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THE LABELLING OF AN AXONAL MEMBRANE COMPONENT WITH 4-(N-MALEIMIDO) BENZYLTRIMETHYLAMMONIUM, A REAGENT CAPABLE OF AFFINITY-LABELLING THE α-SUBUNIT OF THE NICOTINIC ACETYLCHOLINE RECEPTOR

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SUMMARY Membrane vesicles, isolated from crustacean axons, were treated, following disulfide reduction, with ^{3}H-NEM or with ^{3}H-MBTA. SDS polyacrylamide gel electrophoresis showed that exposure to NEM (a nonspecific thiol reagent) resulted in the labelling of several peptide bands, while with MBTA only a single band with a molecular weight of 50,000 was labelled. Reaction with MBTA (believed to be a specific label for the nicotinic acetylcholine receptor) could be largely prevented by preincubation with d-tubocurarine or bromacetylcholine.

INTRODUCTION In several preparations, cholinergic agonists or antagonists may alter the electrical parameters of axonal membranes or of Schwann cells (1-4). Axonal membranes are capable of binding a variety of cholinergic ligands (5-7), while binding sites may be visualized by the use of a conjugate of α-bungarotoxin and horseradish peroxidase (8,9). However, it could be shown that the ligand binding specificity of the axonal membrane component differs from that of classical "nicotinic" or "muscarinic" receptors in several ways (5,7). Fusion of axonal membrane vesicles with a planar phospholipid bilayer produced a large K^{+}-specific conductance increase which was not voltage dependent (10). The conductance increase was highly susceptible to blockade by cholinergic antagonists but not by α-bungarotoxin, while the presence of curare-sensitive and curare-insensitive α-bungarotoxin binding sites in axon plasma membranes was reported (11). The present study is concerned with the interaction of axonal membranes with 4-(N-maleimido) benzyltrimethylammonium (MBTA), a reagent believed to be capable, after prior reduction of a disulfide near the binding

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site, of specific alkylation of the α-subunit of the nicotinic acetylcholine receptor (12,13).

METHODS A generous sample of ^{3}H-MBTA was obtained from Dr. Arthur Karlin of Columbia University; later samples as well as ^{3}H-NEM were purchased from New England Nuclear Corp. Because of the lack of stability of MBTA, fresh solutions were prepared for each experiment. Aliquots were dried under N_2 at 4°C and redissolved in 1.0 mM HCl. For each sample the concentration of MBTA was determined by measuring absorbances at 224, 237, 260 and 290 nm (14). Radiochemical purity was determined by thin-layer chromatography on Eastman Chromatogram Sheet Cellulose using the solvent system: n-butanol: 1N acetic acid: ethanol (3.5 : 1 : 1).

Reagents were purchased from the following sources: d-tubocurarine chloride, atropine sulfate, carbamylcholine chloride, acetylcholine chloride, choline chloride, nicotine free base, procaine chloride, eserine salicylate, PMSF and DTT from Sigma Chemical Co., bromoacetic acid from Aldrich Chemical Co. and 30% H_2O_2 and β-mercaptoethanol from Eastman Kodak Co. Reagents for electrophoresis were bought from Biorad Corp. and Liquiscint aqueous scintillation fluid from National Diagnostic Laboratories. Bromoacetylcholine bromide was synthesized by the procedure of Damle et al. (15). Fresh lobster (Homarus americanus) legs were bought from the J. Hook Co., Boston. A sample of electric organ from Torpedo nobiliana was a gift from Dr. Jonathan Cohen at Harvard University.

Axonal plasma membrane fragments from lobster walking leg nerve bundles were prepared by the procedure of Denburg (16) as modified by Marquis et al. (7). All buffers used during the isolation procedure contained 0.2 mM PMSF, 1 mM EDTA and 0.02% sodium azide. Membranes were stored at 4°C in 10 mM Tris, 1 mM EDTA, 0.2 mM PMSF and 0.02% sodium azide at pH 7.8 and used within one week of preparation.

The conditions for the labelling of axonal membranes with MBTA are similar to those used by Karlin and Cowburn (13) for the labelling of membrane-bound or solubilized nicotinic acetylcholine receptor. The axonal membrane suspension was adjusted to 8 mg protein/ml.

Reduction was carried out by the addition to the suspension of 2 mM DTT to a final concentration of 0.2 mM, followed by incubation for 30 minutes at room temperature. DTT was removed by applying 0.5 mL of membrane suspension to a column of 2 mL coarse Sephadex G-25 in a Pasteur pipette equilibrated with 10 mM NaP_i and 1 mM EDTA, pH 7.0. Elution was carried out using the same buffer. As the membranes passed through the column in the void volume, 1 mL was collected and used immediately for alkylation. Controls showed the reduced membranes to be eluted well before DTT. Alkylation was carried out at room temperature in a reaction volume of 1 mL containing 100 mM NaCl, 10 mM NaP_i and 1 mM EDTA at pH 7.0. Membranes were preincubated with drugs in buffer for 10 minutes. Exposure to MBTA (or NEM) to a final concentration of 1 M for 10 minutes was followed by quenching with 20 μM of 0.125 M β-mercaptoethanol. The mixture was then diluted 1:1 with NaP_i buffer followed by centrifugation in a Beckman 40 rotor at 39,000 rpm for 90 minutes. The tubes were rinsed once with 1 mL of NaP_i buffer and dried. Pellets were redissolved in SDS-PAGE sample buffer. A 20 μL aliquot was removed from each sample for protein assay (17).

SDS polyacrylamide gel electrophoresis was carried out on 9% polyacrylamide slab gels according to the method of Laemmli (18). The sample buffer contained 2% SDS, 62.5 mM Tris (pH 6.8), 10% glycerol and 5% β-mercaptoethanol. Prior to application, samples were heated in boiling water for 5 minutes. Molecular weights were calibrated using phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase and soybean trypsin inhibitor. Gels were stained with 0.1% Coomassie Blue R-250.

Individual lanes were cut into 1.67 mm wide slices and incubated in capped scintillation vials containing 1 mL 15% H_2O_2 at 50°C overnight. Vials were allowed to cool to 4°C and 10 mL aqueous scintillation fluid was added. Radioactivity was measured in a Packard scintillation counter. Specific labelling was assayed by cutting out the major labelled bands in each lane, followed by digestion and counting.

After staining and fixing, gels were impregnated with scintillant (EN^{3}HANCE), New England Nuclear Corp. and exposed to x-ray film (X-Omat. Eastman Kodak) for seven days at -70°C.

RESULTS AND DISCUSSION It can be seen in Fig. 1 that, following reduction with DTT, exposure of axonal membrane fragments to ^{3}H-NEM results in the labelling of several peptide bands. On the other hand, following reduction, exposure to ^{3}H-MBTA, believed to be a specific label for α-subunits of the nicotinic acetylcholine receptor, results in the predominant labelling of a single peptide (Fig. 2). However, the molecular weight of that peptide (50,000 Daltons) differs from that of the α-subunit of the nicotinic acetylcholine receptor (40,000 Daltons). Protection against the labelling of axonal membrane fragments by MBTA can be achieved by pretreatment with 10 μM bromoacetylcholine (Fig. 3), d-tubocurarine and, to a lesser extent, by nicotine and atropine. Only minor protection was afforded by procaine, eserine and α-bungarotoxin and none by 10 μM bromoacetic acid, carbamylcholine and choline. These data are summarized in Table I. A

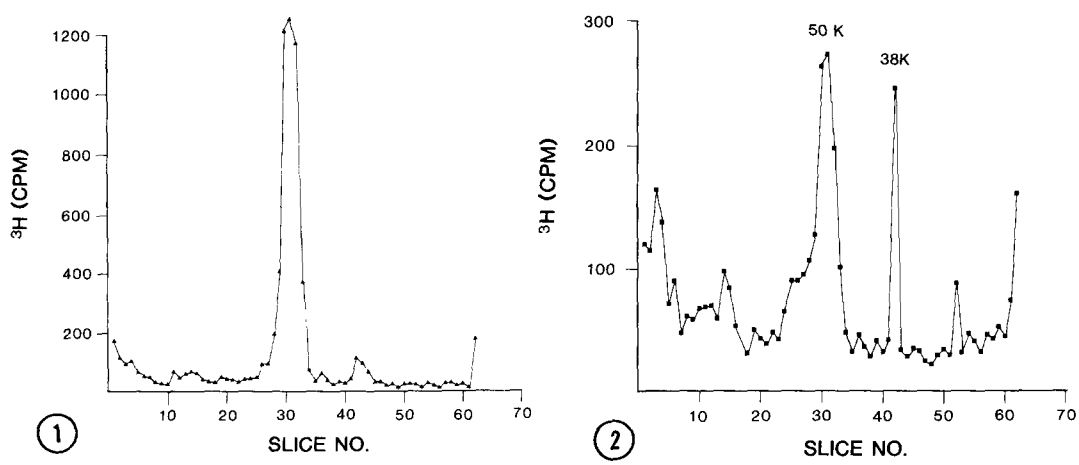

Figure 1. Labelling of several peptide bands with ^{3}H-NEM following reduction of membrane disulfides with DTT.

Figure 2. Labelling of 50 K peptide band with ^{3}H-MBTA, following reduction of membrane disulfides with DTT. Molecular weight was calibrated with phosphorylase B, bovine serum albumin, carbonic anhydrase and soybean trypsin inhibitor.

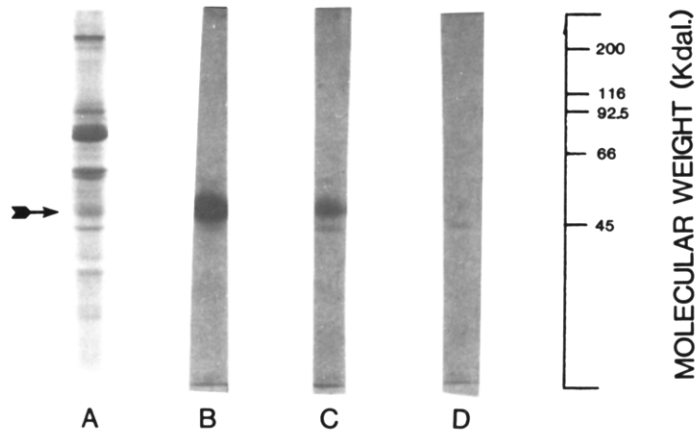

Figure 3. Lobster axon plasma membrane proteins electrophoresed on 9% polyacrylamide slab gels in the presence of SDS.

A. Comassie Blue R-250 stained peptides. MBTA band is marked with an arrow.
B. Visualization of ^{3}H-MBTA labelled peptides with autofluorography (16 day exposure). Labelling of prereduced membranes was carried out in 10 mM Tris, 100 mM NaCl.
C. Same as B except that membranes were preincubated with bromoacetylcholine prior to alkylation with MBTA.
D. Same as B except that membranes were not prereduced with DTT.

dose response for the protection against MBTA labelling by d-tubocurarine is shown in Fig. 4.

Only minor labelling of axonal membrane fragments with ^{3}H-MBTA was achieved without prior disulfide reduction.

While exposure to d-tubocurarine blocks 81% of the labelling of the 50 K peptide by ^{3}H-MBTA, only 40±3% blockade is seen against the labelling of this band by ^{3}H-NEM under equivalent conditions. In addition, it should be noted that while the 50 K peptide band represents most of the radioactivity introduced by labelling with ^{3}H-MBTA, it represents only 38±1% of the radioactivity introduced by labelling with ^{3}H-NEM. The labelling by ^{3}H-NEM of peptides other than the 50 K band is not affected by tubocurarine.

In view of the finding that fusion of axonal membrane fragments to planar lipid bilayers resulted in a potassium-specific increase in conductance (10), the abilities of the potassium channel blockers tetraethylammonium, 4-aminopyridine and 3,4-diaminopyridine to interfere with the binding of MBTA were

Table 1 Inhibition of Labelling of 50 K Peptide Band by ^{3}H-MBTA

Inhibitor	% Inhibition	Number of Experiments
Bromoacetylcholine	75 ± 3	3
Bromoacetic Acid	2 ± 1	3
d-Tubocurarine	82 ± 2	3
Atropine	33 ± 10	3
Nicotine	24 ± 4	4
Procaine	16 ± 6	3
Carbamylcholine	0	3
" (1 mM)	14 ± 4	3
Choline	0	3
α-Bungarotoxin	12 ± 6	3
Eserine	16 ± 4	3
TEA	0	6
Tetramethylammonium	0	6
4-Aminopyridine	0	6

investigated. None of these compounds blocked the binding of MBTA at 10 μM concentration.

The data presented are compatible with the postulate that axonal membranes contain a peptide related to the α-subunit of the nicotinic acetylcholine receptor, although its molecular weight differs from that of the α-subunit. So far,

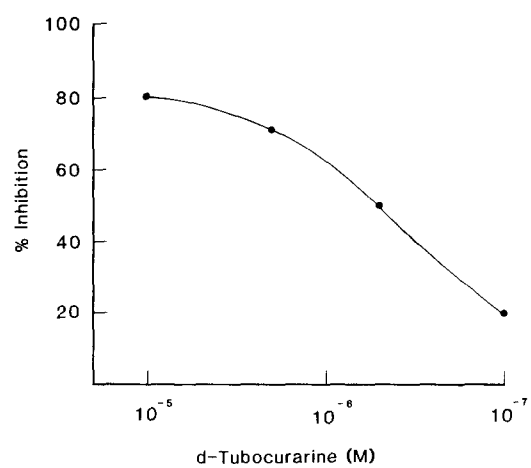

Figure 4. Inhibition of MBTA-labelling of 50 K band by d-tubocurarine.

it has not been possible to induce agonist-induced increases in cation permeability in axonal membrane vesicles (7) analogous to those seen in acetylcholine receptor-rich electroplax membrane vesicles (19). However, cholinergic antagonists lower cation permeability in both preparations. It is possible that the axonal cholinergic binding components may - during isolation - have lost the ability to modulate agonist-induced permeability changes or that they may represent a cholinergic proreceptor in the process of being transported to the nerve terminal.

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