Interaction of cholinergic ligands and local anesthetics with plasma membrane fragments from lobster axon*
(nicotinic-binding fraction/permeability barrier)

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ABSTRACT Isologous local anesthetics containing the ester, thiolester, or selenoester group and their quaternary ammonium analogs were studied for their ability to displace \(^{3}H\)nicotine from plasma membrane fragments of lobster nerve. Tertiary and quaternary analogs were equipotent. The relative ability of oxo, thio, and seleno analogs to displace nicotine was the same as their relative ability to block axonal conduction and synaptic transmission. Among cholinergic ligands, choline and aminochoine, previously shown to be inactive as depolarizing agents, were uniquely unable to displace nicotine. The findings described in detail by Denburg (10). Briefly, the axon plasma membrane fraction with maximal nicotine binding resides in the microsomal (100,000 × g) pellet of a hypotonic (0.32 M sucrose) extract of the nerve tissue. Low levels of nicotine binding can be detected in the tissue homogenate, but not in the 1,000 × g and 10,000 × g pellets and the 100,000 × g supernatant. Typically, 5 kg of lobster yield about 12 mg of protein in the final 100,000 × g pellet. For binding studies, the pellet was resuspended in 10 mM Tris-HCl buffer, pH 7.8. Lobsters were purchased in Boston from the James Hook Co. \(^{3}H\)Nicotine (specific activity 250 mCi/mmol) was obtained from Amersham/Searle. Benzoylcholine analogs were synthesized as described previously (18–20). Acetamidocholine was synthesized by the procedure described by Price et al. (21). All other reagents were of analytical grade.

The binding of radioactive ligands was assayed by equilibrium dialysis using \(\frac{4}{4}\)-inch cellulose tubing. Aliquots (1 ml) of membrane protein (about 1 mg/ml) were dialyzed overnight in a 50 ml bath containing ligand ± inhibitor in 10 mM Tris, pH 7.8. The radioactivities of 100 \(\mu\)l aliquots of both the bag and bath contents were assayed in 5 ml of Aquosol (New England Nuclear), and an aliquot from each bag was assayed for protein by the method of Lowry et al. (22). Acetylcholinesterase (EC 3.1.1.7) was assayed by the Ellman et al. technique (23), choline acetyltransferase (EC 2.3.1.6) by the method of Fonnum (24), and adenosinetriphosphatase (ATPase; EC 3.6.1.3) activities by the method of Fiske and Subbarow (25).

RESULTS

Characterization of the Plasma Membrane Fraction. Table 1 shows the distribution of protein, acetylcholinesterase, choline acetyltransferase, Na\(^{+}\)-K\(^{+}\)-ATPase, and Mg\(^{2+}\)-ATPase measured in the lobster walking leg nerves after differential centrifugation. Although the acetylcholinesterase and ATPase activities differ quantitatively from those reported by Denburg (10) and by Barnola et al. (15), they are in good agreement with the data of Welsch and Dettharn (26) for the same species and the data of Balerna et al. (12) for crab nerves. The observed differences may be due to seasonal variations (26). In addition, all the enzymes, as assayed, were found to be extremely unstable.

In this hypotonic extract of nerve tissue, choline acetyltransferase activity is 100 times higher in the final 100,000 × g supernatant than in the corresponding pellet. Thus, choline acetyltransferase is either an exclusively cytoplasmic enzyme or, as suggested by Welsch and Dettharn (26) and Fonnum (27), a loosely bound membrane enzyme which is readily removed by osmotic shock.

The dissociation constant for the \(^{3}H\)nicotine–receptor complex, as computed from Lineweaver–Burk plots (Fig. 1 A–C), is 1 \(\mu\)M, compared to 0.4 \(\mu\)M in the similar preparation

Abbreviations: AcCh, acetylcholine; ATPase, adenosinetriphosphatase.
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† To whom reprint requests should be addressed.
Table 1. Distribution of protein and enzymatic activities in lobster axon membrane after differential centrifugation

<table>
<thead>
<tr>
<th>Protein</th>
<th>Acetylcholinesterase, µmol ACh/hr per mg of protein</th>
<th>ATPase, µmol ATP/hr per mg of protein</th>
<th>Choline acetyltransferase, µmol ACh/hr per mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td></td>
<td>14.6</td>
<td>0.29 0.45</td>
</tr>
<tr>
<td>1,000 × g P</td>
<td></td>
<td>21.3</td>
<td>0.34 0.38</td>
</tr>
<tr>
<td>10,000 × g P</td>
<td></td>
<td>46</td>
<td>1.00 1.37</td>
</tr>
<tr>
<td>100,000 × g S</td>
<td></td>
<td>0</td>
<td>1.14 0.83</td>
</tr>
<tr>
<td>100,000 × g P</td>
<td></td>
<td>31</td>
<td>0.98 1.23</td>
</tr>
</tbody>
</table>

| Abbreviations: P, pellet; S, supernatant. |

of Denburg et al (16), 0.2 µM (28) and 2.5 µM (29) for Torpedo electroplax, 3.2 µM for housefly brain (30), and 63 nM for Electrophorus electroplax (31). Scatchard analysis (Fig. 1D) suggests that the nicotine-binding fraction contains two distinct populations of receptors for nicotine, a high-affinity population (KD = 0.32 µM) with a maximum capacity of 83 pmol/mg of protein, and a low-affinity population (KD = 4.8 µM) with a maximum capacity of 354 pmol/mg of protein. O'Brien et al. (6), with preparations of purified ACh receptor from both Electrophorus and Torpedo electric tissue, have shown that the binding of individual ligands is generally characterized by dual affinities.

![Fig. 1. (A-C) Lineweaver-Burk plots computed by least squares regression analysis. In each graph, the control is a single representative experiment shown as a broken line. In all four graphs, B = pmol [3H]nicotine bound per mg of protein. K_D for [3H]nicotine = 1 µM. Open symbols = quaternary analog; closed symbols = tertiary analog. (A) 25 µM oxygen isologs, K_1 = 2.9 µM. (B) 10 µM sulfur isologs, K_1 = 0.5 µM. (C) 10 µM selenium isologs, K_1 = 0.16 µM. Each line is computed from the mean of at least three experiments and standard errors are indicated for each point except where the values lie within the area of the symbol used to denote a point. (D) Scatchard analysis of the dose–response data for nicotine. High-affinity sites: K_D = 0.32 µM, maximum capacity = 83 pmol/mg of protein. Low-affinity sites: K_D = 4.8 µM, maximum capacity = 354 pmol/mg of protein. The data were derived from a single representative dose–response curve.](image-url)
The cholinergic Choline, binding methonium, not inhibit choline inhibits a-bungarotoxin did of 100 nicotine carbamoylcholine of activity of the fraction is well found that local anesthetics inhibit nicotine, compounds in inhibit nicotine, specifically, the presence of 0.3 μM ecotriothophate is weak, sulfur isologs were stronger, and the selenium isologs were the most potent inhibitors. The specific differences among the oxygen, sulfur, and selenium compounds are evident only at low concentrations. As the concentration of inhibitors approaches 100 μM, inhibition is close to 100% for all the analogs.

In the intact squid axon, the quaternary analogs benzylocholine, benzoylbiocholine, and benzoylselenochrome exhibited marked reduced inhibitory activity relative to their tertiary analogs (35). These same compounds (tertiary and quaternary) are equipotent inhibitors of carbamoylcholine depolarizing activity in the eel electroplax, a modified synapt prep (G. D. Webb and H. G. Mautner, unpublished observations). Similarly, these tertiary amine and quaternary ammonium compounds, all of which carry a positive charge at physiological pH, were found to be equally effective inhibitors of nicotine binding in the lobster axon plasma membrane fraction (Table 3).

Mechanism of Action of Tertiary and Quaternary Benzylocholine Analogs. The inhibition of nicotine binding over a range of 0.06–1 μM [3H]nicotine by benzylocholine, benzylbiocholine, and benzoylselenochrome and their tertiary analogs is shown in the Lineweaver–Burk plots of Fig. 1 A–C. The straight lines were plotted by least squares regression analysis. A single line was computed for the tertiary and quaternary forms of each isolog, because no statistically significant difference could be observed between the two. K values are as follows: oxygen isologs = 0.9 μM, sulfur isologs = 0.5 μM, and selenium isologs = 0.16 μM. Because the y intercept and the slope of the lines differ from those of the control in the presence of the sulfur and selenium isologs, the inhibition of nicotine binding is of the mixed competitive–noncompetitive type, while the oxygen isologs appear to be competitive inhibitors.

**Discussion**

Based on the observations that labeled quaternary ammonium compounds, in contrast to tertiary amines, failed to penetrate into the axoplasm of squid giant axons (37, 38), Nachmansohn suggested that the inability of quaternary nitrogen compounds to affect electrical activity in axons is due to structural barriers that prevent the compounds from reaching the axon plasma membrane, at which local anesthetics exert their action (17). Later, Bianchi and Strobel (39) suggested that the action of the local anesthetics procaine and lidocaine is directly related to their ability to penetrate to the inside surface of the axon.

Table 2. Inhibition of binding of [3H]nicotine (1 μM) to axon plasma membrane fragments by various compounds related to acetylcholine

<table>
<thead>
<tr>
<th>Compound</th>
<th>% inhibition*</th>
</tr>
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<tbody>
<tr>
<td>Acetylcholine (10 μM)</td>
<td>26</td>
</tr>
<tr>
<td>Carbamoylcholine (10 μM)</td>
<td>27</td>
</tr>
<tr>
<td>Acetamidocholine (10 μM)</td>
<td>22</td>
</tr>
<tr>
<td>d-Tubocurarine (10 μM)</td>
<td>100</td>
</tr>
<tr>
<td>Decamethonium (10 μM)</td>
<td>80</td>
</tr>
<tr>
<td>Hexamethonium (10 μM)</td>
<td>71</td>
</tr>
<tr>
<td>Choline (500 μM)</td>
<td>0</td>
</tr>
<tr>
<td>Homocholine (500 μM)</td>
<td>11</td>
</tr>
<tr>
<td>Methoxycholine (100 μM)</td>
<td>47</td>
</tr>
<tr>
<td>Methylthiocholine (100 μM)</td>
<td>33</td>
</tr>
<tr>
<td>Aminocholine (100 μM)</td>
<td>0</td>
</tr>
</tbody>
</table>

* The data are presented as percentage inhibition of the amount of nicotine bound in the same membrane preparation at 1 μM.

In the presence of 0.3 μM ecotriothophate iodide.

**Cholinergic Specificity of the [3H]Nicotine Binding Site.**

The effects on the nicotine-binding fraction of several compounds related to AcCh are shown in Table 2. At 10 μM, the ligands AcCh (in the presence of 0.3 μM ecotriothophate iodide), carbamoylcholine, acetamidocholine, d-tubocurarine, decamethonium, hexamethonium, and hemicholinium inhibit the binding of 1 μM nicotine by 26, 27, 32, 100, 63, 80, and 71%, respectively. Acetamidocholine has previously been shown to be a good cholinergic agonist in the guinea pig ileum (H. Gold, J. K. Marquis, and H. G. Mautner, unpublished observation). Choline, on the other hand, a molecule with strikingly low cholinergic activity in intact excitable membranes (32, 33), does not inhibit nicotine binding in concentrations as high as 500 μM. Such inactivity contrasts markedly with the inhibitory effects of the closely related analogs methoxycholine and methylthiocholine, which, at 100 μM, inhibit the binding of 1 μM nicotine by 47% and 33%, respectively. Also, 500 μM homocholine inhibits nicotine (1 μM) binding by 11%. Aminocholine at 100 μM has no effect on nicotine binding.

Preliminary investigations of the binding of [125I]-labeled α-bungarotoxin to isolated axon fragments demonstrate levels of toxin binding comparable to the total measurable nicotine binding, i.e., 250–300 pmol of [125I]-labeled α-bungarotoxin/mg of protein. Equivalent levels of toxin binding were measured in the 100,000 × g supernatant which did not bind [3H]nicotine. The characterization of toxin binding is not yet complete.

**Inhibition of Nicotine Binding by Local Anesthetics.**

**Equi-Potent Activity of Tertiary and Quaternary Local Anesthetics.** Benzoylcholine, its tertiary analog, and their sulfur and selenium isologs have previously been shown to differ significantly in their ability to block the depolarizing activity of carbamoylcholine in eel electroplax (34) and the electrical activity of squid giant axons (35). Because their relative activity is well defined in these preparations, their effect on the nicotine-binding fraction was examined.

As previously demonstrated by Denburg et al. (16, 36), it was found that local anesthetics block the binding of [3H]nicotine. 10 μM procaine, for example, completely inhibits the binding of 1 μM nicotine. 2-Dimethylaminoethyl benzoate, benzoylcholine, and analogs in which the side-chain oxygen of both these compounds has been replaced by sulfur or selenium also inhibit nicotine binding. The data are shown in Table 3. The

Table 3. Inhibition by the oxygen, sulfur, and selenium isologs of benzoylcholine and their tertiary analogs (10 μM) of the binding of nicotine (1 μM) to axon plasma membrane fragments

<table>
<thead>
<tr>
<th>Compound</th>
<th>% inhibition of nicotine binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>R = H</td>
</tr>
<tr>
<td>O</td>
<td>9 ± 0.5</td>
</tr>
<tr>
<td>S</td>
<td>23 ± 4</td>
</tr>
<tr>
<td>Se</td>
<td>43 ± 9</td>
</tr>
<tr>
<td>R = CH₃</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>30 ± 6</td>
<td></td>
</tr>
<tr>
<td>47 ± 7</td>
<td></td>
</tr>
</tbody>
</table>

The isologs exhibit the same relative order of potency as previously demonstrated in both squid giant axons and eel electroplax (34, 35). The oxygen isologs were weakest, sulfur isologs were stronger, and the selenium isologs were the most potent inhibitors. The specific differences among the oxygen, sulfur, and selenium compounds are evident only at low concentrations. As the concentration of inhibitors approaches 100 μM, inhibition is close to 100% for all the analogs.
plasma membrane. This hypothesis was supported by experiments with internal perfusion in the squid giant axon (40, 41), which demonstrated that tertiary amine local anesthetics suppressed membrane currents from both sides of the membrane while quaternary compounds were effective only when applied internally. Data on the action of quaternary as compared to tertiary amine local anesthetics on isolated axon plasma membranes (Table 3) as in intact axons and neuromuscular junctions (34, 35). These observations support the hypothesis that, because many drugs are equally selective in blocking axonal conduction and synaptic transmission, similar processes may regulate ion permeability changes in axons and synapses (17, 42). Furthermore, it is possible that the effects of these compounds may be due to interaction with similar cholinergic receptor-like molecules, as emphasized by the competitive nature of the inhibition of nicotine binding by the oxygen isologs. Denburg et al. (16) have also shown that procaine is a competitive inhibitor of nicotine binding. However, the Lineweaver-Burk plots in Fig. 1 A-C show that the action of the sulfur and selenium isologs is at least partially noncompetitive, suggesting a second site of action. Because the selenium isologs have been shown to react with thiol groups (43), they may combine with nonspecific SH groups outside the nicotine-binding sites.

Perhaps the best evidence that the nicotine-binding fraction has specificity for binding cholinergic molecules is the striking inactivity of choline, a compound that is a very poor agonist in the eel electrophlax (33) and which, even after venoms treatment, has little or no effect on the action potential of intact squid axons (32). In addition, the inhibitory effects of all the cholinergic ligands tested on $^3$H]nicotine binding (Table 3) are compatible with the hypothesis that a cholinergic binding macromolecule can be identified in axon plasma membranes (16). In related studies, Villegas has characterized "acetylcholine receptors" in the Schwann cell membrane of the squid nerve fiber (44).

In previous studies, equipotent molar ratios for carbamoylcholine, methylthiocholine, and methoxycholine were measured as an index of the relative activity of these cholinergic agonists in both nicotinic and muscarinic preparations. In the nicotinic eel electrophlax, Webb and Mautner (45) measured equipotent molar ratios of 1:7:70 for carbamoylcholine:methylthiocholine: methoxycholine. In the muscarinic receptors of the guinea pig ileum, ratios of 1:100:100 have been measured (H. Gold, J. K. Marquis, and H. G. Mautner, unpublished observations). From the data of Table 2, equipotent molar ratios close to 1:10:1 can be derived for these compounds. Also, Denburg et al. (16) observed that atropine, a highly specific muscarinic antagonist, strongly inhibits nicotine binding. Thus, it should be emphasized that, although nicotine was chosen as a neurotransmitter for these studies, the axonal binding proteins are not "nicotinic" in the orthodox sense.

These data support the hypothesis that the nicotine-binding fraction of lobster axon plasma membrane fragments has high specificity for cholinergic ligands as well as for local anesthetics. The binding selectivity of isolated axon membrane fragments is similar to that of intact axons and synapses, suggesting that a common receptor mechanism may operate at these sites. Because the activity of analogous tertiary amine and quaternary ammonium compounds is identical in membrane fragments, we conclude that the cholinergic receptor biopolymer in axons is largely masked by permeability barriers.

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