM DTT, the DMPP responses are markedly decreased (*left DTT*). Five-minute incubation with 2×10^{-4} M ACh-SH in 2 mM DTT does not cause a marked increase in subsequent DMPP responses (*ACh-SH*). Treatment again with 2 mM DTT for 20 min does not cause any marked change in the DMPP response (*right DTT*). Further incubation with DMAP-SH (3×10^{-4} M; 5 min in 2 mM DTT) also does not cause a significant increase in the DMPP response (*DMAP-SH*). In contrast, a 2-sec application of 3×10^{-4} M DT-ACh substantially increases subsequent responses of DMPP (*DT-ACh*).

oxidizing agonists is independent of receptor activation. The effects of these oxidizing agonists do appear to be specific for nicotinic receptors, insofar as these agents do not reoxidize DTT-treated NMDA receptors (Fig. 4). Reoxidation occurs at or near the agonist binding site, because occupation by the competitive antagonist DTC prevents reoxidation (Fig. 5) and reoxidation prevents alkylation by the selective affinity reagent BAC (Fig. 6).

The concentration dependence of reoxidation by DT-DMAP or DT-ACh in chick retina is comparable to that reported for cholinedisulfide or homocholinedisulfide (14) at DTT-treated *Electrophorus* electroplax. Those two agents act as competitive antagonists for oxidized receptors (but agonists for reduced receptors) and reoxidize *Electrophorus* receptors with EC_{50} values of about 10^{-7} M, when applied for 5 min. The EC_{50} values for reoxidation by DT-ACh and DT-DMAP in chick retina are also about 10^{-7} M when compounds are applied for 5 min to ^{125}I-NBT binding sites (Fig. 6) but are about 3×10^{-5} M when compounds are applied for 2 sec to intact chick retina (data not shown). This apparent linear increase in potency with time suggests that the agonist binding sites of each reduced receptor must be occupied and reoxidized only once in order to restore normal function to the receptor.

One caveat is that the physiological assay in intact retina potentially activates several subtypes of neuronal nicotinic receptors, whereas the ^{125}I-NBT binding assay may measure redox effects on only one or a few subtypes, possibly making the results of these two assays noncomparable. Northern blot analysis (32) and immunological evidence (33) suggest that the chick retina contains receptors with at least α_3, α_4, and non-α_1 (β_2) subunits. NBT is believed to block α_3-containing receptors in chick ciliary ganglion (34) but not chick α_4-containing receptors expressed in oocytes (35). NBT blocks only 50–90% of the functional receptors in intact retina (23), suggesting heterogeneity in the retinal receptors. However, if this is so, then DT-DMAP and DT-ACh may reoxidize multiple subtypes of receptors in chick retina. The specificity (or lack of it) of DT-ACh and DT-DMAP for defined subtypes of neuronal nicotinic receptors remains to be determined. However, DT-ACh readily reoxidizes receptors from *Torpedo* electroplax,[1] suggesting that DT-ACh, at least, has fairly low selectivity between nicotinic receptor subtypes.

Reoxidation of reduced receptors by DT-DMAP and DT-ACh is proposed to mimic the mechanism of DTNB. DTNB probably reoxidizes by forming a mixed disulfide, followed by an internal thiol-disulfide exchange to yield two molecules of 2-nitro-5-mercaptobenzoic acid and the original receptor disulfide (1) (reaction, RSSR + R′SH → R′SSR + RSH; $R'SSR \xrightarrow{R'SH} R'SSR' + RSH$, where RSSR is DTNB or other disulfides and R′ is one of the cysteinyl residues of the receptor). Not all disulfides reoxidize DTT-reduced receptors in this manner; Czajkowski and Karlin (13) recently demonstrated that *S*-(2-[^{3}H]glycylamidoethyl)dithio-2-pyridine reacts with reduced *Torpedo* receptors to form a stable mixed disulfide. Also, the mechanism proposed above has not yet been demonstrated for any of those disulfides that do oxidize the receptor. If DT-ACh, DT-DMAP, or cholinedisulfide reacts with sufficiently large concentrations of reduced *Torpedo* receptors, it may be possible to demonstrate production of stoichiometric amounts of ACh-SH, DMAP-SH, or thiocholine, to confirm the proposed mechanism (i.e., 2 mol of product/mol of reduced receptor). As a control, alkylation with BAC or other selective agents should block the formation of the reduced compounds to the same extent that the receptor is alkylated.

We did not observe any oxidizing effects of ACh-SH or DMAP-SH similar to those reported for thiocholine (14). Because the medium was bubbled with 95% O_2/5% CO_2, we were very concerned about reoxidation of the reduced agonists. Given the relative potencies and concentrations used, the conversion of <1% of reduced agonist to the oxidizing form could potentially have given a false positive response. Control studies with agonists (Table 2) suggest that, under our conditions, the receptor disulfide should have been protected against reduction by DTT, if the disulfide had been formed by the action of DMAP-SH or ACH-SH. However, the possibility remains that DTT somehow interferes with the unexplained mechanism of reoxidation by free thiols. For instance, it is possible that thiocholine reacts with oxygen to form a sulfenic acid intermediate, which then oxidizes the reduced receptor to form a mixed disulfide (36), which then eliminates thiocholine through a thiol-disulfide exchange to generate the oxidized disulfide in the agonist binding site (reaction, RSH + [O] → [RSOH] $\xrightarrow{R'SH}$ R′SSR + H_2O; $R'SSR \xrightarrow{R'SH} R'SSR' + RSH$, where RSH is thiocholine or other thiols and R′ is a cysteinyl residue of the receptor). The addition of DTT could block such a mechanism by reacting with any sulfenic acid intermediates.

In summary, DT-ACh and DT-DMAP are potent oxidizers of reduced nicotinic receptors, virtually equipotent with oxidizing concentrations of choline disulfide (12, 14). However, choline disulfide is an antagonist, which precludes its use for directly monitoring the physiological responsiveness of nicotinic receptors. The product of choline disulfide upon oxidation of reduced receptors is expected to be thiocholine, which is reported to be a weak nicotinic agonist that, paradoxically, has some oxidizing capacity for reduced receptors (14). In contrast, DT-ACh and DT-DMAP are both agonists, and their proposed products upon oxidation are also agonists, with no redox effects, except the ability to protect nicotinic receptors against reduction with DTT, a property common to all agonists.

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