STABILIZATION OF THE HIGH AFFINITY STATE OF THE MEMBRANE-BOUND ACETYLCHOLINE RECEPTOR FROM *TORPEDO MARMORATA* BY NONCOMPETITIVE BLOCKERS

Evidence for dual interaction and pharmacological selectivity

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1. Introduction

At the neuromuscular junction and the electromotor synapse, a series of compounds referred to as noncompetitive blockers, inhibit the permeability response elicited by acetylcholine (ACh) in a noncompetitive manner (reviews [1,2]). They include, among others, the aminated local anesthetics proadifen and dimethisoquin [3–7], the frog toxin histrionicotoxin (HTX) and its derivatives [8–11], the hallucinogenic drug phencyclidine [12–14]. These pharmacological agents block the physiological response in the μM range and their binding to saturable sites on ACh-receptor-rich membrane fragments isolated from *Torpedo* electric organ has been demonstrated with the radiolabelled derivatives [^{3}H]meproadifen [6], [^{3}H]trimethisoquin [15], [^{3}H]perhydro-HTX [11, 16–18] and [^{3}H]phencyclidine [12,19,20]. A number of structurally unrelated compounds usually considered as primarily interacting with the lipid phase of the membrane, such as the detergents Triton X-100 or Na-cholate [21,22], fatty acids [21,36], phospholipases (reviews [23,27]), general anesthetics and alcohols [7,24] also block the response to ACh in a noncompetitive manner. All of them regulate the affinity of the ACh binding site in an 'allosteric' manner and stabilize, to various extents, the high affinity 'desensitized' state of the ACh-receptor, even in the absence of agonist [5,7]. Additive effects between the detergent Na-cholate and the aminated local anesthetic trimethisoquin [25] as well as between the alcohol 2-propanol and perhydro-HTX [7] have been demonstrated and it was thus suggested that the noncompetitive blockers might exert their effect via two distinct modes of interaction:

(i) They might bind to the saturable high affinity sites identified on the ACh-receptor-rich membranes;
(ii) They might change, in a non-specific manner, the physical properties of the lipid bilayer surrounding the membrane-bound ACh-receptor.

Here, we show that the effects of membrane perturbers such as Triton X-100 or Nonidet P40, at concentrations up to their critical micellar concentrations, can be antagonized by the potent noncompetitive blocker perhydro-HTX, and conversely, that the effects of several local anesthetics among which the aminated local anesthetics dimethisoquin and its quaternary derivative trimethisoquin are insensitive to perhydro-HTX. These data suggest that:

(i) Noncompetitive blockers, in general, may stabilize the high affinity state of the ACh-receptor primarily upon binding to saturable sites;
(ii) There should exist at least two classes of such binding sites with distinct pharmacological selectivity at the level of the ACh-receptor.

Abbreviations: ACh, acetylcholine; HTX, histrionicotoxin; Dns-C_6-Cho, (1-(5-dimethylaminonaphthalene) sulfonamido) *n*-hexanoic acid β-(*N*-trimethylammonium bromide) ethyl ester

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2. Materials and methods

2.1. *Preparation of ACh-receptor-rich membrane fragments*

ACh-receptor-rich membrane fragments were purified from the electric organ of *Torpedo marmorata* as in [26] with modifications to limit proteolysis as in [27], and were kept in liquid nitrogen until use. ACh-receptor sites were quantitated by column filtration as in [26], using ^{125}I-labelled α-bungarotoxin.

2.2. *Rapid kinetics of Dns-C_6-Cho binding*

The fractional concentration of receptors in the high affinity 'desensitized' state was determined by fast kinetic analysis of the binding of the fluorescent cholinergic agonist Dns-C_6-Cho, as in [5,28–30]. Membrane fragments were diluted to a final concentration of ~0.1–0.2 μM α-toxin binding sites in a *Torpedo* physiological solution supplemented or not with given concentrations of noncompetitive blockers, and were rapidly mixed with a solution of Dns-C_6-Cho in the same medium in a Durrum stopped-flow apparatus equipped for fluorescence detection. The fractional amplitude of the relaxation process relative to the binding of Dns-C_6-Cho to the high affinity 'desensitized' receptor sites (the 'rapid' relaxation process, see [5,28,29]) was determined by a non-linear iterative regression analysis as in [29].

2.3. *Chemicals*

Dns-C_6Cho [31,32], trimethisoquin and meproadifen were a gift from G. Waksman who synthetized them. Perhydro-HTX was a gift from Professor J. Daly. Prilocaine, lidocaine and dimethisoquin were a gift from the Laboratoire Roger Bellon (France), and proadifen a gift from Smith, Kline and French. Nonidet P40 was from BDH Chemicals Ltd., dibucaine from K and K Labs. and Triton X-100, Lubrol PX and chlorpromazine from Sigma.

3. Results

As illustrated in fig.1 and extensively described in [5,28–30], rapid mixing of ACh-receptor-rich membranes with the fluorescent cholinergic agonist Dns-C_6-Cho results in an increase of fluorescence intensity which develops in the millisecond to second time

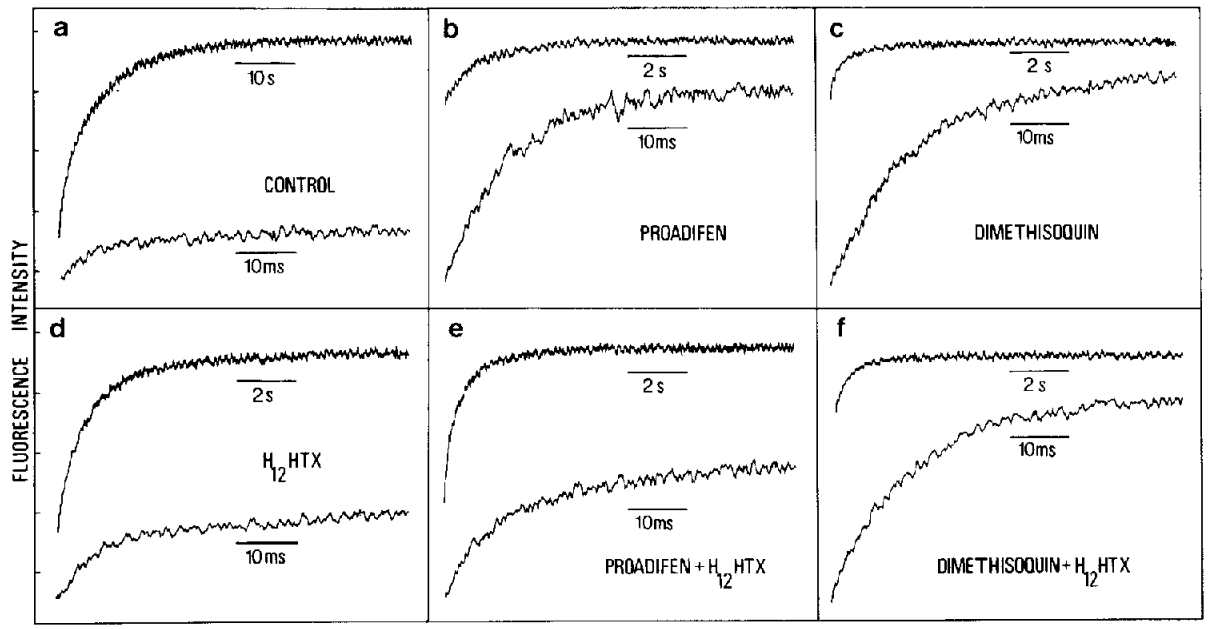

Fig.1. Single-shot traces of stopped-flow experiments: effect of noncompetitive blockers. λ_{ex} = 290 nm, λ_{em} > 540 nm (see [29]); 1:1 mixing of a suspension of ACh-receptor rich membrane fragments (0.2 μM α-toxin sites) in *Torpedo* saline solution (250 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM sodium phosphate (pH 7)) supplemented or not with given concentrations of noncompetitive blockers with a solution of Dns-C_6-Cho (3 μM) in the same medium: (a) control; (b) 12 μM proadifen; (c) 20 μM dimethisoquin; (d) 10 μM perhydro-HTX; (e) 10 μM perhydro-HTX + 12 μM proadifen; (f) 10 μM perhydro-HTX + 20 μM dimethisoquin; 'rapid' relaxation processes are represented on expanded time scales.

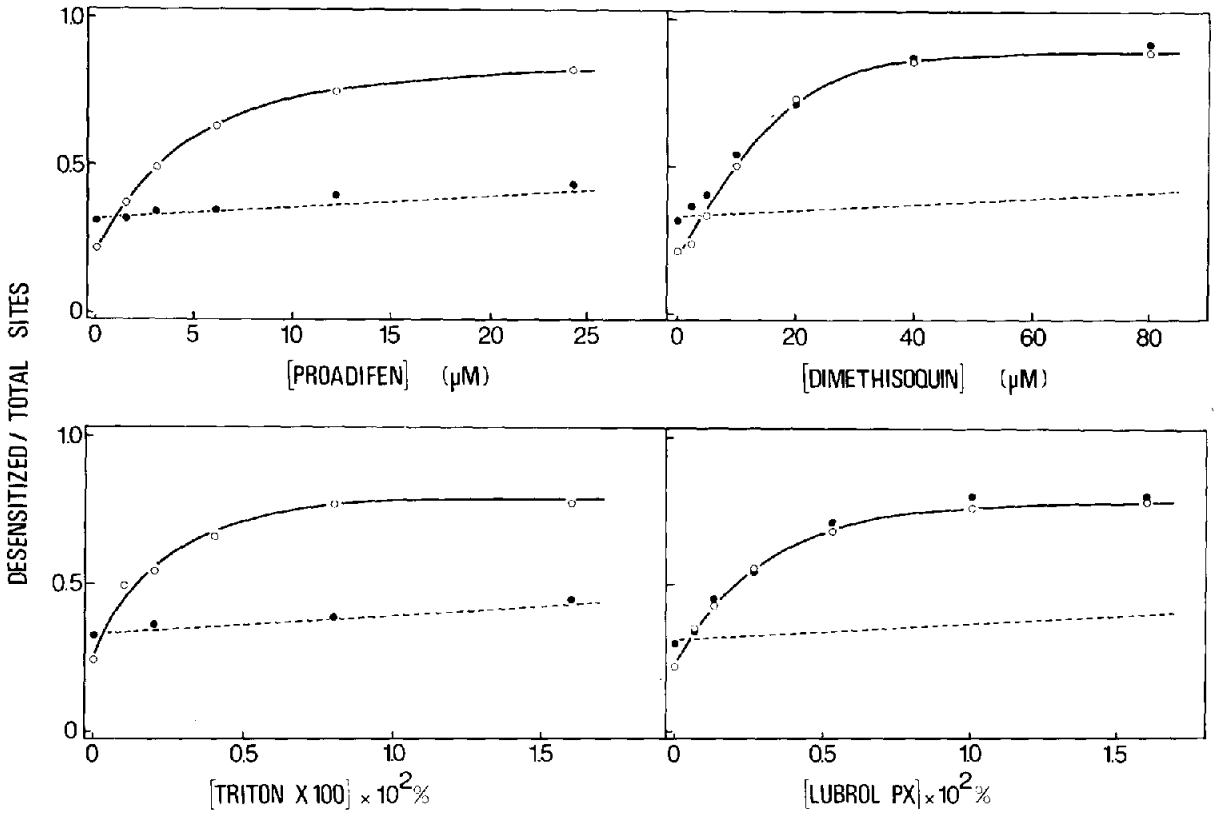

Fig.2. Stabilization of the high affinity state of the receptor by noncompetitive blockers and differential effects of perhydro-HTX. Ordinates: fraction of ACh-receptor sites in the high affinity 'desensitized' state following preincubation with proadifen, dimethisoquin, Triton X-100 and Lubrol PX, in the absence (○) or presence (●) of 10 μM perhydro-HTX, as a function of the concentration of these noncompetitive blockers. The dotted lines (– – –) are the theoretical values, assuming a unique site of action for noncompetitive blockers, and calculated according to eq. (1) (see text) with a K_d-value for perhydro-HTX equal to 0.3 μM ([16], unpublished). The fraction of ACh-receptor sites in the high affinity state was determined from the fractional amplitude of the 'rapid' relaxation process (see text and [5,29]) and experimental conditions are the same as in fig.1.

range, according to, at least, 3 distinct processes: rapid, intermediate and slow. At saturating concentration of agonist, the relative amplitude of the 'rapid' relaxation process (represented with an expanded time scale in fig.1) can be taken as an index of the fraction of ACh-receptor sites which exist, prior to agonist addition, in a high affinity 'desensitized' state and bind the agonist following a rapid and diffusion-controlled reaction [29]. According to this criterion, in the native membranes under resting conditions (see fig.1a), ~20% of the receptor sites are in a high affinity state, in reversible equilibrium with the remaining 80% of sites, of lower affinity, which account for the physiological response.

Preincubation of the ACh-receptor-rich membrane fragments with noncompetitive blockers such as proadifen or dimethisoquin (fig.1b,c) results in an increase of the amplitude of the rapid relaxation process monitored upon mixing with Dns-C_6-Cho. These noncompetitive blockers, thus shift the equilibrium between the low and high affinity states of the ACh-receptor towards the high affinity state (see [5] for a detailed analysis). Quantitation of this effect (see fig.2) yields values for half-maximum effect, in the 3–30 μM range for proadifen, dimethisoquin, Triton X-100 and Lubrol PX. Values of maximum effect are close to unity for the 4 noncompetitive blockers of fig.2, but can be smaller for instance in the case of Na-cholate [25], phospholipasic toxins [5], HTX [5], and also, as illustrated in fig.1d (see also [7]), in the case of its hydrogenated derivative perhydro-HTX.

Preincubation of the ACh-receptor-rich membrane fragments with perhydro-HTX at concentrations as high as 10 μM (i.e. ~50-times its equilibrium dissocia-

tion constant, see [16,17]) results only in a limited increase of the amplitude of the rapid relaxation process (from 21± 2%–33± 2%). This limited effect, which can simply be accounted for by assuming that perhydro-HTX non-exclusively binds (see [33]) to both the resting and desensitized states (see [5] for a theoretical treatment in the case of HTX) renders possible a quantitative analysis of the effect of non-competitive blockers under conditions of perhydro-HTX site occupancy.

Membrane fragments were therefore preincubated with a saturating concentration of perhydro-HTX (10 μM, see fig.1d–f) supplemented with increasing concentrations of noncompetitive blockers. In these conditions, two classes of effects were observed:

(i) With some noncompetitive blockers such as dimethisoquin or the detergent Lubrol PX, the stabilization of the high affinity state was not affected by perhydro-HTX; in particular, as shown in fig.2, the values for half-maximum stabilization by the noncompetitive blockers tested did not change.

(ii) On the contrary, with others, such as proadifen but also the detergent Triton X-100, perhydro-HTX almost completely abolished the stabilization of the high affinity state (see fig.1e,2).

A variety of noncompetitive blockers were tested by this method and are listed in fig.3. Compounds which possess the structure of aminated local anesthetics or compounds known as detergents may exert anyone of the two classes of effects.

Assuming that the allosteric regulation of the ACh-receptor by a noncompetitive blocker is mediated via a unique class of sites, the presence of perhydro-HTX should result in a shift of the dose–response curves for stabilization of the high affinity state (fig.2) toward the high concentration range, with values for half-maximum stabilization, K_{app} equal to:

$$K_{app} = K^{o}_{app}\,(1 + (H_{12}\ HTX)/K_d) \qquad (1)$$

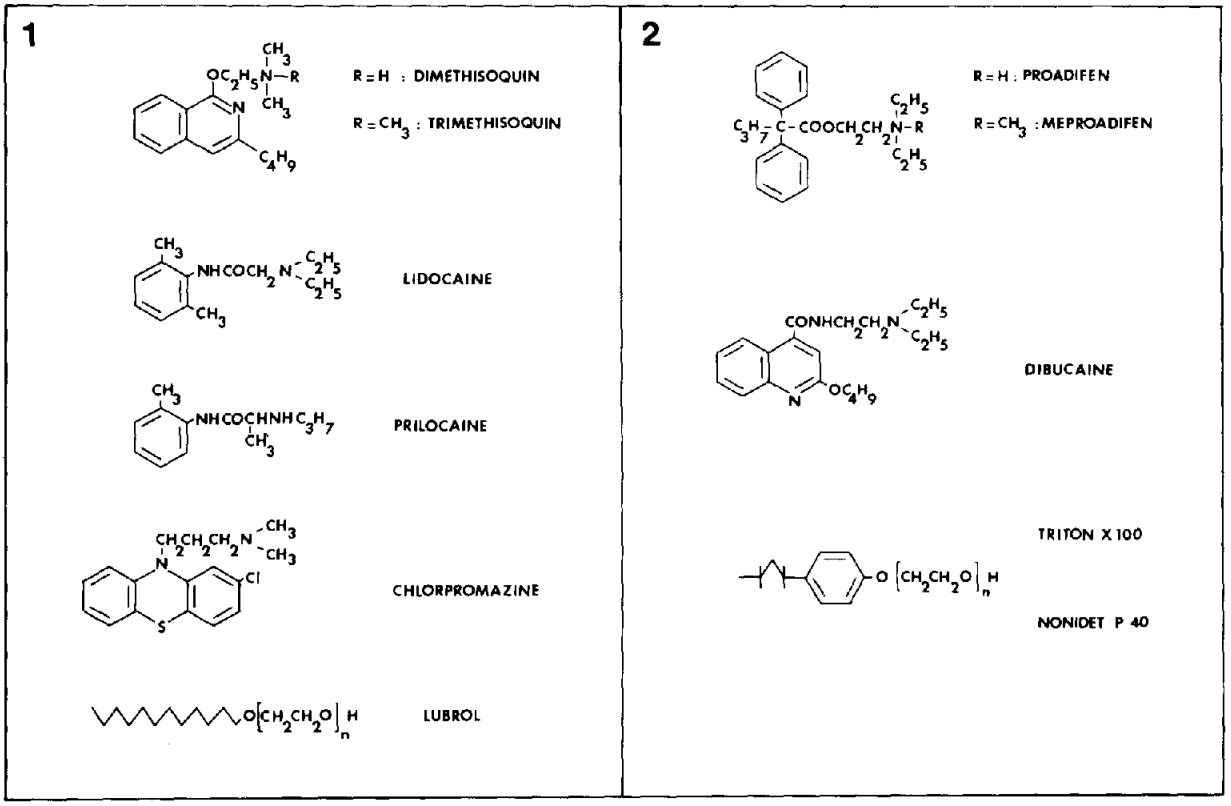

Fig.3. Pharmacology of the two classes of noncompetitive blockers. Noncompetitive blockers were tested as in fig.1,2. They fall into two groups: (1) dimethisoquin and noncompetitive blockers whose effects are insensitive to perhydro-HTX; (2) proafiden and noncompetitive blockers whose effect are blocked by perhydro-HTX.

where K_d is the equilibrium dissociation constant for the binding of perhydro-HTX, and K^o_{app} the value for half-maximum stabilization in the absence of perhydro-HTX. Accordingly, K_{app} should be ~50-times higher than K^o_{app}. Clearly, this is not the case for dimethisoquin and homologues, whereas for proadifen, Triton X-100 and homologues such an interpretation appears plausible (fig.2, dotted line).

4. Discussion

These data disclose that several local anesthetics such as di- and trimethisoquin, lidocaine and prilocaine, stabilize the high affinity state of the ACh-receptor in a manner which is insensitive to perhydro-HTX. On the other hand, several membrane perturbers, such as the detergents Triton X-100 or Nonidet P40 at levels up to their critical micellar concentrations, exert similar effects that perhydro-HTX blocks. A plausible interpretation of these data, then, is that at least two classes of saturable binding sites for non-competitive blockers, 'perhydro-HTX-sensitive' and 'perhydro-HTX-insensitive', exist on the membrane bound ACh-receptor and that both typical local anesthetics and general membrane perturbers differentially interact with these sites. In their low concentration range, dimethisoquin, lidocaine, Lubrol PX and homologues (fig.3) would stabilize the high affinity state of the ACh-receptor upon binding to the perhydro-HTX-insensitive sites, whereas proadifen, Triton X-100 and homologues (fig.3) would exert their action at the level of the perhydro-HTX-sensitive ones. These data, of course, do not exclude that effectors from one class interact with the alternate class of sites, either in a non-exclusive manner or/and in a distinct concentration range. Perhydro-HTX itself might bind to the sites where dimethisoquin and homologues primarily exert their action but, in a higher concentration range.

This interpretation is compatible with the observed displacement of radiolabelled aminated local anesthetics and perhydro-HTX by detergents below their critical micellar concentration [7,15]. It might account for the heterogeneity of binding reported for [^{3}H]trimethisoquin with ACh-receptor-rich membrane fragments, and for the differential effects of HTX on this binding [15]. It could also explain the results obtained upon covalent labelling by UV irradiation of the polypeptide chains of the ACh-receptor protein with radioactive noncompetitive blockers [34]. With all of them, the δ chain (66 000 M_r) was found to be primarily labelled but with some noncompetitive blockers such as [^{3}H]chlorpromazine, other chains were also found radioactive, a result compatible with the occurrence of more than one category of binding sites for these effectors on the ACh-receptor protein. An attractive hypothesis would be that each of the 4 ACh-receptor polypeptide chains, which exhibit important sequence homologies [35], carries a site to which the noncompetitive blockers would bind with distinct affinities and selectivities. Following this scheme, one may consider the possibility that these sites are in fact homologous to the ACh-receptor site carried by the α-chain, and even contribute to the physiological response upon binding ACh in a high concentration range. Binding studies with radiolabelled noncompetitive blockers are further developed to identify and characterize these sites, to determine their stoichiometry, and to understand their physiological significance.

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