Infected animals came from several localities in the provinces of Panama and Colon on both sides of the Isthmus, and from the Canal Zone. In some of these localities human leishmaniasis has almost disappeared during the last few years, mainly because of deforestation, while in other areas the forest is still untouched and human infections are frequent when man enters the jungle.

The two-toed sloth has a higher rate of infection with *L. braziliensis* (14.1 percent) than any forest mammal so far found infected in Panama. Moreover, infection has been demonstrated consistently throughout the period of study, which indicates the potential importance of this edentate in the epidemiology of cutaneous leishmaniasis.

ARISTIDES HERRER
SAM R. TELFORD, JR.
Gorgas Memorial Laboratory, Panama, R. P., Apartado 6991

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Acetylcholine Receptor: Covalent Attachment of Depolarizing Groups at the Active Site

Abstract. *Following reduction of the acetylcholine receptor in the electroplax with dithiothreitol, the quaternary ammonium compounds bromoacetylcholine bromide and the* p-*nitrophenyl ester of* (p-*carboxyphenyl*)*trimethylammonium iodide react near the active site probably with a sulfhydryl group. The covalently attached quaternary ammonium moieties additionally interact with the active site noncovalently to activate the receptor and cause depolarization of the cell.*

The acetylcholine receptor transduces the binding of acetylcholine into a permeability change. Although the detailed mechanism is unknown, the phenomenology has been extensively studied. One class of ligands, acetylcholine and its congeners, reversibly activate the receptor, while another class, including compounds such as *d*-tubocurarine, competitively inhibit activation. The electroplax of the electric eel, *Electrophorus electricus*, has been a useful preparation for quantitating these phenomena (*1–4*). It has been possible, using this same preparation, to modify the receptor chemically *in situ* and in this way to infer some of its properties (*5*). The receptor appears to contain both sulfhydryl and disulfide groups (*5*), which supports the early suggestion that the receptor is a protein (*6*).

The presence of a disulfide group on the receptor is indicated by the following evidence. Brief application of a low concentration of the reducing agent dithiothreitol to the innervated membrane of the electroplax causes subsequent inhibition of depolarization by receptor activators. This inhibition is completely reversed by application of an oxidizing agent such as 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB); if, however, an alkylating agent such as *N*-ethylmaleimide is applied after reduction, the inhibition can no longer be reversed by DTNB. The demonstration that a quaternary ammonium maleimide derivative, 4-(*N*-maleimido)phenyltrimethylammonium iodide (MPTA), alkylates the sulfhydryls formed by reduction, three orders of magnitude faster than either its tertiary amine analog or *N*-ethylmaleimide, is strong evidence for the location of the reducible disulfide bond on the receptor and in fact close to the active site (*7*). This large enhancement of rate, characteristic of *affinity labels* (*8*), is presumably due to the quaternary ammonium group binding reversibly to the negative subsite of the active site bringing one of the reactive ethylenic carbons of the maleimide group in approximate juxtaposition to one of the sulfhydryls of the receptor. This increases manyfold the probability of a successful collision. Additional evidence that MPTA is covalently bound near the active site is that upon reaction MPTA causes a small depolarization (approximately 1 mv) which is not reversed when the unreacted MPTA is washed out (*7*). Of three other closely related and approximately equally reactive quaternary ammonium maleimide derivatives, one depolarizes upon reaction slightly more than MPTA (approximately 2 mv) and two cause no depolarization (*9*). A compound of another type, *p*-(trimethylammonium)benzenediazonium fluoborate, has been reported to be an affinity label of the acetylcholine-receptor in the electroplax and not to cause any depolarization upon reaction (*10*). We report here two quaternary ammonium compounds which, like the maleimide derivatives, react covalently only with the reduced receptor. The resultant covalently attached quaternary ammonium groups interact with the active site causing in both cases a considerably greater depolarization than that obtained with the other compounds.

Bromoacetylcholine bromide (BAC) is an analog of acetylcholine which will react with nucleophiles substituting for bromide on the α-carbon of the acetyl group. BAC is also a substrate for acetylcholinesterase, as was previously reported (*11*), and in the following experiments BAC was applied to the electroplax in the presence of 50 μM eserine to inhibit the endogenous cholinesterase. Added to the innervated side of the electroplax, BAC acts as a receptor activator (Fig. 1). Repetitive application of BAC seems to have no irreversible effect on the electroplax. Following reduction of the receptor by dithiothreitol, application of BAC results in a response somewhat smaller than the response before dithiothreitol; however, the response is only partially reversed after BAC is washed out (Fig. 1). Furthermore, if the dithiothreitol-treated electroplax is treated with *N*-ethylmaleimide to alkylate the sulfhydryls formed by reduction, then BAC added subsequently still depolarizes, but in this case completely reversibly. Finally, the portion of the depolarization caused by the application of BAC to the dithiothreitol-treated electroplax which is not reversed by prolonged washing is reversed by $10^{-4}M$ *d*-tubocurarine (Fig. 1). With the removal of the *d*-tubocurarine the cell depolarizes again. We conclude that unreacted BAC is an activator of the receptor, that BAC reacts in the vicinity of the active site of the reduced receptor probably with a sulfhydryl group, and that the quaternary ammonium group of the covalently bound moiety interacts reversibly with the negative subsite activating the receptor and causing depolarization of the membrane. The reversible portion of the depolarization following BAC addition to the dithiothreitol-treated electroplax is then presumably due to BAC reversibly binding to unreduced receptors.

The second compound, the *p*-nitrophenyl ester of (*p*-carboxyphenyl)trimethylammonium iodide (NPTMB), is an active ester which can acylate a nucleophile such as a sulfhydryl group, releasing *p*-nitrophenol. Added to the innervated membrane of the electroplax, $10^{-4}M$ NPTMB (Fig. 2) elicits a barely detectable depolarization of about

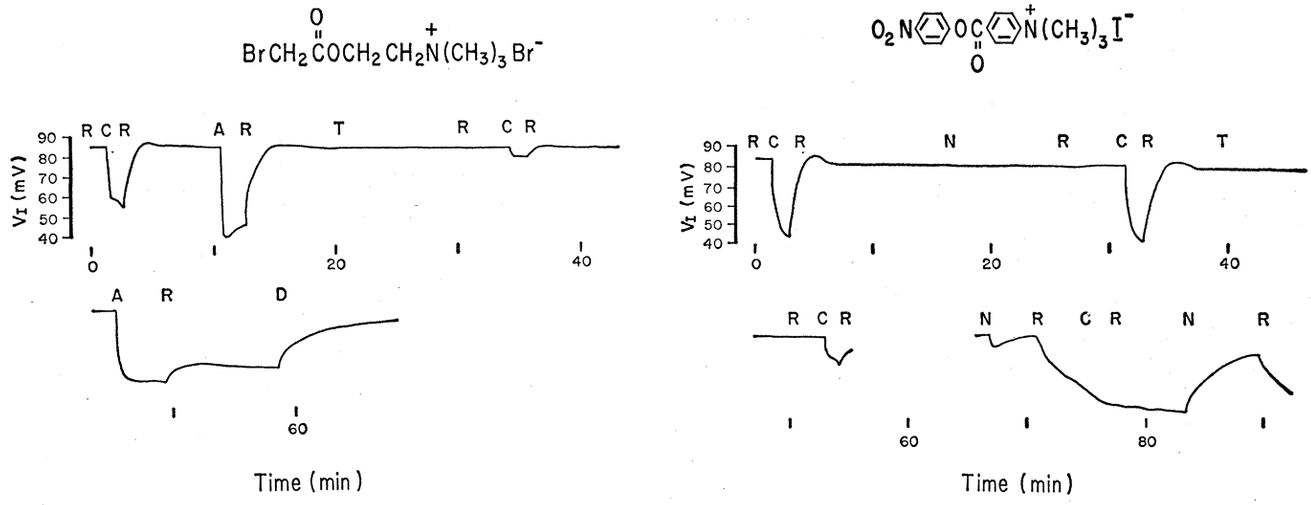

Fig. 1 (left). Effect of bromoacetylcholine bromide (BAC) on the dithiothreitol-treated electroplax. The cell was mounted and solutions were applied to the innervated membrane as previously described (*4*, *9*). The potential difference, outside minus inside, across the innervated membrane (V_I) was measured between an intracellular glass microelectrode and an agar bridge in the outside solution. The following solutions were applied to the innervated membrane where indicated: R, modified Ringer solution (*4*); C, 40 μM carbamylcholine in R; T, 1 mM dithiothreitol in tris-Ringer solution (*p*H 8.0); A, 20 μM BAC in R; D, 100 μM *d*-tubocurarine in R. All solutions except T contain 50 μM eserine. Fig. 2 (right). Effect of the *p*-nitrophenyl ester of (*p*-carboxyphenyl)trimethylammonium iodide (NPTMB) on the dithiothreitol-treated electroplax. The following solutions were applied to the innervated membrane where indicated: R, C, and T as in Fig. 1; N, 100 μM NPTMB in R. None of these solutions contain eserine.

0.5 mv which is reversed upon removal of NPTMB. However, NPTMB is a potent competitive inhibitor of the receptor, having a K_I of approximately $6 \times 10^{-6} M$. This inhibition is completely reversed when the NPTMB is washed out. In contrast to the reversible effect observed on the untreated electroplax, application of $10^{-4} M$ NPTMB after dithiothreitol-treatment causes a depolarization initially of a few millivolts which increases upon removal of the unreacted NPTMB. Reapplying $10^{-4} M$ NPTMB reverses the depolarization to the level obtained in the presence of the original NPTMB solution. Adding $10^{-5} M$ NPTMB after dithiothreitol results in a larger initial depolarization than with $10^{-4} M$ NPTMB, but the increase in depolarization after the unreacted NPTMB is washed out is smaller. NPTMB has no irreversible effect on the dithiothreitol-treated electroplax if preceded by *N*-ethylmaleimide. We conclude that NPTMB reacts near the active site of the reduced receptor probably with a sulfhydryl group and that the *p*-trimethylammoniumbenzoyl group thereby covalently attached interacts with the active site, activating the receptor. Unreacted NPTMB, however, is a potent reversible inhibitor of the receptor. Therefore, in the presence of excess NPTMB, the unreacted reagent competes with the covalently attached *p*-trimethylammoniumbenzoyl moiety for the active site. Although $10^{-4} M$ NPTMB reacts more rapidly with the reduced receptor than $10^{-5} M$ NPTMB, the $10^{-4} M$ NPTMB causes less of an initial depolarization than $10^{-5} M$ NPTMB because of competitive inhibition by the higher concentration of unreacted reagent. As with BAC, $10^{-4} M$ *d*-tubocurarine can reverse the depolarization due to the covalently attached moiety of NPTMB.

The action of BAC and of NPTMB on the dithiothreitol-treated electroplax is additional evidence for the contention that dithiothreitol reduces a disulfide bond close to the negative subsite of the receptor. The extent of depolarization resulting from the reaction of NPTMB, BAC, and the quaternary ammonium maleimide derivatives with the reduced receptor can be correlated with the length of the covalently attached moieties, and this correlation suggests that conformational changes of the active site accompany activation (*9*). In addition, the present experiments suggest that the active site of the receptor, at least after a disulfide bond has been reduced, is sufficiently flexible to permit competition between a free inhibitor and a covalently attached activator, assuming that they bind to the same site. The competitive inhibitors must be added at considerably greater concentrations to compete effectively with the covalently attached activators than with noncovalently attached activators. NPTMB (Fig. 2) is added at a 16-fold higher concentration and *d*-tubocurarine (Fig. 1) at a 400-fold higher concentration than their apparent K_I's (for *d*-tubocurarine, $2.5 \times 10^{-7} M$, see *2*) for the unreduced receptor. Flexibility of the specific binding sites of proteins has been postulated, and evidence for this concept has been obtained (for review, see *12*).

ISRAEL SILMAN*
ARTHUR KARLIN
Department of Neurology,
College of Physicians and Surgeons,
Columbia University, New York 10032

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* Present address: Department of Biophysics, Weizmann Institute of Science, Rehovoth, Israel.

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