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# Binding of Five Cholinergic Ligands to Housefly Brain and *Torpedo* Electroplax

## Relationship to Acetylcholine Receptors

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SUMMARY

The binding of radioactive muscarone, nicotine, decamethonium, dimethyl *d*-tubocurarine, and atropine to preparations of *Torpedo* electroplax and housefly brain has been studied at concentrations near 1 μM. Factors examined include binding constants and amounts, competition by cholinergic and noncholinergic drugs, reversibility, and sensitivity to hydrolytic enzymes. In *Torpedo* electroplax, muscarone, nicotine, and decamethonium appeared to bind, with binding constants between 0.4 and 2.5 μM, to the same macromolecules. These were phospholipoproteins present in the amount of 1.0–2.1 nmoles/g, fresh weight; their properties were similar to those of a nicotinic receptor. In the brain of the housefly, the same three ligands and also atropine appeared to bind, with binding constants between 0.2 and 3.2 μM, to the same macromolecules, which were proteins present in the amount of 2.2–3.2 nmoles/g, fresh weight. The latter exhibited properties similar to those of both nicotinic and muscarinic receptors.

## INTRODUCTION

Binding of cholinergic ligands to subcellular fractions has been used as the main criterion for identifying the acetylcholine receptor(s) and following it through subsequent purification procedures. The nature of such binding macromolecules has included acidic mucopolysaccharides (1), proteins (2, 3) isolated from the electric organ of the electric eel, *Electrophorus electricus*, and nucleoproteins isolated from human skeletal muscle (4, 5). These macromolecular components occurred in relatively large quantities and had low affinities for cholinergic ligands. The binding was irreversible in the eel and occurred in synapses as well as other areas, as shown by immunohistochemical or radioautographic methods. The claims that mucopolysaccharides and proteins were acetylcholine receptors were later withdrawn (6, 7). Recently, proteolipids were isolated from cerebral cortex and electroplaxes of the skate *Torpedo marmorta* and the electric eel *Electrophorus*, and were identified as acetylcholine receptors by virtue of their binding of cholinergic ligands (8–12).

Binding alone is an insufficient criterion for identification of receptors, because many nonspecific acidic groups in macromolecules react with the positively charged cholinergic ligands. To avoid isolating nonspecific macromolecules, one has to demonstrate that the binding under study has appropriate affinity and location, is reversible, and responds to suitable drugs which correspond to the physiological properties of the acetyl-

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choline receptor. We have previously examined (13–15) the binding of $^3$H-muscarone, which was selected because of its close structural similarity to acetylcholine and the facts that it cannot be hydrolyzed by cholinesterase and is able to excite both nicotinic and muscarinic acetylcholine receptors (16). Muscarone was bound reversibly and with high affinity to subcellular preparations of *Torpedo* electroplax and brain of the housefly, *Musca domestica* L., and the binding was blocked by cholinergic but not by noncholinergic drugs. In the brain of the housefly the binding macromolecules were proteins and exhibited both nicotinic and muscarinic characteristics, whereas in the electroplax these macromolecules were phospholipoproteins endowed with nicotinic binding characteristics alone. The present report deals with an extension of the investigation of the properties of binding of other cholinergic ligands to subcellular preparations from *Torpedo* electroplax and the brain of the housefly.

## MATERIALS AND METHODS

The lyophilized precipitate from *Torpedo* electroplax and the supernatant fraction from housefly head homogenates were used as previously described (14, 15). *N*-Methyl-$^3$H-DL-muscarone iodide (specific activity, 81 mCi/mmole), referred to as "muscarone," was prepared as described (13). $^3$H-Nicotine (specific activity, 295 mCi/mmole), $^3$H-decamethonium (specific activity, 178 mCi/mmole), $^3$H-atropine (specific activity, 500 mCi/mmole), and $^{14}$C-dimethyl *d*-tubocurarine iodide ("dimethylcurare") (specific activity, 83.3 mCi/mmole) were purchased from Amersham-Searle (Arlington, Ill.). The radiochemical purity of the compounds was periodically checked by thin-layer chromatography.

Binding was studied by equilibrium dialysis. One milliliter of the supernatant fraction obtained from 0.2 g of housefly heads or containing lyophilized precipitate from 0.5 g of electroplax was placed in 0.25-inch dialysis tubing, which then was tied at both ends and suspended in 50 volumes of a modified Krebs-Ringer solution, pH 7.4 (15), containing the radioactive ligand at 1 $\mu$M unless otherwise stated. The system was shaken overnight at 4°. Five samples of 0.1 ml were drawn from the bath and from the contents of the bag and counted in a Packard Tri-Carb liquid scintillation spectrometer (model 3375). The excess counts of the samples of the bag represented the amount bound. A toluene mixture containing 4.75 g of 2,5-diphenyloxazole (PPO), 0.32 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (dimethylPOPOP), and 40 ml of Beckman (BBS-3) solubilizer per liter was used, and gave counting efficiencies of 40% for $^3$H and 70% for $^{14}$C. Each determination was corrected for quenching. Duplicate experiments were performed on different tissue preparations, giving 10 values for each test point. The mean and standard deviation for each set of values were calculated and subjected to a *t*-test. The competitive blockade by other drugs and the effect of enzyme preparations on ligand binding and the reversibility of binding were determined as previously described (15).

## RESULTS

The binding constants of five cholinergic ligands to subcellular preparations of *Musca* brain and *Torpedo* electroplax were determined by measuring the binding as a function of concentration. The concentration range was chosen between 0.1 and 1 $\mu$M, because the ligands tested are known to be physiologically active on acetylcholine receptors in this range of concentrations (17–19). The Lineweaver-Burk plots were linear, as previously shown for muscarone (14, 15) and nicotine (20). Figure 1 illustrates this linearity for decamethonium, and contrasts the two tissues. From the Lineweaver-Burk plots, the binding constants ($K$) (Table 1) and the concentrations of the binding sites were computed by the weighted regression method of Wilkinson (21). It is of interest that atropine, which is a muscarinic blocker, showed a low affinity for binding to macromolecules in *Torpedo* electroplax, which contains neuromuscular nicotinic acetylcholine receptor (22, 23). Decamethonium exhibited the highest binding affinity, with binding constants of 0.19 and

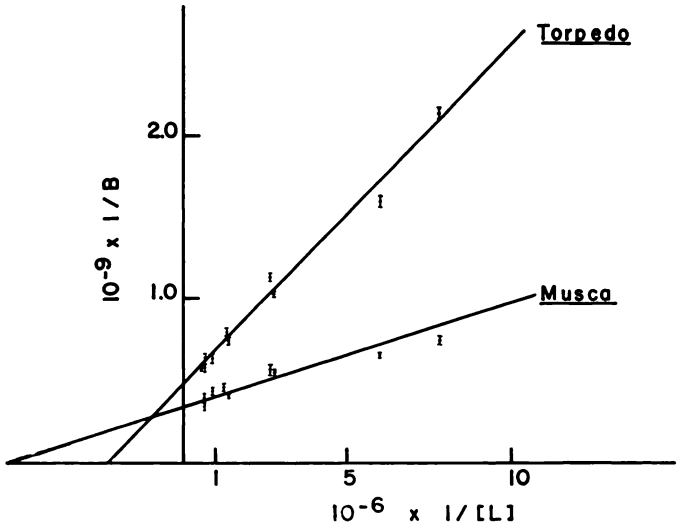


FIG. 1. *Lineweaver-Burk plot of binding of decamethonium (B, in moles per gram of fresh tissue) to subcellular preparations from housefly brain (Musca) and Torpedo electroplax, as a function of molar concentration of the ligand decamethonium, [L]*

The line was computed by the weighted regression method of Wilkinson (21). Vertical lines represent the standard deviation for five replicates at each concentration.

TABLE 1

*Binding constants (K) of cholinergic ligands and concentrations of binding sites (B) in Musca brain and Torpedo electroplax*

| Ligand | K | | B | |
|---|---|---|---|---|
| | Musca | Torpedo | Musca | Torpedo |
| | *μM* | *μM* | *nmoles/g, fresh wt* | |
| Muscarone | 2.4 ± 0.4[a] | 0.72 ± 0.06[b] | 3.2 ± 0.3[a] | 1.0 ± 0.03[b] |
| Nicotine | 3.2 ± 1.7[c] | 2.5 ± 0.5 | 2.3 ± 1.1[c] | 1.3 ± 0.2 |
| Decamethonium | 0.19 ± 0.02 | 0.43 ± 0.03 | 3.0 ± 0.07 | 2.1 ± 0.04 |
| Dimethylcurare | 1.3 ± 0.2 | 2.3 ± 0.6 | 23.3 ± 2.3 | 1.3 ± 0.3 |
| Atropine | 2.1 ± 0.8 | 31.0 ± 77.2[d] | 2.2 ± 0.6 | 4.6 ± 13.7[d] |

[a] From Eldefrawi and O'Brien (15).

[b] From O'Brien *et al.* (14)

[c] From Eldefrawi *et al.* (20).

[d] The high variance of atropine binding to *Torpedo* is due to the poor affinity, which leads to measuring small amounts of binding at high concentrations, and hence computing a small number by subtraction of two large values.

0.43 μM for receptor molecules present in brain and electroplax, respectively. The concentrations of the binding macromolecules within any one tissue were essentially similar, except for dimethylcurare for brain and atropine for electroplax. This agreement fits the hypothesis that one group of four ligands binds to a common site(s) in the housefly and a different group of four ligands binds to a different common site(s) in *Torpedo*.

If the ligands examined are bound to the same sites on macromolecules in each preparation, they should compete for each other's binding sites. Table 2 shows the results when binding of each radioactive ligand was blocked by nonradioactive ligands. It is clear that the noncholinergic

TABLE 2

*Blockade of ligand binding to Musca brain (M) and Torpedo electroplax (T)*

| Competitive ligand (100 μM) | Blockade of ligand (1μM) binding | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Muscarone | | Nicotine | | Decamethonium | | Dimethylcurare | | Atropine | |
| | M | T | M | T | M | T | M | T | M | T |
| | % | % | % | % | % | % | % | % | % | % |
| Nicotine | 74 | 100 | | | 44 | 50 | 15 | 44 | 49 | (−18)[a] |
| Decamethonium | 76 | 77 | 75[b] | 65 | | | 27 | 36 | 47 | (10) |
| Curare | 54[c] | 85 | 55[b] | 61 | 51 | 57 | | | 43 | (15) |
| Atropine | 72[c] | 21[c] | 80[b] | 51 | 51 | 29 | 28 | (3) | | |
| Pilocarpine | 84[c] | 0[c] | 84[b] | (−13) | 51 | 9 | (4) | (−8) | 52 | −33 |
| γ-Aminobutyrate | (−7)[c] | 0[c] | (5)[b] | −24 | 0 | (−6) | (−1) | (−9) | −23 | −36 |
| Serotonin | (13)[c] | 0[c] | (15)[b] | −30 | (16) | (3) | (1) | (−1) | (8) | (−22) |

[a] Values in parentheses are not significant as judged by the *t*-test. Negative values indicate greater binding in the presence of the drug.

[b] From Eldefrawi *et al.* (20).

[c] From Eldefrawi and O'Brien (15).

ligands, γ-aminobutyrate and serotonin, did not compete with any of the cholinergic ligands for the binding sites. In some cases the noncholinergic ligands even enhanced the cholinergic ligand binding significantly, as reported for electroplax previously (14). The binding of atropine to electroplax was not much affected by the nicotinic ligands, although it blocked the binding of muscarone, nicotine, and decamethonium. The latter three ligands and dimethylcurare showed variable degrees of competition in brain and electroplax.

The reversibility of binding was determined by performing dialysis as usual and transferring the bag to ligand-free Ringer's solution for 24 hr. The calculated reversibility was determined by extrapolation from the Lineweaver-Burk plots. The five ligands bound reversibly to the preparations of brain and electroplax (Table 3), with atropine and dimethylcurare exhibiting only partial reversibilities (minimally 55%).

To determine roughly the chemical nature of the binding macromolecules in the subcellular preparations of brain and electroplax, these preparations were treated with hydrolytic enzymes and their binding of the ligands was measured (Table 4). With the proteolytic enzymes trypsin and chymotrypsin, binding of all ligands was much reduced, except in the case of the binding of dimethylcurare to electroplax. Phospholipase C (EC 3.1.4.3) reduced binding of three of the five ligands to electroplax, but only dimethylcurare and decamethonium binding to fly brain.

TABLE 3

*Reversibility of binding of ligands to Musca brain and Torpedo electroplax*

| Ligand | Reversibility | | | |
|---|---|---|---|---|
| | Musca | | Torpedo | |
| | Observed | Calculated | Observed | Calculated |
| | % | % | % | % |
| Muscarone | 91[a] | 98[a] | 79 | 97 |
| Nicotine | 84[b] | 98[b] | 86 | 97 |
| Decamethonium | 80 | 90 | 79 | 89 |
| Dimethylcurare | 69 | 93 | 70 | 94 |
| Atropine | 73 | 98 | 55 | 88 |

[a] From Eldefrawi and O'Brien (15).

[b] From Eldefrawi *et al.* (20).

TABLE 4

*Effect of enzyme treatment on binding of cholinergic ligands by Musca brain (M) and Torpedo electroplax (T)*

Controls showed significant binding of decamethonium to the three enzymes, and of dimethylcurare to trypsin and phospholipase C. Values in the table were corrected accordingly.

| Ligand (1 μM) | Reduction of binding | | | | | |
|---|---|---|---|---|---|---|
| | Trypsin | | Chymotrypsin | | Phospholipase C | |
| | M | T | M | T | M | T |
| | % | % | % | % | % | % |
| Muscarone | 85[a] | 67[b] | 79[a] | 80[b] | (5)[a, c] | 48[b] |
| Nicotine | 56[d] | 49 | 55[d] | 58 | (−4)[d] | 16 |
| Decamethonium | 77 | 80 | 94 | 80 | 14 | 25 |
| Atropine | 63 | 44 | 48 | 33 | (−5) | (21) |
| Dimethylcurare | 25 | (7) | 43 | 12 | 7 | (5) |

[a] From Eldefrawi and O'Brien (15).

[b] From O'Brien *et al.* (14).

[c] Values in parentheses are not significant as judged by the *t*-test. Negative values indicate greater binding after enzyme treatment.

[d] From Eldefrawi *et al.* (20).

## DISCUSSION

All five ligands have been shown to bind with high affinity to preparations which presumably contain high levels of acetylcholine receptor (Table 1). If all were bound to acetylcholine receptor sites only, the *B* values should be the same for any one tissue. The data suggest that for housefly brain, dimethylcurare was bound to additional sites, and perhaps the same is true for atropine binding to *Torpedo* electroplax (but the variance is high). It is precisely these combinations that displayed a substantial degree of irreversible binding (Table 3). Furthermore, dimethylcurare binding to both tissues had an anomalously low sensitivity to trypsin (Table 4). It would seem that binding to 1 μM dimethylcurare is not a good index of acetylcholine receptor in housefly brain. Similarly, for *Torpedo* electroplax, binding of 1 μM atropine is not a good index of the presence of acetylcholine receptor.

There have been only a few estimates of the concentration of acetylcholine receptor in tissues. A value of 0.1 nmole/g was estimated for *Electrophorus* electric organ (24), and 18 nmoles/g for rat brain (13). The concentrations of the macromolecules binding atropine were 3.3 nmoles/g of dog intestine (25), but 1.15 nmoles/g of guinea pig intestine (26). The latter concentration represented two binding sites, one of 0.18 nmole/g of tissue, representing macromolecules having a high binding affinity for atropine ($K$ = 1 nM). The concentrations of the receptor macromolecules in our preparations (Table 1) were in the same range as the above calculations.

Not many comparisons of our biochemical binding constants with physiological findings are possible. For *d*-tubocurarine ("curare"), $K$ = 0.42 μM was reported with skeletal muscle of the frog (17), and $K$ = 0.24 μM with *Electrophorus* electroplax (18). These are reasonably similar to our biochemical values for dimethylcurare of 1.3 μM for housefly brain and 2.3 μM for *Torpedo* electroplax, in spite of the unsuitability of using the value for dimethylcurare binding to housefly brain, as indicated above. For atropine, Paton and Rang (26) found physiologically a $K$ value of 1.1 nM for smooth muscle of guinea pig intestine, a figure far smaller than our values, once again indicating that atropine in the concentrations we have employed may provide a poor index of acetylcholine receptor in the two tissues we have studied. However, one should exercise caution in

interpreting these results, in view of the finding that dissociation constants were 140–510 times higher for cellular acetylcholinesterase than for the purified enzyme preparation (27).

The competition studies of Table 2 provide data compatible with the view that some or all of the five radioactive ligands bind to acetylcholine receptors. Nicotine, decamethonium, and curare strongly blocked the binding of one another, except that nicotine blocked only weakly the binding of dimethylcurare to housefly brain. Curare blocked the binding of all radioactive ligands strongly (except for atropine in *Torpedo*); yet most compounds were not very effective blockers of the binding of radioactive dimethylcurare. This confirms the view voiced above that dimethylcurare binds not only to acetylcholine receptor but to other macromolecules as well. Binding of atropine to *Torpedo* was not blocked by any agent, supporting the above view that it is a poor index of acetylcholine receptor in this tissue. By contrast, in housefly brain preparations, atropine binding was blocked by all four cholinergic drugs. Pilocarpine and atropine discriminated well between the nicotinic receptor of *Torpedo* and the mixed nicotinic-muscarinic receptor which has been considered to be present in housefly brain (15). Thus pilocarpine and atropine blocked quite strongly the binding of muscarone, nicotine, and decamethonium to housefly brain, but blocked very weakly the binding to *Torpedo*.

These extended studies with five cholinergic ligands confirm our earlier reports, made with nicotine and muscarone only, that the majority of noncholinergic agents do not block binding of cholinergic ligands. When we have obtained more highly purified preparations, we propose to explore other kinds of specificity, including effects of varying chain length and optical isomerism.

Although the binding data of Table 1 and the competition data of Table 2 are in good qualitative agreement, there are quantitative discrepancies. For example, nicotine in housefly brain has a binding constant of 3.2 $\mu$M, but even at 100 $\mu$M it causes only 15–74% blockade of binding of other ligands. Such discrepancies, and also some differing effects of proteolytic enzymes on the binding of the five ligands (such as the relative insensitivity of dimethylcurare binding), may be partly explained by the involvement of multiple binding sites rather than a single one, and we have preliminary evidence suggesting such a possibility.

These studies with five labeled ligands confirm the following principal contrasts between cholinergic binding activity from *Torpedo* electroplax (14) and housefly heads (15), deduced from experiments on labeled muscarone. Binding to *Torpedo* electroplax appears to involve phospholipoprotein(s) (or a complex of protein and phospholipid), whose properties parallel those of a neuromuscular nicotinic acetylcholine receptor. The fly material is a protein whose properties parallel those of both muscarinic and nicotinic types of acetylcholine receptors. There is about twice as much binding activity in the preparation of housefly brain as in the electroplax, on a fresh weight basis. These macromolecules may possess a single binding site having the affinities and concentrations reported above. It is also possible that several sites may contribute to the total concentration and that only the affinities of those sites that bind best in the 0.1–1.0 $\mu$M concentration range were observed.

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