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[^{3}H]Acetylcholine Binding Sites in Brain

Effect of Disulfide Bond Modification

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SUMMARY

Nicotinic cholinergic receptor recognition sites have been measured in rat brain using [^{3}H]acetylcholine. Modification of these sites *in vitro* with the disulfide bond reducing agent, dithiothreitol, resulted in a decrease in the density (B_{max}) of [^{3}H]acetylcholine binding sites while the affinity of these sites was unaltered. Reoxidation of the reduced sites with 5,5′-dithiobis(2-nitrobenzoic acid) reversed the effects of dithiothreitol. The reoxidation effect was partially prevented by *p*-chloromercuribenzoic acid, which generates thiol complexes with exposed sulfhydryl groups. Reduction of disulfide bonds had no effect on the ability of nicotinic cholinergic agonists or antagonists to compete for the remaining [^{3}H]acetylcholine binding sites. In addition, pretreatment of cortical homogenates with acetylcholine or nicotine did not alter the effects of dithiothreitol on [^{3}H] acetylcholine binding, suggesting that the disulfide bonds which are critical for [^{3}H] acetylcholine binding are not located directly at the recognition site.

INTRODUCTION

[^{3}H]Acetylcholine binds to a site that has characteristics of a nicotinic cholinergic receptor recognition site in rat brain membranes (1). This site has high affinity (1–12 nM) for cholinergic agonists such as nicotine, cytisine, and carbachol, but relatively low affinity for antagonists of peripheral nicotinic cholinergic receptors (1). The pharmacological characteristics of the binding site suggest that the brain receptor may differ from either of the two types of peripheral nicotinic cholinergic receptors found in ganglia and skeletal muscle. In addition, both the pharmacological characteristics and the brain regional distribution of the [^{3}H]acetylcholine binding site suggest that it is distinct from the α-bungarotoxin binding site in brain (1).

In the electroplax and in skeletal muscle, modifications of disulfide bonds or sulfhydryl groups with dithiothreitol or with PCMB[2] decrease the potency of agonists at nicotinic cholinergic receptors (2–5). Dithiothreitol has also been reported to decrease synaptic transmission at frog sympathetic ganglia (6). Karlin (7) has concluded that reducible disulfide bonds near acetylcholine recognition sites may be commonly associated with nicotinic acetylcholine receptors from a variety of tissues. In rat brain homogenates, modification of disulfide bonds or sulfhydryl groups does not alter the binding of [^{3}H]α-bungarotoxin, a peripheral nicotinic cholinergic receptor antagonist, but does decrease the potency of agonists in competing for the binding of the radiolabeled antagonist (8).

We have used [^{3}H]acetylcholine to label nicotinic cholinergic binding sites in rat brain membranes and to study directly the effects of modifications of disulfide bonds and sulfhydryl groups on the receptor recognition site for agonists. When disulfide bonds are reduced to sulfhydryl groups, there is an apparent decrease in the number of binding sites. This effect is at least partially reversed by reoxidation of sulfhydryl groups. The data presented here thus indicate that a disulfide bond is essential for acetylcholine to bind to the membrane recognition site.

MATERIALS AND METHODS

In vitro modifications. Cerebral cortex from male Sprague-Dawley rats was homogenized in 50 mM Tris-HCl buffer (pH 8.5, 0°) containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 1.5 μM atropine sulfate using a Brinkmann Polytron. The homogenates were pretreated with dithiothreitol to reduce disulfide bonds, with PCMB to form thiol complexes with sulfhydryl groups, or with DTNB to reoxidize sulfhydryl groups. In each case, the homogenates were preincubated with the

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[2] The abbreviations used are: PCMB, *p*-chloromercuribenzoic acid; DFP, diisopropyl fluorophosphate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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modifying agent for 30 min at 0° and pH 8.5. The preincubation was terminated by dilution with cold buffer and washing by centrifugation at 49,000 × *g* for 10 min. The pellets were either resuspended in buffer at pH 8.5 and preincubated with another modifying agent or resuspended in buffer at pH 7.4 at 0° and prepared for the assay of [^{3}H] acetylcholine binding sites. In all cases, control tissues were carried through the same number of preincubation procedures under similar conditions but without modifying agents.

Binding assays. The [^{3}H]acetylcholine binding assay was carried out as previously described (1). Briefly stated, the tissues were homogenized with a Brinkmann Polytron in 50 mM Tris-HCl buffer containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 1.5 μM atropine sulfate (pH 7.4 at 0°). The homogenates were centrifuged twice at 49,000 × *g* for 10 min with intermediate washes with buffer. A cholinesterase inhibitor, DFP (100 μM), was added to the final volume of homogenate prior to adding the tissue to the assay tube. Aliquots of tissue (5–10 mg) were added in sextuplicate to assay tubes containing the indicated concentration of [^{3}H]acetylcholine and incubated for 40 min at 0°. One-half of the tubes contained 100 μM carbachol for the determination of nonspecific binding. The reaction was stopped by rapid filtration over Whatman GF/C filters which had been treated with 0.05% polyethyleneimine to eliminate displaceable binding to the filters. The filters were placed in Liquiscint scintillation cocktail and counted in a Searle Mark III liquid scintillation counter at approximately 40% efficiency. Specific binding was defined as the difference between total and nonspecific binding. Under these conditions, specific binding of 10 nM [^{3}H]acetylcholine to 10 mg of cerebral cortex (equivalent to 650 μg of protein) is approximately 3000 dpm and represents 60%–70% of total binding.

[^{3}H]Acetylcholine of high specific activity (80 Ci/mmole) was synthesized by acetylation of [^{3}H]choline (80 Ci/mmole; New England Nuclear Corporation) as described previously (1). All other drugs and reagents were obtained from commercial sources.

Statistics. Differences between groups were analyzed statistically by Student's *t*-test or by Duncan's new multiple-range test when more than two groups were compared.

RESULTS

In vitro modifications of [^{3}H]acetylcholine binding sites. Preincubation of cerebral cortical homogenates with dithiothreitol decreased specific binding of [^{3}H]acetylcholine. The decrease was due to a loss in specific binding; nonspecific binding was unaltered. The IC_{50} for reduction of binding by dithiothreitol preincubation was approximately 500 μM. Saturation analyses by Scatchard plots (9) indicated that the decrease in binding was attributable entirely to an apparent decrease in the number of binding sites (B_{max}) without alterations in the affinity of the sites (K_D) for [^{3}H]acetylcholine (Fig. 1).

The most prominent chemical effect of dithiothreitol is reduction of disulfide bonds to sulfhydryl groups. If the presence of disulfide bonds is critical to the binding of [^{3}H]acetylcholine, then reoxidation of the sulfhydryl groups should restore binding sites. Preincubation with the oxidizing agent DTNB alone had no effect on [^{3}H] acetylcholine binding (Table 1). However, the decrease in binding following dithiothreitol pretreatment could be reversed substantially by subsequent treatment with 1 mM DTNB (Table 1). This effect was due to a restoration of the number of binding sites by DTNB (data not shown). When 1 mM dithiothreitol was added to the [^{3}H] acetylcholine binding assay without prior incubation there was no change in binding, indicating the importance of the preincubation conditions (pH 8.5 at 0°). This requirement for an alkaline pH during the modification of [^{3}H]acetylcholine binding sites in brain is similar to

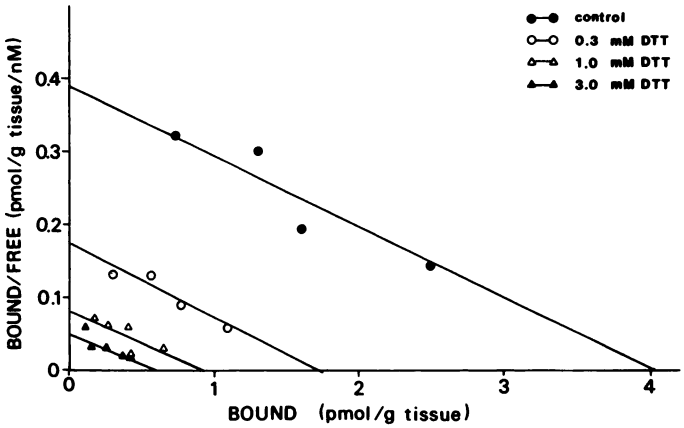

FIG. 1. *Scatchard analysis of [^{3}H]acetylcholine binding in dithiothreitol-treated cerebral cortex*

Cortical homogenates were incubated with 0.3, 1.0, or 3.0 mM dithiothreitol (*DTT*) for 30 min at 0° (pH 8.5). The reactions were terminated by dilution with cold buffer and centrifugation at 49,000 × *g* for 10 min. The treated homogenates were then prepared for the [^{3}H] acetylcholine binding assay as described in the text using 2.5–30.0 nM [^{3}H]acetylcholine. The scatchard plots are representative of 2–7 experiments. The K_D and B_{max} were determined by least-squares linear regression. The B_{max} value for the control was 3.7 ± 0.2 pmoles/g of tissue. Pretreatment with 0.3, 1.0, and 3.0 mM dithiothreitol decreased the B_{max} values to 1.8 ± 0.2, 1.3 ± 0.2, and 0.7 ± 0.1 pmoles/g of tissue, respectively. The K_D value for the control was 11.1 ± 1.3 nM. The K_D values following pretreatment with 0.3, 1.0, and 3.0 mM dithiothreitol were 11.4 ± 1.8, 12.1 ± 1.2, and 13.6 ± 2.6 nM, respectively.

that reported recently for peripheral nicotinic cholinergic receptors (6).

The effects of generating thiol complexes with the reduced sites were investigated by preincubation of the homogenates with PCMB. Preincubation with PCMB alone (1 mM) had no effect on [^{3}H]acetylcholine binding (Table 1), and preincubation with dithiothreitol followed by a second preincubation with PCMB reduced binding to approximately the same extent as dithiothreitol preincubation alone (Table 1). However, when dithiothreitol-treated tissue was exposed to PCMB, the restoration of

TABLE 1

Effects of disulfide bond and sulfhydryl reagents on [^{3}H] acetylcholine binding in cerebral cortex

Cortical homogenates were incubated with 1 mM dithiothreitol, DTNB, or PCMB for 30 min at 0° (pH 8.5) and either washed for subsequent treatment with another reagent or prepared for [^{3}H]acetylcholine binding (10 nM) as described in the text. Control binding = 2.11 ± 0.08 pmoles/g of tissue. Data are expressed as the mean ± standard error of the mean of four experiments.

	Pretreatment	% of control
Control		100 ± 3.8
A.	1 mM dithiothreitol	24.4 ± 8.0[a]
B.	1 mM DTNB	90.0 ± 8.0
C.	1 mM dithiothreitol followed by 1 mM DTNB	90.6 ± 4.1
D.	1 mM PCMB	107.6 ± 3.7
E.	1 mM dithiothreitol followed by 1 mM PCMB	33.7 ± 1.2[a]
F.	1 mM dithiothreitol followed by 1 mM PCMB followed by 1 mM DTNB	71.4 ± 5.7[b]

[a] $p < 0.01$ compared with control.

[b] $p < 0.01$ compared with control and with Treatments C and E.

binding sites by DTNB was partially prevented (Table 1). This indicates that interaction of the sulfhydryl groups with PCMB retards or prevents reoxidation by DTNB. (It is possible that a longer preincubation period with PCMB would have complexed more sites, thus preventing more sites from being reoxidized by DTNB.)

Scatchard analyses indicated that the apparent affinity of the sites for [^{3}H]acetylcholine was unchanged following dithiothreitol treatment (Fig. 1). The effects of disulfide bond reduction on agonist and antagonist drug affinities for [^{3}H]acetylcholine binding sites were also studied by comparing competition curves in control and dithiothreitol-treated brain membranes. Among nicotinic cholinergic antagonist drugs which we have tested, dihydro-β-erythroidine had the highest apparent affinity for the [^{3}H]acetylcholine binding site in brain. Its IC_{50} of 215 nM indicated that it is 400 times more potent than the neuromusclar antagonist *d*-tubocurarine and more than 2000 times more potent than the ganglionic antagonist hexamethonium in competing for these brain sites (Table 2; see also ref. 1). Reduction of disulfide bonds by preincubation with dithiothreitol did not alter the apparent affinity of dihydro-β-erythroidine for the remaining sites (Table 2; Fig. 2, *left*). Similar results were obtained using *d*-tubocurarine and hexamethonium as the competing antagonist (Table 2). Cytisine, a ganglionic-stimulating drug, had high affinity for [^{3}H]acetylcholine binding sites (Table 2). Following reduction of disulfide bonds with dithiothreitol cytisine competed for the remaining sites with approximately the same affinity as in membranes not treated with dithiothreitol (Table 2; and Fig. 2, *right*). In addition, the maximal decrease in [^{3}H]acetylcholine binding by antagonists or agonists was unaltered (Fig. 2). Thus, although reduction of disulfide bonds markedly decreased the number of [^{3}H]acetylcholine binding sites, the remaining sites appeared to retain the native properties which determine the affinities of agonists and antagonists.

TABLE 2

Effect of dithiothreitol pretreatment on agonist and antagonist competition for [^{3}H]acetylcholine binding sites in cerebral cortex

Cortical homogenates were incubated with 300 μM dithiothreitol for 30 min at 0° (pH 8.5). The reaction was terminated by dilution with cold buffer. The washed homogenates were washed and subsequently incubated with 10 nM [^{3}H]acetylcholine and several concentrations of competing drugs for 40 min at 0° as described in the text. The concentration of drug which decreased specific binding of [^{3}H]acetylcholine by 50% (IC_{50}) was determined graphically by inspection. Each value is the mean ± standard error of the mean of two to four experiments.

Competing drug	IC_{50}	
	Untreated	Dithiothreitol-treated
	nM	
Dihydro-β-eryhtroidine	215 ± 39	214 ± 31
d-Tubocurarine	38,000 ± 2,500	25,300 ± 5,800
Hexamethonium	630,000 ± 60,000	530,000 ± 30,000
Cytisine	5.0 ± 0.1	7.3 ± 0.3

To determine whether agonist occupation of the binding site could protect the disulfide bond from cleavage by dithiothreitol, preincubation with dithiothreitol was carried out in the presence of agonists. The presence of 100 μM acetylcholine or nicotine (concentrations which are approximately 10,000 times their K_D values) before and during the preincubation with dithiothreitol (1 mM) failed to prevent the reduction in [^{3}H]acetylcholine binding (Table 3). To determine whether acetylcholine can bind under the incubation conditions which favor the action of dithiothreitol, [^{3}H]acetylcholine binding was

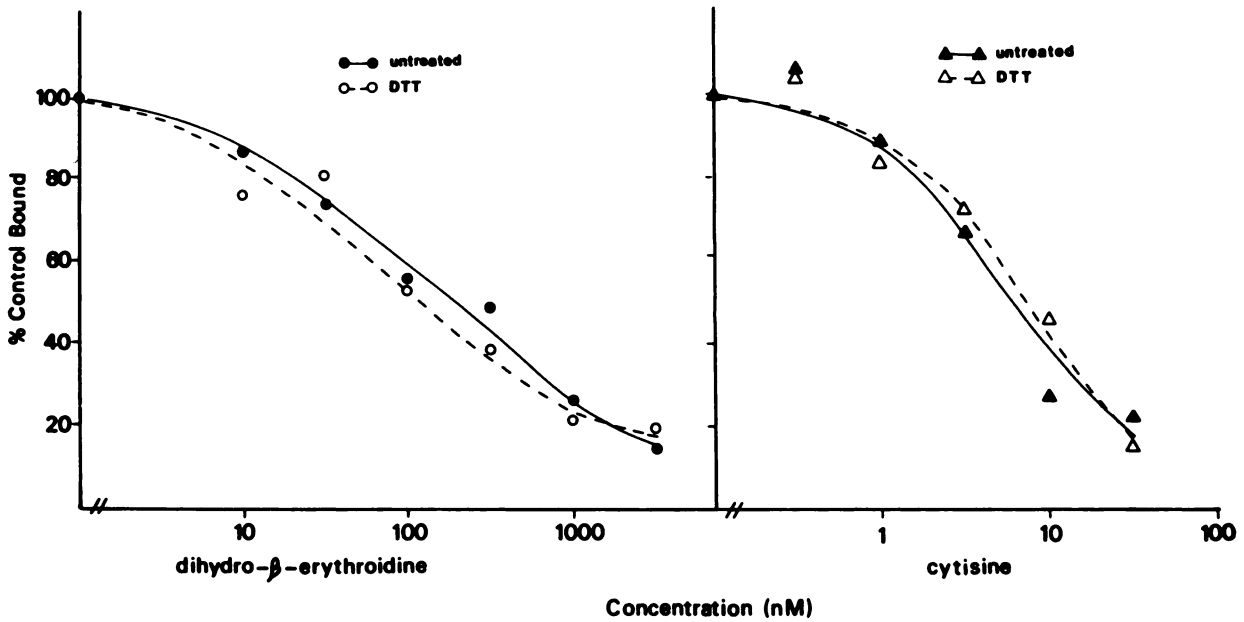

FIG. 2. *Effect of dithiothreitol pretreatment on antagonist and agonist competition for [^{3}H]acetylcholine binding sites in cerebral cortex*

Cortical homogenates were incubated with 300 μM dithiothreitol (*DTT*) for 30 min at 0° (pH 8.5). The reaction was terminated by dilution with cold buffer. The washed homogenates were subsequently incubated with 10 nM [^{3}H]acetylcholine and several concentrations of either (*left*) the antagonist dihydro-β-erythroidine or (*right*) the agonist cytisine for 40 min at 0° as described in the text. The data are representative of two to four experiments.

TABLE 3

Effect of nicotinic cholinergic agonist pretreatment on the reduction of disulfide bonds by dithiothreitol in rat cerebral cortex

Cortical homogenates were incubated in the presence or absence of 100 μM acetylcholine (in the presence of 100 μM DFP) or 100 μM (−)-nicotine for 30 min at 0°. Subsequently, 1 mM dithiothreitol was added and the total mixture was incubated for an additional 30 min at 0° (pH 8.5). The reaction was terminated by dilution with cold buffer, and the homogenates were washed two times. [^{3}H]Acetylcholine binding (10 nM) was assayed in the washed homogenates as described in the text. Control binding = 1.81 ± 0.22 pmoles/g of tissue. The data are expressed as the mean ± standard error of the mean of three experiments.

Pretreatment	% of control
Control	100 ± 12.2
A. 1 mM dithiothreitol	40.7 ± 4.6[a]
B. 100 μM acetylcholine	99.3 ± 8.9
C. 100 μM (−)-nicotine	106.6 ± 5.6
D. 100 μM acetylcholine + 1 mM dithiothreitol	32.5 ± 2.1[a]
E. 100 μM (−)-nicotine + 1 mM dithiothreitol	43.0 ± 4.5[a]

[a] $p < 0.01$ compared with control.

carried out at pH 8.5, 0°. [^{3}H]Acetylcholine binding at this pH was comparable to that at pH 7.4. Thus, receptor occupation by acetylcholine during dithiothreitol treatment was unaffected by the higher pH required for the effect of dithiothreitol, and agonist occupation did not protect the disulfide bond from cleavage.

DISCUSSION

The [^{3}H]acetylcholine binding site described here has properties of a nicotinic cholinergic receptor recognition site in brain (1). The reversible decrease in the number of binding sites following treatment with dithiothreitol suggests that membrane disulfide bonds are required for the binding of acetylcholine to nicotinic cholinergic receptors in brain. The restoration of the number of binding sites by DTNB, which reoxidizes free sulfhydryl groups to disulfide bonds, and the prevention of this binding site restoration by PCMB, which combines with free sulfhydryl groups, provide further evidence of the requirement for disulfide bonds at or near the [^{3}H]acetylcholine recognition site. The lack of effect of DTNB alone or PCMB alone on [^{3}H]acetylcholine binding indicates that the essential disulfide bonds are not in equilibrium with reduced sulfhydryl groups, since it would be expected that such an equilibrium would be shifted by DTNB (toward disulfide formation) and PCMB (away from disulfide formation) with consequent increases and decreases, respectively, in the binding.

The presence of high concentrations of agonists during preincubation with dithiothreitol did not prevent decreases in binding. This suggests that the critical disulfide bond is not directly at the recognition site but is close enough that, when reduced to the sulfhydryl group, it renders the site incapable of binding [^{3}H]acetylcholine. It is possible that breakage of the disulfide bond causes a change in the conformation of the receptor molecule so that agonists can no longer bind to the recognition site.

Although dithiothreitol preincubation reduces the number of brain [^{3}H]acetylcholine binding sites, the affinities of agonists and antagonists for the remaining sites appear to be unaltered, indicating that these remaining sites retain the native properties which determine the recognition of agonists and antagonists. This is in contrast to the effects of dithiothreitol on nicotinic cholinergic receptor-mediated responses in skeletal muscle and in electroplax, where reduction of disulfide bonds decreases the apparent affinity of agonists, but does not appear to alter the maximal response (2–5) or the number of α-bungarotoxin binding sites (5). It is also in contrast to the effects of dithiothreitol on α-bungarotoxin binding sites in brain, where reduction of disulfide bonds decreases the apparent affinity of the binding site for agonists but does not alter the number of binding sites (8).

The [^{3}H]acetylcholine recognition site has an unusual pharmacological profile. Nicotinic agonists are 10–10,000 times more potent than antagonists in competing for [^{3}H]acetylcholine binding (1). One explanation may be that the [^{3}H]acetylcholine binds to a desensitized site during the assay incubation. The desensitized site would have high affinity for agonists, whereas antagonists may not compete as effectively for desensitized sites. [^{3}H]Nicotine binding in rat brain has also been characterized by high agonist and low antagonist potencies (10). In electroplax, although the affinity of [^{3}H]acetylcholine for binding to nicotinic cholinergic receptors is approximately 3 nM (11), the EC_{50} for acetylcholine to induce ion permeability responses is in the micromolar range (12, 13). The high affinity binding has been explained by the existence of a desensitized site or state of the receptor which has high affinity for the agonist (11–14). In brain, the EC_{50} for acetylcholine to elicit biochemical and electrophysiological responses is also in the micromolar range (15, 16). Thus, the low K_D for [^{3}H]acetylcholine and the high agonist affinities for the [^{3}H]acetylcholine binding site in brain are consistent with a desensitized state.

Thus, although the [^{3}H]acetylcholine binding site has properties of a nicotinic cholinergic receptor recognition site, it differs from skeletal muscle and electroplax nicotinic cholinergic receptors and from brain α-bungarotoxin binding sites in its pharmacological characteristics (1) and in the apparent effect which chemical modification of its disulfide bond has on its kinetics. In addition to these pharmacological and chemical differences, the distributions of [^{3}H]acetylcholine binding sites and α-bungarotoxin binding sites in brain are different (1).

The function of these [^{3}H]acetylcholine recognition sites is not yet clear. Nicotinic cholinergic receptor-mediated release of catecholamines from rat brain tissue slices has been reported (15), and nicotinic cholinergic agonists are reported to elicit excitatory electrophysiological responses in rat brain (16). In addition, we have recently reported that repeated administration of DFP to rats decreases [^{3}H]acetylcholine recognition sites in brain (17). Thus, these sites appear to be innervated by cholinergic neurons and appear to be regulated by the synaptic concentrations of acetylcholine.

The *in vitro* modification of the [^{3}H]acetylcholine binding sites by alterations of disulfide bonds should be a valuable tool for determining the relationship between the [^{3}H]acetylcholine binding site and nicotinic cholinergic receptor-mediated functions in brain.

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REFERENCES

1. Schwartz, R. D., R. McGee, Jr., and K. J. Kellar. Nicotinic cholinergic receptors labeled by [^{3}H]acetylcholine in rat brain. *Mol. Pharmacol.* **22**:56–62 (1982).
2. Karlin, A., and E. Bartels. Effects of blocking sulfhydryl groups and of reducing disulfide bonds on the acetylcholine-activated permeability system of the electroplax. *Biochim. Biophys. Acta* **126**:525–535 (1966).
3. Lindstrom, J. M., S. J. Singer, and E. S. Lennox. The effects of reducing and alkylating agents on the acetylcholine receptor activity of frog sartorius muscle. *J. Membr. Biol.* **11**:217–226 (1973).
4. Ben-Haim, D., E. M. Landau, and I. Silman. The role of a reactive disulfide bond in the function of the acetylcholine receptor at the frog neuromuscular junction. *J. Physiol. (Lond.)* **234**:305–325 (1973).
5. Walker, J. W., R. J. Lukas, and M. G. McNamee. Effects of thio-group modifications on the ion permeability control and ligand binding properties of *Torpedo californica* acetylcholine receptor. *Biochemistry* **20**:2191–2199 (1981).
6. Sasaki, K., and W. K. Rikerl. Effects of dithiothreitol, 5,5′-dithiobis-(2-nitrobenzoic acid) and N-ethylmaleimide on synaptic transmission at sympathetic ganglion cells of frog. *Neuropharmacology* **21**:1365–1373 (1982).
7. Karlin, A. Molecular interactions of the acetylcholine receptor. *Fed. Proc.* **32**:1847–1853 (1973).
8. Lukas, R. J., and E. L. Bennett. Chemical modification and reactivity of sulfhydryls and disulfides of rat brain nicotinic-like acetylcholine receptors. *J. Biol. Chem.* **255**:5573–5577 (1980).
9. Scatchard, G. The attractions of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.* **51**:660–672 (1949).
10. Romano, C., and A. Goldstein. Stereospecific nicotine receptors on rat brain membranes. *Science (Wash. D. C.)* **210**:647–649 (1980).
11. Boyd, N. D., and J. B. Cohen. Kinetics of binding of [^{3}H]acetylcholine to *Torpedo* postsynaptic membranes: association and dissociation rate constants by rapid mixing and ultrafiltration. *Biochemistry* **19**:5353–5358 (1980).
12. Moreau, M., and J. P. Changeaux. Studies on the electrogenic action of acetylcholine with *Torpedo mamorata* electric organ. I. Pharmacological properties of the electroplaque. *J. Mol. Biol.* **106**:457–467 (1976).
13. Popot, J. L., S. Hiroyuki, and J. P. Changeaux. Studies on the electrogenic action of acetylcholine with Torpedo marmorata electric organ. II. The permeability response of the receptor-rich membrane fragments to cholinergic agonists *in vitro*. *J. Mol. Biol.* **106**:469–483 (1976).
14. Conti-Tronconi, B. M., S. M. J. Dunn, and M. A. Raftery. Independent sites of low and high affinity for agonists on *Torpedo californica* acetylcholine receptor. *Biochem. Biophys. Res. Commun.* **107**:123–129 (1982).
15. Gioguieff, M. F., M. L. Le Floche, T. C. Westfall, J. Glowinski, and M. J. Besson. Nicotinic effect of acetylcholine on the release of newly synthesized [^{3}H]dopamine in rat striatal slices and cat caudate nucleus. *Brain Res.* **106**:117–131 (1976).
16. McLennan, H., and T. P. Hicks. Pharmacological characterization of the excitatory cholinergic receptors of rat central neurons. *Neuropharmacology* **17**:329–334 (1978).
17. Schwartz, R. D., and K. J. Kellar. Nictoinic cholinergic receptor binding sites in brain: *in vivo* regulation. *Science (Wash. D. C.)* **220**:214–216 (1983).

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