The ionic channel of the nicotinic acetylcholine receptor is unable to differentiate between the optical antipodes of perhydrohistrionicotoxin

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The enantiomers of perhydrohistrionicotoxin were studied in their effects on endplate currents recorded at the junctional region of sartorius muscles of *Rana pipiens*. The two optical antipodes progressively decreased the peak amplitude of the endplate currents and were indistinguishable from each other at all times. The enantiomers shortened equally the time constants for endplate current decay, but did not alter their voltage sensitivities. Although perhydrohistrionicotoxin contains 4 chiral centers, complete steric inversion does not alter its effects on the acetylcholine receptor–ion channel complex. By contrast the recognition site of the AcChR is extremely sensitive to any change in the chirality of agonists.

Perhydrohistrionicotoxin *Nicotinic acetylcholine receptor* *Ion channel stereospecificity* *Endplate current* *Neuromuscular junction*

1. INTRODUCTION

Among toxins produced by animals, the histrionicotoxins have a spiro linkage and (in the unsaturated members) alkyne and allene bonds that make them chemically unique [1]. Historically these toxins were among the first alkaloids shown to block the nicotinic acetylcholine receptor (AChR)–ion channel complex by altering the kinetics that govern the opening and closing of the ionic channel instead of competing for the agonist at the recognition site (e.g., [2–9]). These toxins cause a voltage-dependent depression of the peak amplitudes of endplate currents (EPCs), and they shorten the time constants for their decay. Various histrionicotoxins have been tested with the intent of discerning structure–action relationships [6]. All were found to be qualitatively equivalent, though their potencies varied. The hypothesis emerged that hydrophobic forces were decisive in the actions of these and other non-competitive blockers of the AChR (e.g., [6,10]). Recently, the enantiomer of the natural (−)-H_{12}-HTX has been synthesized [11]. These toxins possess 4 chiral centers (see fig.1) that are attachment points for the major functional groups of the molecule. If stereoselective binding to the AChR protein exists, then one expects different activities for these enantiomers. On the other hand, if hydrophobic forces

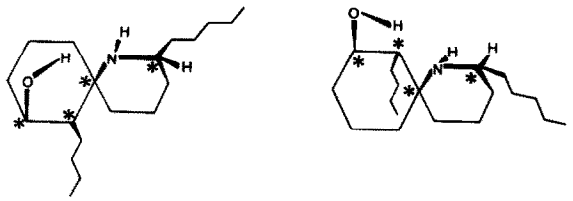

Fig.1. The structures of natural (−)-H_{12}-HTX and its enantiomer, (+)-H_{12}-HTX. The 4 chiral centers are marked with asterisks.

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predominate, or if the toxins bind to a site with no intrinsic asymmetry, one expects no difference in activity between enantiomers. The objective of this study was to test whether or not the AChR can discriminate between these enantiomers of H_{12}-HTX.

2. MATERIALS AND METHODS

The frog Ringer's solution had the following composition (mM): NaCl, 116; KCl, 2.0; $CaCl_2$, 1.8; Na_2HPO_4, 1.3 and NaH_2PO_4, 0.7. The enantiomers of H_{12}-HTX were synthesized by Dr K. Takahashi et al. [11]. Sartorius muscles with the sciatic nerve were dissected from the frog *Rana pipiens*. Both muscles from each frog were always used, one for each enantiomer. These matched pairs of muscles were used because the toxin washes out of muscle so slowly that tests of the two enantiomers on the same muscle are impossible. To prevent twitch, glycerol shock was used [12–14].

The technique for EPC experiments and analysis is described elsewhere [13,14]. It has been shown [6] that 10 μM H_{12}-HTX reduces EPC peak amplitude to about 50% of the control value. Although higher concentrations deepen the blockade and enhance the difference from control values, one also finds fibers that are totally blocked or have EPCs too small to be detectable, especially at incubation times of 1 h or more. For this reason the 10 μM concentration of toxins was used for all EPC experiments.

3. RESULTS

3.1. *Effects of (+)- and (−)-H_{12}-HTX on parameters of the EPC*

Peak amplitudes of the EPCs were plotted as a function of membrane potential (fig.2). The peak amplitude at −150 mV, especially in the presence of H_{12}-HTX, departs from the linear portion of the current-voltage graph. The plot of ln τ *vs* membrane potential was linear both in the presence and in the absence of the toxins (see also fig.2). The logarithms of all peak amplitudes of the EPC obtained under control conditions at −150 mV are shown as a histogram in fig.3. A Gaussian curve with the same mean, standard deviation and area (79 fibers) is superimposed to show that the

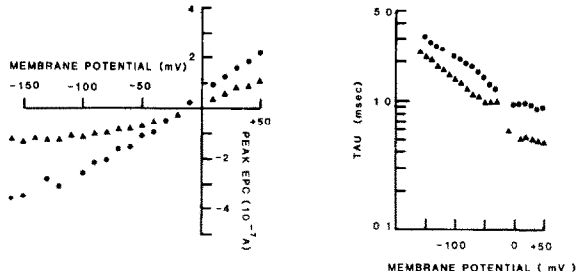

Fig.2. Two families of EPC peak amplitudes (left) and decay time constants (right) obtained under control conditions (●) and in the presence of 10 μM (+)-H_{12}-HTX (▲). These graphs illustrate the means by which EPC data were taken as measures of toxin effect. These measures were: (1) the slope of the regression line (slope conductance) of the current-voltage plot (left) calculated for points between +50 and −50 mV; (2) the EPC peak amplitude at −150 mV; (3) the linear regression parameters for the ln τ *vs* mV plot (right) evaluated between −50 and −150 mV. Each curve is from a single muscle fiber.

amplitudes were lognormally distributed. The slope conductances also had a lognormal distribution (not shown). Therefore, the logarithmic transforms of EPC peak amplitudes (at −150 mV)

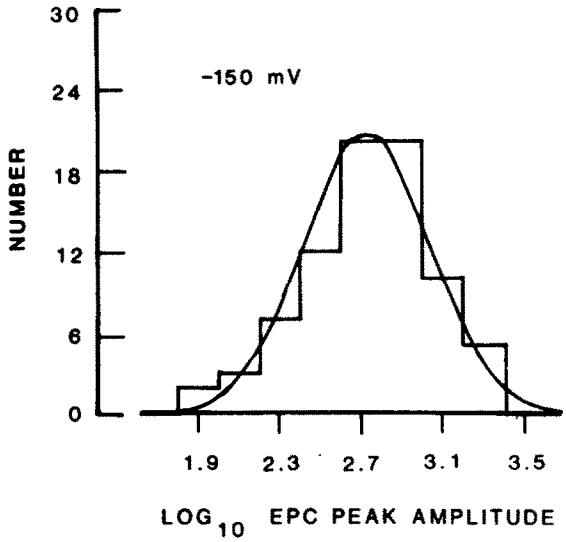

Fig.3. Histogram of EPC peak amplitudes obtained at −150 mV under control conditions. A Gaussian curve with the same mean, standard deviation and area (79 fibers) is superimposed to show that the amplitudes were lognormally distributed.

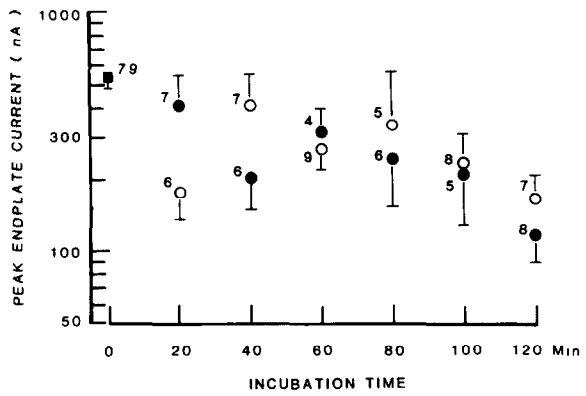

Fig.4. Peak amplitudes of EPCs at −150 mV under control condition (■) and in the presence of (+)- (●), and (−)-H_{12}-HTX (○) plotted as functions of incubation time. Each point represents the (geometric) mean of the number of fibers shown; bars represent standard errors.

and slope conductances (in the range +50 and −50 mV, at which peak amplitudes are linear functions of membrane potential) were used to calculate means and standard errors shown in fig.4 and 5, respectively.

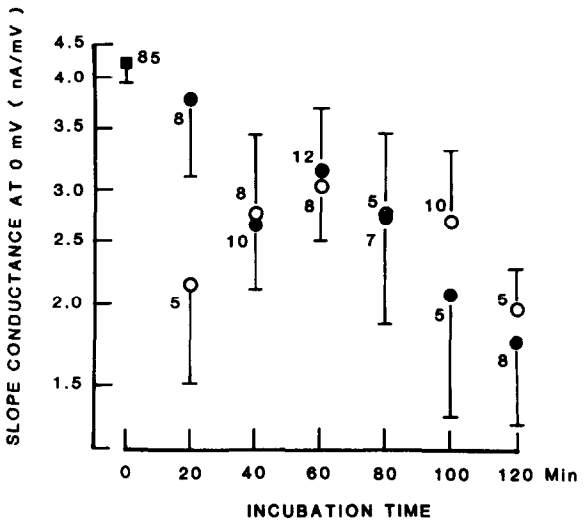

Fig.5. Slope conductances of EPC peak amplitudes under control conditions (■) and in the presence of (+)- (●), and (−)-H_{12}-HTX (○) plotted as functions of incubation time. Each point represents the (geometric) mean of the number of fibers shown; bars represent standard errors.

As shown in fig.4, the peak EPC amplitudes at −150 mV were progressively blocked by both (+)- and (−)-H_{12}-HTX. The blockade developed slowly and apparently as an exponential function of time (the semilog plot is roughly linear) with a half-time of around 70 min. By 120 min the EPC peak amplitude was about 30% of control. Throughout this time, however, the peak amplitudes under treatment with (+)- and (−)-H_{12}-HTX intermingled, and no statistically significant difference could be discerned between them. The slope conductance (fig.5) also decreased slowly with time after adding the toxins. The half-time was somewhat greater (around 110 min) and the degree of blockade at 120 min was less (around 45% of control) than the corresponding half-time and blockade of peak amplitude at −150 mV. Again no significant difference between enantiomers could be seen at any time. Both enantiomers depressed the time constant (τ) for decay of the EPC. Slopes of plots of ln τ *vs* membrane potential were unchanged by either toxin; the plots were simply shifted downward while remaining parallel to controls (fig.2). The estimated (from the regression line) τ_{EPC} at

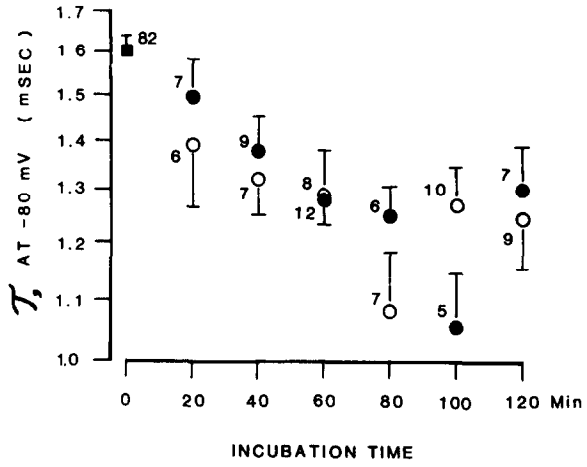

Fig.6. Time constants (τ) for EPC decays at −80 mV under control conditions (■) and in the presence of (+)- (●), or (−)-H_{12}-HTX (○) plotted as a function of incubation time. The ln τ-value for each fiber was estimated from linear regression of ln τ on membrane potential. At 10 μM (+)- or (−)-H_{12}-HTX these plots were parallel to control plots, but they were shifted downward. The τ at −80 is a measure of this downward shift. Each point represents the (geometric) mean of the number of fibers shown; bars represent standard errors.

−80 mV, taken as a measure of this vertical shift, is shown in fig.6 under control conditions and in the presence of the enantiomers. The depression in τ_{EPC} progressed until around 60 min, when (in contrast to the peak amplitude and slope conductance) equilibrium seemed to have been reached. At no time, however, could the effects of (+)- or (−)-H_{12}-HTX be distinguished from one another.

4. DISCUSSION

We have shown that the enantiomers of H_{12}-HTX indistinguishably depressed the peak amplitudes and decay time constants of junctionally recorded EPCs. Dispersion of data was decreased by using matched muscles and by pooling data according to duration of treatment by H_{12}-HTX. Although the dispersion that remained could still obscure a small difference between the enantiomers (a 'type II error'), one can conclude safely that stereochemical recognition of a non-competitive antagonist, such as H_{12}-HTX, by the AChR is far less than it is for an agent that binds to the agonist recognition site (see below). In agreement with the present results, authors in [15] reported no difference between enantiomers of pentobarbital in their ability to block carbachol-stimulated ^{22}Na influx in cultured chick muscle cells. On the other hand, the recognition site of the AChR can show high stereoselectivity for agonists. Thus (+)-anatoxin-a is twice as potent as a racemic mixture [16] and the enantiomers of *trans*-3-acetoxy-1-methylthioniacyclohexane differ in potency by >1000:1 [17]. Competitive antagonists also bind stereoselectively, though less markedly. For instance, (+)-tubocurarine is 30–60-times more potent than (−)-tubocurarine [18]. Testing a variety of synthetic, competitive antagonists, potency ratios of about 2:1 for pairs of enantiomers were found in [19–22]. Higher potency ratios could have been found if the compounds tested were purely competitive, which is unlikely (e.g., [10]).

Several mechanisms have been proposed to account for non-competitive blockade of the AChR (e.g., [6,10]). Almost certainly several different modes and sites of action are involved in the actions of various compounds whose observable endpoint, the blockade, is the same (e.g., see [10,23]). A single dissociation constant for (±)-H_{12}-HTX at the AChR has been estimated by its effect on the time constant of the decay of EPCs to be about 10 μM [6] and by direct binding assays of the tritiated derivative to be about 0.1–1 μM [7–9,23]. These constants correspond to a free energy for binding of around 7–9 kcal/mol. In a molecule such as H_{12}-HTX, with its two aliphatic side chains and its two saturated rings, this energy may easily be accounted for by purely hydrophobic factors [24] in which binding to the biophase results more from the exclusion of the molecule from water than from attraction to the biophase. Though H_{12}-HTX contains two polar functional groups (NH and OH), these lie in close proximity to one another and, in the crystal phase at least, are joined by an intramolecular hydrogen bond [1]. They may be viewed, therefore, as a single polar center on an aliphatic mass. The absence of stereoselectivity for the H_{12}-HTX enantiomers at the AChR may be due to a functional mirror plane (through the molecule) defined by the two polar functional groups and a third point such as the spiro linkage. The two flexible, hydrophobic chains can dangle on this plane into a hydrophobic region of the biophase. The absence of alkyl chains makes the remaining molecule a much weaker antagonist [25,26].

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REFERENCES

[1] Daly, J.W., Karle, I., Myers, C.W., Tokuyama, T., Waters, J.A. and Witkop, B. (1971) Proc. Natl. Acad. Sci. USA 68, 1870–1875.

[2] Albuquerque, E.X., Barnard, E.A., Chiu, T.H., Lapa, A.J., Dolly, J.O., Jansson, S.-E., Daly, J. and Witkop, B. (1973) Proc. Natl. Acad. Sci. USA 70, 949–953.

[3] Albuquerque, E.X., Kuba, K., Lapa, A.J., Daly, J.W. and Witkop, B. (1973) in: Exploratory Concepts in Muscular Dystrophy (Milhorat, A.T. ed) vol.2, pp.585–600, Excerpta Medica, Amsterdam.

[4] Albuquerque, E.X., Kuba, K. and Daly, J. (1974) J. Pharmacol. Exp. Ther. 189, 513–524.
[5] Lapa, A.J., Albuquerque, E.X., Sarvey, J.M., Daly, J. and Witkop, B. (1975) Exp. Neurol. 47, 558–580.
[6] Spivak, C.E., Maleque, M.A., Oliveira, A.C., Masukawa, L.M., Tokuyama, T., Daly, J.W. and Albuquerque, E.X. (1982) Mol. Pharmacol. 21, 351–361.
[7] Elliott, J., Dunn, S.M.J., Blanchard, S.G. and Raftery, M.A. (1979) Proc. Natl. Acad. Sci. USA 76, 2576–2579.
[8] Eldefrawi, M.E. and Eldefrawi, A.T. (1979) in: Advances in Cytopharmacology (Ceccarelli, B. and Clementi, F. eds) vol.3, pp.213–223, Raven, New York.
[9] Aronstam, R.S., Eldefrawi, A.T., Pessah, I.N., Daly, J.W., Albuquerque, E.X. and Eldefrawi, M.E. (1981) J. Biol. Chem. 256, 2843–2850.
[10] Spivak, C.E. and Albuquerque, E.X. (1982) in: Progress in Cholinergic Biology: Model Cholinergic Synapses (Hanin, I. and Goldberg, A.M. eds) pp.323–357, Raven, New York.
[11] Takahashi, K., Witkop, B., Brossi, A., Maleque, M.A. and Albuquerque, E.X. (1982) Helv. Chim. Acta 65, 252–261.
[12] Gage, P.W. and Eisenberg, R.S. (1967) Science 158, 1702–1703.
[13] Kuba, K., Albuquerque, E.X., Daly, J. and Barnard, E.A. (1974) J. Pharmacol. Exp. Ther. 189, 499–512.
[14] Masukawa, L.M. and Albuquerque, E.X. (1978) J. Gen. Physiol. 72, 351–367.
[15] Barker, J.L., Huang, L.-Y.M., McDonald, J.F. and McBurney, R.N. (1980) in: Molecular Mechanisms of Anesthesia (Fink, B.R. ed) Prog. Anesth. vol.2, pp.79–93, Raven, New York.
[16] Spivak, C.E., Waters, J., Witkop, B. and Albuquerque, E.X. (1983) Mol. Pharmacol. 23, 337–343.
[17] Lambrecht, G. (1981) Arzneim.-Forsch. 31, 634–640.
[18] King, H. (1947) J. Chem. Soc. 936–937.
[19] Erhardt, P.W. and Soine, T.O. (1975) J. Pharm. Sci. 64, 53–62.
[20] Genenah, A.A., Soine, T.O. and Shaath, N.A. (1975) J. Pharm. Sci. 64, 62–66.
[21] Soine, T.O., Hanley, W.S., Shaath, N.A. and Genenah, A.A. (1975) J. Pharm. Sci. 64, 67–70.
[22] Erhardt, P.W. and Soine, T.O. (1979) J. Pharm. Sci. 68, 522–524.
[23] Heidmann, T., Oswald, R.E. and Changeux, J.-P. (1983) Biochemistry 22, 3112–3127.
[24] Tanford, C. (1973) in: The Hydrophobic Effect: Formation of Micelles and Biological Membranes, Wiley, New York.
[25] Witkop, B. and Brossi, A. (1984) in: Natural Products and Drug Development, Alfred Benzon Symposium 20, Munksgaard, Copenhagen.
[26] Spivak, C.E. and Albuquerque, E.X. (1983) in: Natural Products and Drug Development, cf. abstracts p.22, Alfred Benzon Symposium 20, August 7–11, Copenhagen.