*AFFINITY LABELING OF THE ACETYLCHOLINE-RECEPTOR**

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The monocellular electroplax of *Electrophorus* is a preparation particularly well suited for the study of the mechanism of action of specific ligands on the electrical parameters of an excitable membrane.[1] In the presence of low concentrations of compounds possessing a quaternary nitrogen function like acetylcholine (ACh), carbamylcholine (Carb), phenyltrimethylammonium (Pta), or decamethonium (Deca), the membrane potential of the cell decreases from -75 ± 15 millivolts (mV) in the resting cell to a steady-state value which depends on the nature and the concentration of the compound but is never lower than -15 ± 5 mV at saturating levels of the ligand. The effect of these receptor activators is specifically blocked by other quaternary nitrogen-containing compounds which will be referred to as receptor inhibitors like d-tubocurarine (d-Tubo), flaxedil, and several others, which stabilize the membrane potential at its resting value. All of them are postulated to bind to the same site on the membrane—the ACh-receptor site.[2] In order to account for the remarkable specificity of recognition involved in the action of these ligands, the ACh-receptor site was further postulated to be part of a macromolecule, presumably a protein, and compared with the active center of an enzyme[2] or the regulatory site of an allosteric protein.[3]

On the basis of these analogies it is of interest to study the ACh-receptor site with some of the methods which provided fruitful information about the amino acid side-chains constituting the active centers of enzymes and antibodies. In an *affinity-labeling* experiment[4] a reagent is used which (1) exhibits a high affinity for the binding site; (2) forms an irreversible, covalent bond with certain amino acid residues of the site. This technique has been used to label specifically the active sites of several enzymes[5] and antibodies[4, 6] and it has been suggested as a means of labeling and identifying specific proteins of membranes.[7]

In this communication, observations are reported and discussed about the effect of p-(trimethylammonium)benzenediazonium fluoroborate (Tdf) on the electroplax synaptic membrane. This compound, which has been used successfully as an affinity-labeling reagent for anti-p-azotrimethylphenylammonium antibodies[8] and for bovine erythrocyte ACh-esterase,[9] possesses the following characteristics: (1) it is a structural analogue of phenyltrimethylammonium, a typical and potent activator of the ACh-receptor;[10] (2) it has a reactive diazonium group which forms a covalent bond mainly with tyrosine, histidine, and lysine side-chains in proteins. Evidence is presented here that the ACh-receptor site of the electroplax synaptic membrane can be specifically and irreversibly labeled by Tdf.

Results.—(*I*) Figure 1 shows the result of a 20-minute exposure of an electroplax to 10^{-4} *M* Tdf. The resting potential and the resistance of the innervated membrane remain constant during the treatment. After extensive washing, exposure of the cell to solutions of a variety of receptor activators, at concentrations about 10 times larger than their apparent dissociation constants, is not followed by any

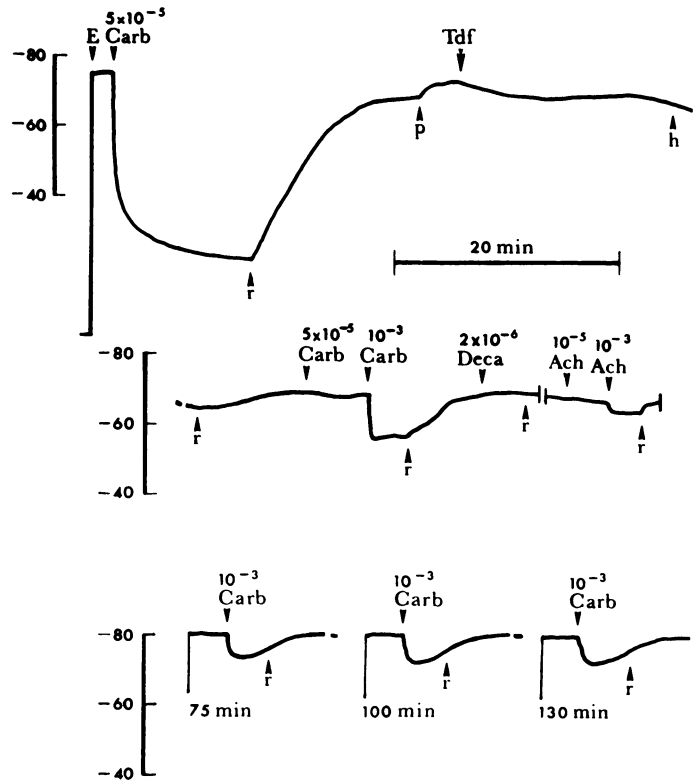

FIG. 1.—Irreversible abolition of the sensitivity to receptor activators after a 20-min exposure to Tdf. Isolated electroplax.[1] Ordinate, membrane potentials in mV; abscissa, time. *E*, empalement; *r*, Ringer's solution; *p*, Ringer's solution supplemented with 5×10^{-2} *M* Na-phosphate pH 5.9; *Tdf*, 10^{-4} *M* in p; *h*, 10^{-3} *M* l-histidine in Ringer's solution to quench unreacted Tdf; *Carb*, carbamylcholine chloride; *Deca*, decamethonium bromide; *ACh*, acetylcholine bromide. Solutions (moles/liter) in r. Tdf prepared by the method of Traylor and Singer,[7] stored as a 2×10^{-3} *M* solution in 0.01 *N* HCl at −20°C and diluted in p immediately before use. *Lower line:* 10^{-3} *M* Carb is applied 75, 100, and 130 min., after the end of the Tdf exposure.

significant change of the membrane potential or of the membrane resistance. The Tdf treatment renders the cell insensitive to receptor activators. However, with concentrations of these activators as large as 10^{-3} *M* (more than 100 times their apparent dissociation constant) a residual depolarization of about 10 mV is still observed. This residual response may be conveniently used to follow the Tdf modification as a function of time and, in particular, to test whether the cell recovers its sensitivity or not. Figure 1 illustrates that the residual response is exactly the same 20 minutes after the end of the exposure to Tdf and 130 minutes later. The modification of the cell is irreversible.

In order to test the possibility that this modification is due to the experimental procedures (e.g., perfusion with a Ringer's solution supplemented with 5×10^{-2} *M* Na phosphate pH 5.9) rather than to a specific reaction of Tdf with the membrane, the same exposure was performed with an unreactive derivative of Tdf. This derivative is obtained by adding to a 10^{-4} *M* Tdf solution at pH 5.9 an excess of histidine ($10^{-2}M$). After 19 hours at room temperature the diazonium group of

TABLE 1

SPECIFICITY OF THE EFFECT OF TDF

	Control response (%)
(1) 10^{-4} *M* Tdf	0
(2) 10^{-5} *M* Tdf	106
(3) 10^{-4} *M* Ddf	94
(4) 10^{-4} *M* Tdf + 10^{-4} *M* histidine (after 1 min)	0
(5) 10^{-4} *M* Tdf + 10^{-2} *M* histidine (after 19 hr)	87
(6) 10^{-4} *M* Tdf + 10^{-5} *M* d-tubocurarine	83
(7) 10^{-4} *M* Tdf + 10^{-5} *M* flaxedil	64
(8) 10^{-4} *M* Tdf + 10^{-4} *M* dimethyl-d-tubocurarine	60
(9) 10^{-4} *M* Tdf + 10^{-4} *M* DFP	0
(10) 10^{-4} *M* Tdf + 10^{-4} *M* phospholine	0

Same experimental procedure as in Fig. 1. The response of the cell to 5×10^{-5} *M* Carb is tested after the indicated treatment and expressed as per cent of the control response measured before the treatment. (3) Ddf, p-dimethylaniline diazonium zinc chloride, (4) and (5) Tdf and histidine were mixed 1 min (4) or 19 hours (5) before the treatment; (6–10) before exposure to Tdf + inhibitor the cells were preincubated 5–10 min with a solution of inhibitor in p.

Tdf has reacted with histidine giving p-(trimethylammonium)benzeneazo histidine. A standard exposure to this mixture does not modify the cell which still responds normally to carbamylcholine (Table 1). The irreversible abolition of the sensitivity to receptor activators is thus caused by the covalent bonding of Tdf to the membrane.

(*II*) The irreversible effect of Tdf on the sensitivity to ACh might be due (1) to the permanent occupation of the ACh receptor site by Tdf covalently bonded to an amino acid side-chain belonging to or adjacent to this site, or (2) to the block of an intermediate step in the sequence of macromolecular processes mediating the interaction *between* the ACh-receptor site and the structures which support the membrane potential. In order to distinguish between these two possibilities the effect of various reversible receptor activators and inhibitors on the reaction of Tdf with the membrane was tested. Figure 2 shows the effect of phenyltrimethlam-

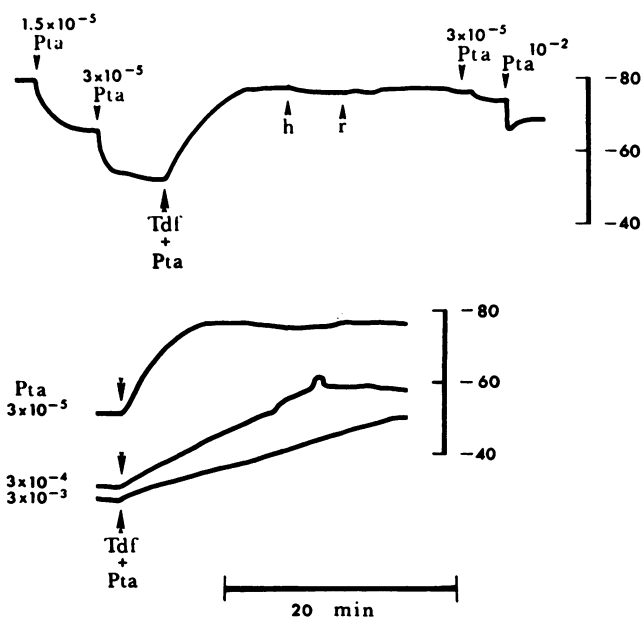

FIG. 2.—Antagonism between Tdf and a reversible receptor activator phenyltrimethylammonium (Pta). *Upper:* Irreversible repolarization upon addition of Tdf. Cell first equilibrated with p; solutions of Pta in p. Tdf + Pta: 10^{-4} *M* Tdf is added *in the presence* of 3×10^{-5} *M* Pta. *Lower:* Rates of irreversible repolarization in the presence of increased Pta concentrations. The cell is first equilibrated with Pta in p at the indicated concentration, then 10^{-4} *M* Tdf is added without changing the Pta concentration. For abbreviations see Fig. 1.

monium (Pta), a receptor activator which is a structural analogue of Tdf. In the presence of Pta the membrane potential decreases and reaches a steady state. If, at this point, Tdf is added, without changing the concentration of Pta, a repolarization of the membrane occurs. As illustrated by Figure 2, this repolarization is irreversible and is thus the consequence of the covalent bonding of Tdf. Tdf antagonizes the action of Pta. If both compounds are competing for the same binding site on the membrane, then the role of the Tdf-induced repolarization should depend upon the concentration of Pta: the rate should decrease with increased concentrations of Pta. Figure 2 shows that this is indeed the case.

A similar competition is observed between Tdf and a typical and potent receptor inhibitor: d-tubocurarine (Table 1, Fig. 3). In the presence of this compound the membrane potential does not change. Added with Tdf, 10^{-5} *M* d-Tubo is a concentration sufficiently high to protect almost completely against the irreversible effect of a 10 times larger concentration of Tdf. The same result is observed with two other specific receptor inhibitors: dimethyl d-Tubo and flaxedil (Table 1). The interpretation of these last results is not without ambiguity since at least one of these effectors can react with Tdf. Pta, flaxedil, dimethyl d-Tubo are unreactive, but d-Tubo contains phenolic rings which could form a bond with the diazonium group of Tdf. Fortunately, the rates of reaction of Tdf and d-Tubo in solution, at the concentrations used in this experiment, are considerably slower than the rate of reaction of Tdf with the membrane. Nevertheless, the following control experiment was performed: the cell is treated with Tdf for 20 minutes according to the standard procedure except that the 10^{-4} *M* solution of Tdf is mixed with 10^{-4} *M* histidine immediately prior to the incubation period. The data of Table 1 clearly show that, even in this case, the presence of 10^{-4} *M* histidine in the Tdf solution has not been followed by a decrease of Tdf reactivity within the time limits of the experiment. Thus the protective effect exerted by various receptor inhibitors and activators cannot be accounted for by direct reaction with Tdf, but must be due to competition with Tdf at the level of the ACh-receptor site.

A specific bonding of Tdf to the ACh-receptor site is further supported by an experiment performed with the tertiary analogue of Tdf, p-dimethylanilinediazonium zinc chloride (Ddf). The substitution of the quaternary nitrogen of ACh by a tertiary one is accompanied by a 250-fold decrease of the potency of the compound. Similarly, Table 1 shows that a standard treatment of the cell by 10^{-4} *M* Ddf has almost no effect on the chemosensitivity of the cell. Tdf thus possesses the structural features of a compound reacting specifically with the ACh-receptor.

In Figure 3 are presented titration curves by carbamylcholine of electroplax exposed for various periods of time to 10^{-4} *M* Tdf. A characteristic parameter of such titration curves is the amplitude of the maximal depolarization recorded at saturating levels of the activator. This maximal response is generally considered to be quantitatively related to the total number of ACh-receptor sites occupied by the ligand.[11] Assuming this interpretation to be correct, if each receptor covalently bound to a Tdf molecule is inactive, then the maximal response to a receptor activator should decrease progressively as a function of the time of exposure. Figure 3 shows that this is indeed the case, a further indication in favor of the selective inactivation of the ACh-receptor site by Tdf.

(*III*) The active center of ACh-esterase and the ACh-receptor site have some

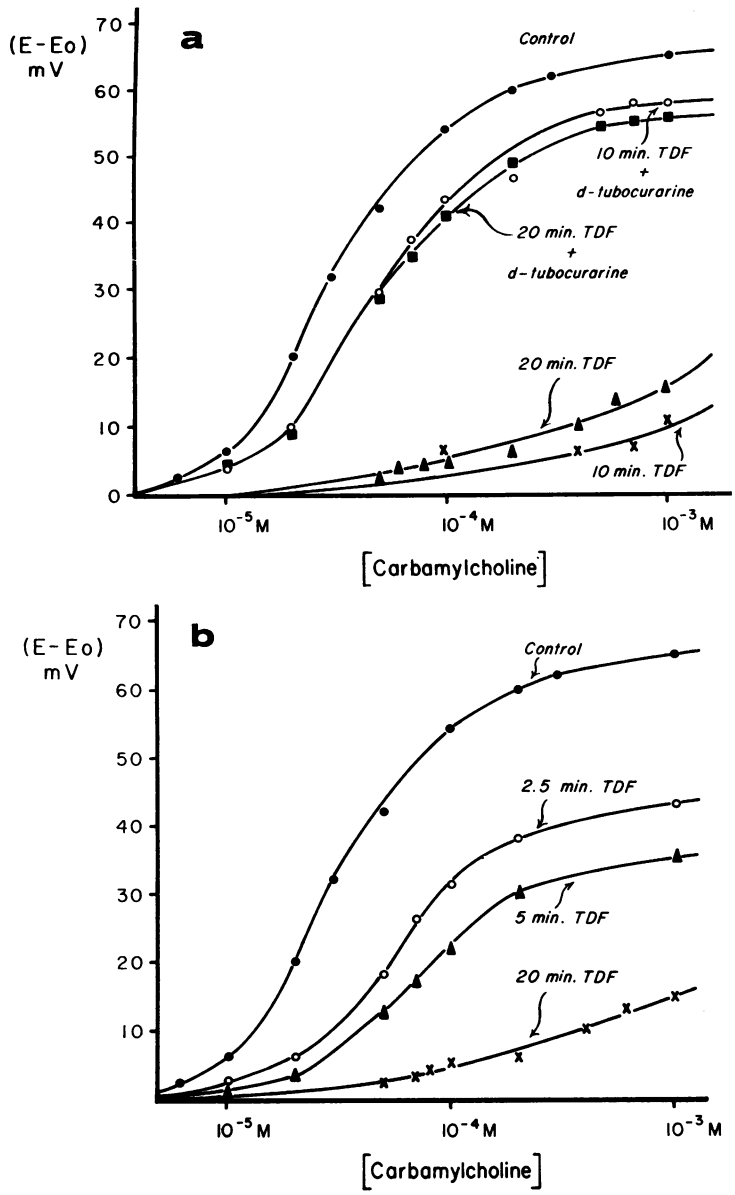

FIG. 3.—Response of electroplax to increased concentrations of carbamylcholine after various Tdf exposures. *Ordinate*: E, steady-state potentials recorded in the presence of a given concentration of Carb; E_0, resting potential in the absence of Carb (-75 mV $\pm$ 10 mV). *Abscissa:* log Carb concentrations. (*a*) Cells exposed 10 and 20 min to 10^{-4} M Tdf in the presence or absence of 10^{-5} M d-Tubo (see Table 1). (*b*) Cells exposed to 10^{-4} M Tdf from 2.5 to 20 min.

structural properties in common but differ in several respects.[10, 12] Since Tdf reacts with the ACh-receptor site, it constitutes a useful tool to investigate the relationships between the ACh-esteratic site and the ACh-receptor site and, in particular, to test whether or not the esteratic site of the enzyme is directly involved in the cell sensitivity to ACh. We therefore studied the effect of irreversible

inhibitors of ACh-esterase on the reaction of Tdf with the ACh-receptor site. If the ACh-receptor site and the esteratic site are either closely related or identical, then the block of the esteratic center by an irreversible inhibitor should prevent,

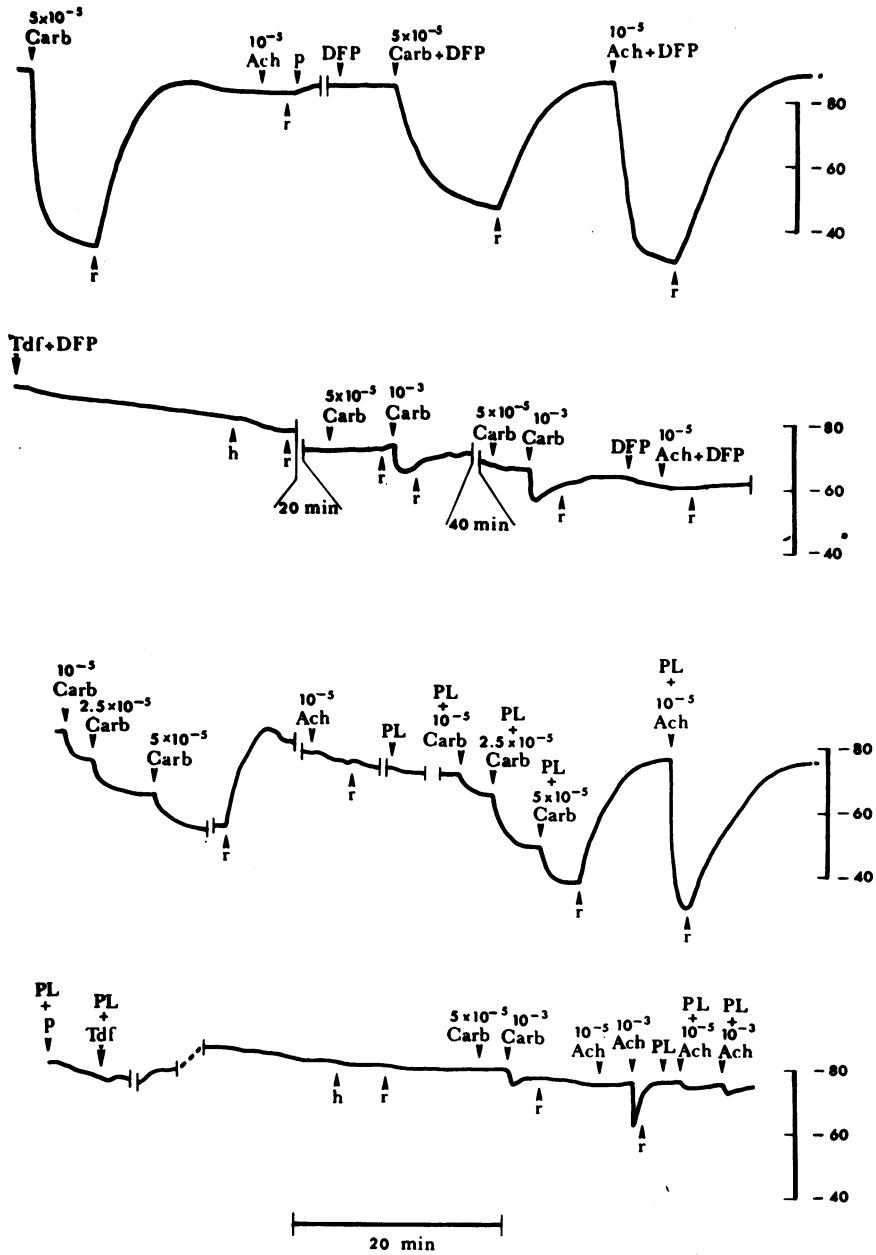

FIG. 4.—Absence of antagonism between Tdf and two irreversible inhibitors of ACh-esterase: DFP and phospholine. *First and third lines:* 10^{-4} *M* DFP or 10^{-4} *M* phospholine (PL) potentiate the response to ACh without changing the response to Carb. *Second and fourth lines:* Presence of 10^{-4} *M* DFP or PL does not prevent the action of 10^{-4} *M* Tdf. For abbreviations see Fig. 1.

or at least decrease, the loss of sensitivity caused by the Tdf treatment. Diisopropylphosphorofluoridate (DFP) and diisopropylphosphorylthiocholine (phospholine) were used as substrates of ACh-esterase which give a stable acylation of the enzyme.[13] The effective block of ACh-esterase in the membrane by these inhibitors was ascertained by comparing the effects of ACh before and during the exposure to DFP or phospholine. Figure 4 shows that in the presence of an ACh-esterase inhibitor the action of ACh is strongly potentiated, but that under the same conditions, there is no change in the sensitivity of the cell to receptor activators such as carbachol which are not ACh-esterase substrates. Finally, it illustrates that even when ACh-esterase is irreversibly inhibited, the loss of sensitivity proceeds as fast as in a normal cell. The stable acylation of the esteratic site of ACh-esterase does not interfere with the binding of Tdf to the ACh-receptor site. This observation, in agreement with previous experimental results,[12] suggests that the esteratic center of ACh-esterase and the ACh-receptor site are, at least partially, distinct binding sites.

Discussion.—Exposure of the synaptic membrane of the electroplax to 10^{-4} *M* Tdf for 20 minutes abolishes irreversibly its sensitivity to ACh and to related receptor activators. Since d-Tubo and several specific receptor inhibitors or activators strongly protect against Tdf action, while irreversible acylation of the catalytic center of ACh-esterase does not interfere with it, it is concluded that Tdf is an affinity-labeling reagent of the ACh-receptor site, a site topographically distinct from the esteratic site of ACh-esterase.[12–14] Moreover, diazonium derivatives like Tdf are known to establish covalent bonds mainly with tyrosyl, histidyl, and lysyl residues. It is likely that Tdf attaches covalently to an amino acid of the ACh-receptor site, thus supporting the earlier suggestion that the ACh-receptor macromolecule is a protein.[2, 15] Conclusive evidence in favor of this last point, however, requires the isolation and purification of the component to which Tdf is bound.[16]

Appendix.—The present observations are a further important support of the analogy already mentioned between excitable membranes and regulatory enzymes.[3, 17] Both categories of biological structures are "excitable" in the sense that they respond to the binding of specific ligands by characteristic changes of their biological-electrical or catalytic-activity; in addition, both of them exhibit a complex combination of cooperative phenomena.[17] The effect of Tdf on the electroplax synaptic membrane is remarkably similar, in its consequences, to the "desensitization phenomenon" described with regulatory enzymes.[18, 19] Tdf abolishes the sensitivity to ACh and to other receptor activators without altering the resting potential and the resistance of the synaptic membrane. Similarly, regulatory enzymes which have been desensitized no longer respond to their metabolic signal but are still fully active. Several molecular mechanisms have been invoked to account for the desensitization of regulatory enzymes.[19] For instance, the structure of the site at which the regulatory signal binds can be locally modified in such a way that it no longer recognizes this ligand.[20] The same interpretation is valid in the present case, and we may say that Tdf "desensitizes" the membrane by blocking selectively the ACh-receptor site considered as a "regulatory site." It was concluded that the catalytic site of these enzymes is entirely distinct from their regulatory site. Similarly it becomes plausible that the ACh-receptor site and the sites on the membrane which are directly responsible for the resting potential are topographically distinct and thus that the interaction between these sites is an indirect or "allosteric" interaction.

The analogy between regulatory enzyme and excitable membranes may be extended even further to the mechanism of these indirect interactions. It was proposed by Nachmansohn more than ten years ago that the changes of membrane potential caused by the binding of a receptor activator were triggered by a change of conformation of the macromolecular receptor of ACh.[21] This mecha-

nism would be equivalent to the allosteric transition which mediates the indirect interactions between topographically distinct sites in regulatory enzymes.[19] A two-state model[22] has been conveniently used in this latter case for the quantitative description of the transition and seems adequate for the interpretation of the present results.[3, 17] One of the conformational states of the receptor macromolecule would correspond to a depolarized state of the membrane and would be preferentially stabilized by receptor activators; the other, corresponding to the polarized state of the membrane, would be stabilized by receptor inhibitors. This model accounts for several aspects of the mode of action of receptor activators and inhibitors and for their mutual interactions.[17] In this framework, Tdf would not only occupy permanently the ACh-receptor site but would stabilize irreversibly the receptor macromolecule, and thus the membrane, in the polarized conformation.

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[14] This observation is still consistent with the hypothesis that the ACh-receptor site might be some allosteric site of the ACh-esterase molecule.[3]

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