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Conference Paper

Actions of Histrionicotoxin on Acetylcholine Receptors

G. Kato

Neurobiologie Moléculaire, Institut Pasteur, Paris 75015, France
and

M. Glavinovic, J. Henry, K. Krnjevic, E. Puil, and B. Tattrie

Department of Research in Anaesthesia, Mc Gill University, Montréal, Canada

The effects of histrionicotoxin have been evaluated at the rat neuromuscular junction, the eel electroplaque and on the central nervous system of the cat, and have been shown to reduce markedly the response to acetylcholine or carbamylcholine. This toxin does not inhibit the binding of acetylcholine to *Torpedo* membrane fragments rich in acetylcholine receptor protein, but rather augments its affinity for the receptor site. These findings suggest an accentuation of a desensitization phenomenon or a block of ionic channels.

INTRODUCTION

Most of the articles in this volume are concerned with efforts to characterize the nicotinic acetylcholine receptor and acetylcholinesterase of skeletal muscle and electric organs and the relationship between these two proteins in the cell membrane. It is evident from these papers that the α-toxins from snake venoms have provided ideal reagents for assaying, isolating, purifying and characterizing these receptors and as a result progress in this area of research has been rapid in the past few years[1,2]. Although our knowledge of the structure and function of the second protein in the post-synaptic surface, acetylcholinesterase, is progressing rapidly, the exact location of this enzyme is currently in doubt. The most recent evidence suggests that it is only loosely associated with the post-synaptic surface and that it may not constitute a part of the post-synaptic membrane. The third component of the post-synaptic membrane, the acetylcholine ionophore has only had brief mention although it appears likely that extensive efforts will be devoted in the next few years to an attempt to understand the factors that control the flow of ions across membranes.

HISTRIONICOTOXIN

Histrionicotoxin (HTX), an alkaloid toxin[3] present in the skin of the Colombian frog *Dendrobates histrionicus* blocks miniature end-plate potentials and the depolarizing action of acetylcholine at synapses in vertebrate skeletal muscle[4,5]. The effect of the toxin and its analogs, dihydroisohistrionicotoxin (DHTX) and perhydroisohistrionicotoxin (PHTX) on neuromuscular transmission is for practical purposes reversible. Biochemical studies indicate that at concentrations of 80 μg/ml PHTX or *d*-tubocurarine partially (65%) protect

the binding of α-bungarotoxin at rat diaphragm muscle[4]. Electrophysiological studies indicate that *d*-tubocurarine inhibits the binding of α-bungarotoxin and also prevents the irreversible blockade of end-plate potentials elicited by α-bungarotoxin. Protection by PHTX, however, does not prevent the blocking action of α-bungarotoxin on end-plate potentials.

All of these observations taken together suggest that the synaptic excitation by acetylcholine involves at least two sites; the acetylcholine receptor site which is blocked competitively by α-bungarotoxin and *d*-tubocurarine and a second site which binds PHTX and α-bungarotoxin. According to Albuquerque *et al.*[4,5] HTX paralyzes skeletal neuromuscular transmission not by a curare--like effect, but rather by a specific block of the second site, the acetylcholine ionophore or »ion conductance modulator«.

The interaction of HTX with the acetylcholine ionophore appeared to be an interesting approach to the studies on the relationship of the acetylcholine receptor with the opening of membrane pores. In order to determine more precisely the mechanism by which HTX blocks the synaptic excitation by acetylcholine a combined electrophysiological and biochemical approach was undertaken[6].

ELECTROPHYSIOLOGICAL ANALYSIS

Peripheral structures

Studies on the rat diaphragm[7] confirmed the potent neuromuscular blocking action reported by Albuquerque *et al.*[4]. In concentrations of 10—30 μM both HTX and DHTX blocked contractions and miniature end-plate potentials within 30—40 min. A predominantly post-synaptic site of action was indicated by these experiments due to the absence of any marked changes in the frequency of the miniature potentials before they disappeared. The depolarizing effect of acetylcholine (30 μM, applied in the muscle bath) was also abolished by 10—30 μM HTX or DHTX. A slow recovery occurred after washing for 1.5—2 h.

HTX does not have any nerve blocking properties since conduction in frog sciatic nerve was only little blocked after three hour exposure to concentrations of 500 μM HTX.

Monocellular electroplaque of Electrophorus electricus

Fig. 1 shows the membrane response of a single cell (electroplaque) isolated from the Sachs organ of the electric fish *Electrophorus electricus*[8]. The changes in membrane potential in response to various concentrations of carbamylcholine are shown in Fig. 1A. The relationship between potential change and carbamylcholine concentration is sigmoid, and is confirmed in Fig. 1B, where the data are plotted in a Hill plot. The Hill coefficient n_H, was 1.9. The competition between HTX and carbamylcholine is distinctly non-competitive, as indicated by the reduction in the maximum response of carbamylcholine in the presence of HTX. 1 μM HTX or DHTX reduced the maximum response to carbamylcholine (100 μM) by 70 and 50% respectively. Fig. 1B also shows that HTX (1 μM) reduced the cooperative behaviour of the membrane. The Hill coefficient was reduced from 1.9 to 1.4.

Our studies on the time-course of inhibition[6] showed that a maximum block by 1 μM HTX or DHTX (in the presence of 15 μM carbamylcholine) was produced in 20 min (± 5 min). After repeatedly washing the cell with

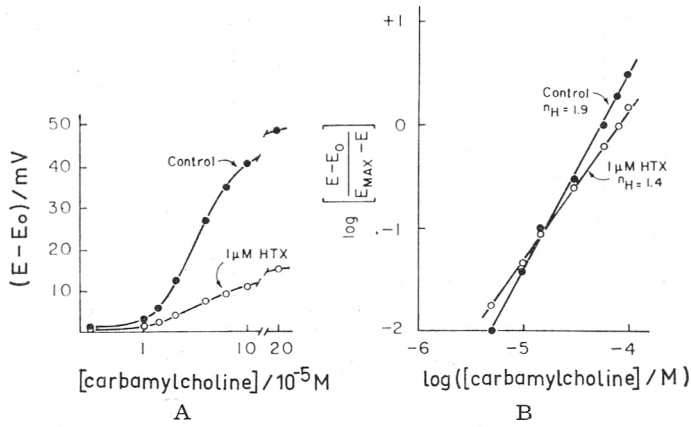

Fig. 1. Pharmacological action of HTX *in vivo*
A: Effect of HTX (1 μM) on response of *Electrophorus* isolated electroplax to increased concentration of carbamylcholine. The ordinate shows steady-state membrane depolarization recorded in the presence of a given concentration of carbamylcholine; E_o, resting potential (— 80 mV). The bath solution was *Electrophorus* Ringer's solution.
B: Hill plot of the same data (taken from reference 6).

physiological solution for two hours, only about 50% of the initial response to carbamylcholine could be recorded.

Half-maximum block was produced by 0.6 and 1 μM HTX and DHTX respectively; at concentrations of 20 μM HTX or DHTX, the response to 15 μM carbamylcholine was completely abolished and recovered only partially (30%) after repeatedly washing the cell for 1.5 h[6].

BIOCHEMICAL ANALYSIS

Investigation of the biochemical and physiological correlates of events occurring at the receptor level have been advanced by the use of a membrane fraction rich in acetylcholine receptors. A plasma membrane fraction of the electric tissue of *Torpedo* is prepared by a modification of a procedure of Cohen *et al.*[9]. The new procedure[6,10] permits the processing of about 500 g of tissue at a time and results in vesicles containing approximately 30—40% receptor material with specific activity ranging between 500—1500 nmol [^{3}H]α-toxin from *Naja nigricollis*/g membrane protein. The number of acetylcholinesterase catalytic sites is approximately 12 ± 5 nmol/g protein.

Fig. 2A shows the binding of [^{3}H]acetylcholine to membrane fragments rich in acetylcholine receptor. The binding curve of [^{3}H]acetylcholine is sigmoid in shape whereas that with DHTX present is hyperbolic. The Hill coefficients (Fig. 2B) with and without 8 μM DHTX are 1.05 and 1.40 respectively. Neither HTX nor DHTX blocks the binding of [^{3}H]acetylcholine to the receptor site even at concentrations of 500 μM. Rather, both toxins increase the affinity of acetylcholine for the receptor site at concentrations which block the carbamylcholine effect on the electroplaque. The dissociation constant K_D for acetylcholine was 8 and 16 nM with and without the presence of DHTX. HTX or DHTX (8 μM) increased the affinity of acetylcholine for the receptor site two to three fold. The assumption that [^{3}H]acetylcholine binds to the receptor site in these membrane fragments is confirmed by the complete (100%) inhibition of acetylcholine binding by 1 μM α-toxin from *Naja nigricollis* (Fig. 2A).

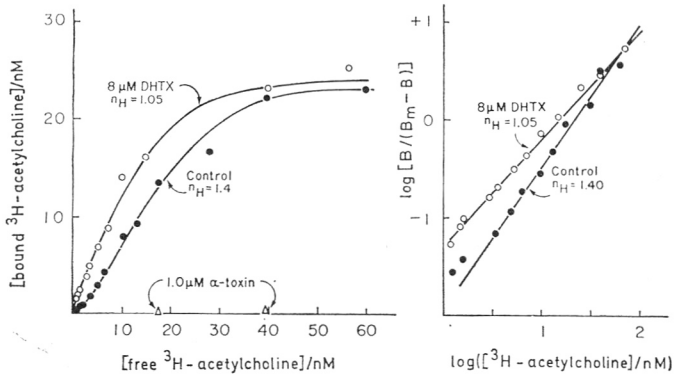

Fig. 2. Effect of DHTX on the binding of [^{3}H]acetylcholine to membrane fragments
A: —●—●, *Torpedo* membrane fragments (10 μM *Naja* α-toxin binding sites, 5 g of protein/l) were diluted 500 fold in physiological solution plus 0.1 mM Tetram. ○—○, dilution in the same medium plus 1 μM DHTX. Double reciprocal plot of the data gave a value of 30 mM for the total number of [^{3}H]acetylcholine binding sites. The concentration of [^{3}H]α-toxin binding sites was 20 nM.
B: Hill plot of data shown in A. B is the concentration of bound [^{3}H]acetylcholine; B_m is the total concentration of its binding sites determined from double-reciprocal plots (taken from reference 6).

Both HTX and DHTX increase the binding of [^{3}H]acetylcholine to about the same extent in the range of concentrations where they block the response of *Electrophorus* electroplaque to carbamylcholine (Fig. 3). Only at much higher concentrations (50—1000 times the apparent dissociation constant *in vivo*) do these two toxins inhibit [^{3}H]acetylcholine binding.

A comparison of the effect of DHTX on the carbamylcholine response of the electroplaque preparation and on [^{3}H]acetylcholine binding to membrane

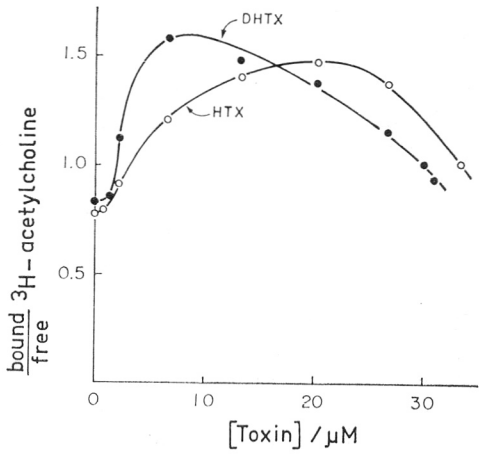

Fig. 3. Effect of the concentration of HTX or DHTX on binding of [^{3}H]acetylcholine to receptor-rich membrane fragments.
A membrane suspension (3 μM *Naja* α-toxin binding sites; 6 g protein/l) was diluted 155 fold in physiological Ringer's solution plus 0.1 mM Tetram and indicated concentrations of HTX or DHTX. Binding was measured in the presence of 20 nM total [^{3}H]acetylcholine. In the absence of HTX or DHTX the concentration of bound [^{3}H]acetylcholine was 12 nM. The concentration of [^{3}H]α-toxin binding sites was 20 nM (taken from ref. 6).

fragments from *Torpedo* is shown in Fig. 4. The response to carbamylcholine was reduced 50% by 0.8 μM DHTX whereas the half-maximum potentiation of [^{3}H]acetylcholine binding occurred at 2 μM DHTX. This small discrepancy in the concentrations of toxin required to produce half-maximal effects may be due to the different sources of electric tissues used in the two types of experiments. It should be pointed out that a successful monocellular electroplaque preparation from *Torpedo* has not yet been developed.

Local Anaesthetics

The ability to increase the affinity of acetylcholine for its receptor site is not a unique property of HTX. Certain local anaesthetics, prilocaine, dimethisoquin, as well as SKF 525A[11] also share this property in common with HTX. They differ only in the concentrations at which they produce an increase in the affinity of [^{3}H]acetylcholine for the receptor site. Maximum potentiation of [^{3}H]acetylcholine binding occurs at concentrations of prilocaine, dimethisoquin and DHTX of 100, 10 and 2μM respectively[6]. Similar experiments have been described elsewhere[11] and our results with prilocaine are presented in Fig. 5A. If prilocaine and DHTX bind to the same site in the membrane, they should neither compete with nor potentiate each other. Indeed, the potentiation of [^{3}H]acetylcholine binding by 1 mM prilocaine was unaffected by 30 μM DHTX (Fig. 5B). At only higher concentrations (150 μM) did DHTX abolish the potentiating effect of prilocaine. At these concentrations, DHTX also inhibits the binding of acetylcholine to the receptor site.

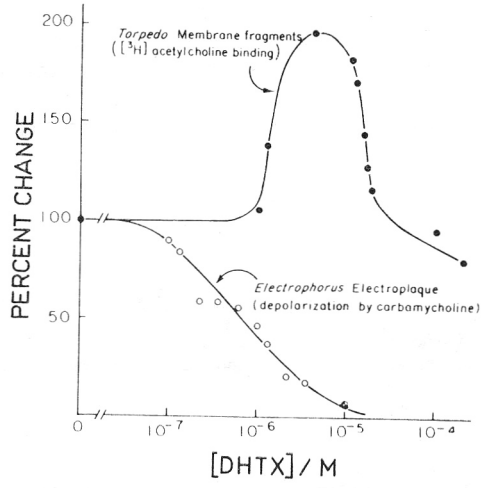

Fig. 4. Comparison of the effect of DHTX concentrations on the carbamylcholine response of the electroplaque and on the binding of [^{3}H]acetylcholine to *Torpedo* membrane fragments. *Lower graph:* A fixed concentration of DHTX was incubated with the innervated face of the electroplaque for 15 min and the response to 15 μM carbamylcholine was measured. After a steady-state is reached, the bath is washed with eel Ringer's solution until a steady membrane potential is reached. The above procedure is repeated using increasing concentrations of DHTX.

Upper graph: Data were taken from Fig. 3 for DHTX and plotted as a % change (taken from reference 6).

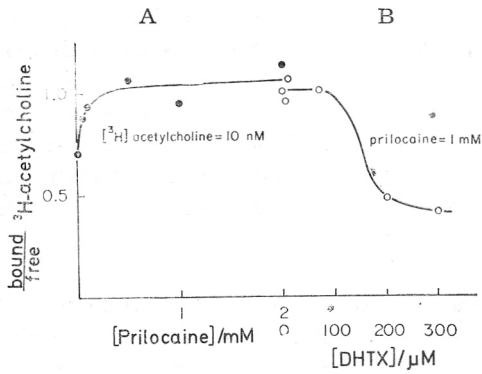

Fig. 5. Effect of DHTX concentration on the potentiation of [³H]acetylcholine binding to membrane fragments from *Torpedo* by prilocaine.

Left (A): A membrane suspension (45 μM *Naja* α-toxin binding sites; 4.7 g of protein/l) was diluted 225 fold in physiological Ringer's solution plus 0.1 mM Tetram and indicated concentrations of prilocaine. Binding was measured in the presence of 10 nM total [³H]acetylcholine. In the absence of prilocaine the concentration of bound [³H]acetylcholine was 8.2 nM. The concentration of [³H]α-toxin binding sites was 20 nM.

Right (B): The above membrane suspension contained 10 nM [³H]acetylcholine and 1 mM prilocaine and the concentration of DHTX was varied (taken from reference 6).

EFFECTS ON THE CENTRAL NERVOUS SYSTEM

Spontaneous discharges

Microiontophoretic techniques were used to study the effects of HTX on single units in the cerebral cortex or the spinal cord[4].

HTX had a powerful depressant effect on central neuronal firing whether spontaneous or evoked. Very small iontophoretic currents (< 14 nA) releasing HTX from the microelectrode were required to produce a partial or a complete block of the spontaneous discharge of cortical neurones (Fig. 6). The effect was rapid in onset (5—10 sec) and reversible. Comparable changes were observed when procaine was applied (with currents < 126 nA) although recovery after procaine was quicker (1—5 sec). The depressions of spontaneous firing were observed both in the cortex and the spinal cord (Fig. 7B).

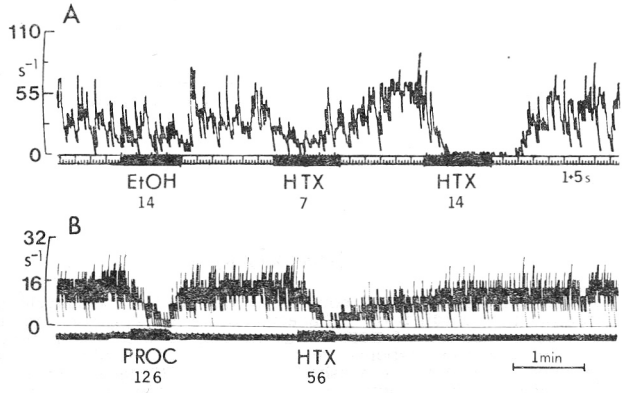

Fig. 6. Rate-meter records of the effects of ethanol (EtOH), procaine (Proc) and histrionicotoxin (HTX) on two different cortical neurones (A, B). Bars signal periods of drug application. In all figures iontophoretic currents are indicated in nA (taken from reference 7).

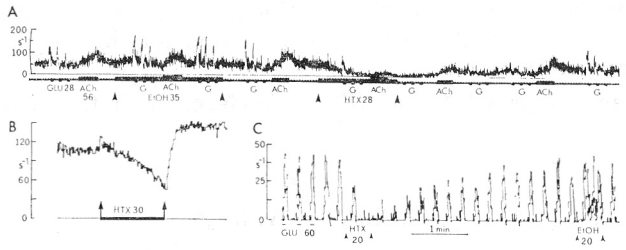

Fig. 7. Rate-meter records of the effects of ethanol (EtOH) and histrionicotoxin (HTX) on ACh — or glutamate — (Glu or G) evoked firing of a cortical (Aa) and a spinal (C) neurone, and on the spontaneous discharge of another spinal unit (B). Time marks in A indicates 5 s; B and C have a common time scale. Record C is distorted by electrical noise generated during ethanol application (taken from reference 7).

Evoked responses

Although acetylcholine-evoked responses were depressed by HTX, it became evident that the depressive effect of HTX or DHTX was not specific for acetylcholine sensitive cells. Both acetylcholine and glutamate-evoked responses were depressed by HTX (Fig. 7A) and in certain instances glutamate--evoked firing was even more effectively depressed. Small applications of HTX were particularly effective in depressing glutamate-evoked firing of non-acetylcholine-sensitive neurones. In Fig. 7C, the responses of a spinal interneurone to repeated applications of glutamate were completely abolished by HTX and they recovered only gradually. Interestingly, HTX itself had no acetylcholine-like excitatory action.

CONCLUSIONS

The effect of HTX has been evaluated at the rat neuromuscular junction, the eel electroplaque and on the CNS of the cat and has been shown to reduce markedly the response to acetylcholine or carbamylcholine. This toxin does not inhibit the binding of acetylcholine to *Torpedo* membrane fragments rich in acetylcholine receptor protein but rather augments its affinity for the receptor site. These findings suggest an accentuation of a desensitization phenomenon (12—14) or a block of ionic channels (2, 3).

Accentuation of desensitization

The first step in the reaction sequence leading to depolarization of the muscle membrane is the combination of acetylcholine with its receptor (R) site, leading to the formation of an intermediate inactive complex, $AChR_i$:

$$ACh + R \rightleftharpoons AChR_i \rightleftharpoons AChR_a \rightarrow AChR_d$$

This form is rapidly changed to $AChR_a$ which is the active form of the receptor causing depolarization. If acetylcholine is continually present, the reaction slowly goes to $AChR_d$ which is an inactive desensitized receptor. Acetylcholine can combine with both forms of the receptor (R_a and R_d) but R_d is inactive.

One possible interpretation of our results is that HTX accentuates the desensitization phenomenon. That is, HTX converts the receptor into an inactive form which still binds acetylcholine but can no longer activate the ionophore. We found that this form of the receptor has an increased affinity for acetyl-

choline. The above theory is attractive since desensitization in muscle is also accompanied by a higher affinity of acetylcholine for the receptor site[14].

Block of ionic channels

It has been suggested[2,3] that HTX interferes with the reaction

$$\begin{array}{ccc} & I_c \quad I_o & \\ & \nwarrow\nearrow & \\ AChR_i & \rightleftarrows & AChR_a \end{array}$$

in the reaction sequence described above (where I_c and I_o are the closed and open forms of the acetylcholine ionophore respectively) by binding directly to the acetylcholine ionophore thus preventing the opening of the ionic channels. We showed that HTX reduces the effect of acetylcholine on muscle, Renshaw cells[7] and cortical neurones to acetylcholine, as well as glutamate evoked firing. This surely indicates an unspecific mechanism of block and we may rule out a direct blocking effect on the acetylcholine ionophore.

The resemblance between the effects of HTX and local anaesthetics seems significant. These similarities may indicate a common mode of action. To sum, we suggest that local anaesthetics, SKF 525A and HTX bind to a site at the receptor-phospholipid interface in the cell membrane. This interaction leads to the conversion of the receptor into an inactive or desensitized state which has a high affinity for acetylcholine but which can no longer produce the necessary changes in the receptor leading to the activation of the ionophore.

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DISCUSSION

R. D. O'Brien:

(a) Have you examined the effect of cold histrionicotoxin upon [^{3}H]-acetylcholine binding by solubilized receptors? (b) Is not the observed affinity increase for acetylcholine of up to 3 times rather small to be associated with the proposed conversion of a receptor to a quite different desensitized form?

G. Kato:

(a) We have not done this yet, but are in the process of doing it now. (b) I agree that it is a rather small increase in affinity but since the apparent dissociation constants for histrionicotoxin for both the carbamylcholine block on the monocellular electroplax and for the augmentation of [^{3}H]-acetylcholine binding to membrane fragments are similar, it was suggested that this small increase in affinity may explain the electroplax results. It may be that the receptor is already in a desensitized state and that we cannot push this state much further with histrionicotoxin. In other words this increase in affinity may in reality be much larger if the receptor were in an active state with a K_D for acetylcholine closer to 10^{-7} M rather than 10^{-8} M.

SAŽETAK

Učinci histrionikotoksina na receptore acetilkolina

G. Kato, M. Glavinovic, J. Henry, K. Krnjevic, E. Puil i B. Tattrie

Istraženi su učinci histrionikotoksina na neuromuskularnim sinapsama štakora, elektromotornoj ploči jegulje i centralnom nervnom sistemu mačke. Utvrđeno je znatno sniženje osjetljivosti na acetilkolin ili karbamilkolin. Vezanje acetilkolina za fragmente membrane *Torpeda* bogate proteinima acetilkolinskih receptora nije inhibirano ovim toksinom, već on povećava afinitet za receptorsko mjesto. Rezultati ovih istraživanja upućuju na važnost fenomena desenzibilizacije ili bloka ionskih kanala.

MOLEKULARNA NEUROBIOLOGIJA,
PASTEUROV INSTITUT,
PARIS 75015, FRANCUSKA
i
ZAVOD ZA ISTRAŽIVANJA ANESTEZIJE,
SVEUČILIŠTE MCGILL,
MONTREAL, KANADA