The Mechanism of Tetrodotoxin on the in situ Acetylcholinesterase in the Vagal Heart System of *Rana pipiens*

Tetrodotoxin (TTX) isolated from puffer fish has a strong effect on neuromuscular transmission as well as membrane potentials[1-4]. The neuromuscular transmission has been primarily a membrane phenomenon which has been proved to be intimately related to the activity of acetylcholinesterase (AChE)[5,6]. However, there seems to be no profound effect of TTX on the isolated in vitro AChE materials[7]. There are several reports of in vivo experiments that TTX has hypotensive effects and bradycardia[8,9], which can be regarded as lowering the in vivo AChE activity. Since the effect of a terminal depolarization in accelerating the release of acetylcholine (ACh) is not impaired by this toxin[2], it would be of interest to see whether this powerful toxin has any effect on the in situ AChE. The method used for this study is described in detail somewhere else[10,11].

Figure 1 shows the vagal heart inhibition before and after application of 2.4×10^{-10} g/ml of TTX. The rate of hydrolysis of the introduced acetylcholine either by vagal stimulation or by injection of ACh into the sinus is 2 to 3 times lower in the TTX treated heart than in the control. TTX at a concentration of 10^{-10} g/ml inactivates the in situ AChE by 50–70% (Table). Figure 2 shows a typical Lineweaver and Burk plot, 1/v versus 1/(S), before and after treatment with 2 different concentrations of TTX. As the concentration of TTX increases, the slope of the

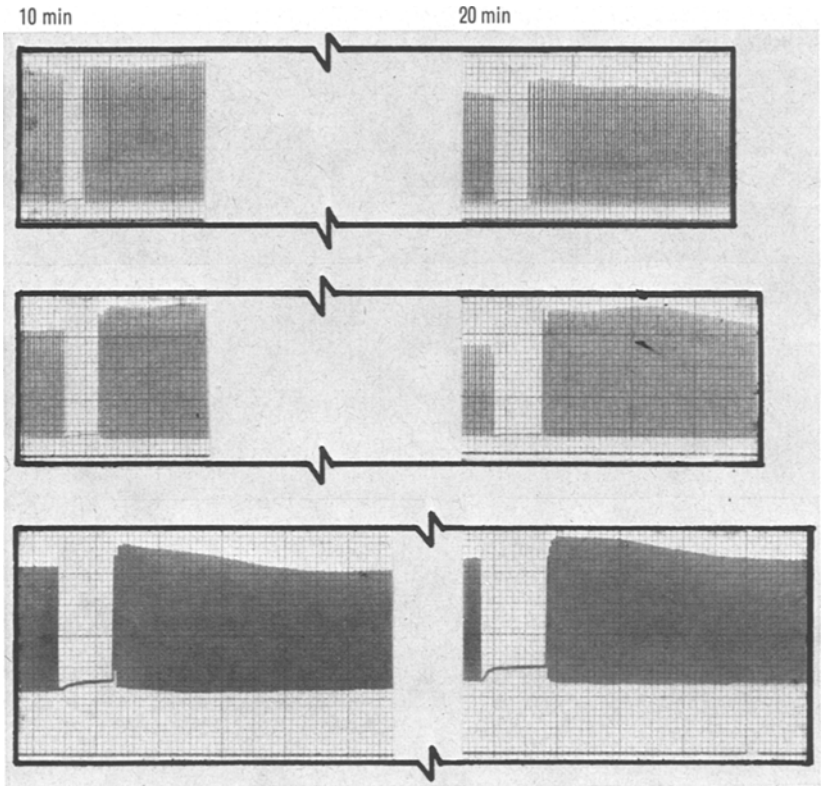

Fig. 1. Effect of vagal stimulation on the cessation of the heart contraction. The number at the top of the tracings indicates the time interval during which vagus nerve was stimulated. Frequency, 30 pulses/sec. Duration of the pulse, 4 msec. Voltage of the pulse, 4 V. The stimulations were spaced 20 min to allow the necessary recovery. The perfusing fluid, i.e. the reaction volume, was 2 ml. Tetrodotoxin was added to the perfusion solution after the control experimental period. The concentrations of TTX of the top, middle and bottom row of tracings are 0, 2.4×10^{-10}, and 4.8×10^{-10} g/ml respectively.

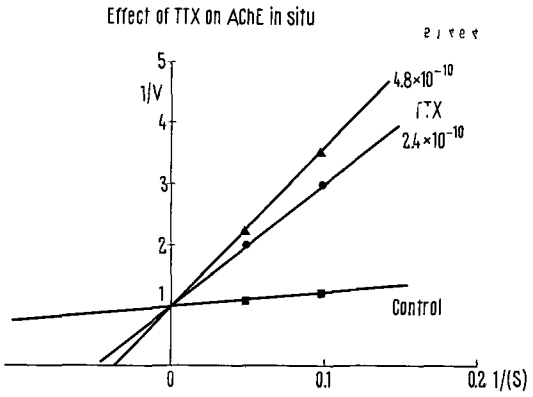

Fig. 2. Lineweaver and Burk plot. The reciprocal of velocity of hydrolysis versus the reciprocal of the substrate concentration. The concentrations of TTX in the experiments are indicated in the curve.

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The effect of tetrodotoxin on the rate of hydrolysis of acetylcholine in the isolated vagal heart system

Acetylcholine (g)	Rate of hydrolysis Without TTX (g/sec)	With TTX (g/sec)	% of control
1×10^{-8}	8.3×10^{-10}	2.8×10^{-10}	34
1×10^{-8}	7.9×10^{-10}	3.3×10^{-10}	42
1×10^{-8}	8.1×10^{-10}	4.8×10^{-10}	59
1×10^{-8}	8.3×10^{-10}	4.8×10^{-10}	57
1×10^{-8}	8.3×10^{-10}	3.9×10^{-10}	47
2×10^{-8}	9.4×10^{-10}	6.5×10^{-10}	69
2×10^{-8}	8.9×10^{-10}	6.6×10^{-10}	74
2×10^{-8}	9.2×10^{-10}	4.9×10^{-10}	53
2×10^{-8}	9.3×10^{-10}	5.2×10^{-10}	56

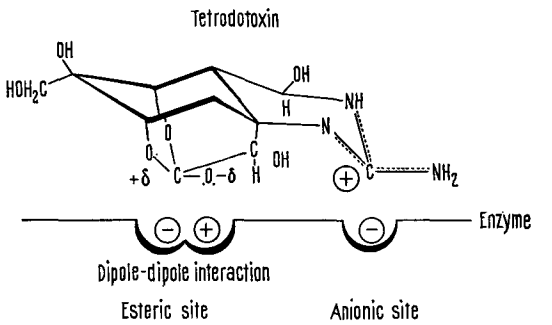

Fig. 4. Model of binding of TTX molecule for active sites of acetylcholinesterase by dipole-dipole interaction at the esteric site and electrostatic forces at the anionic site.

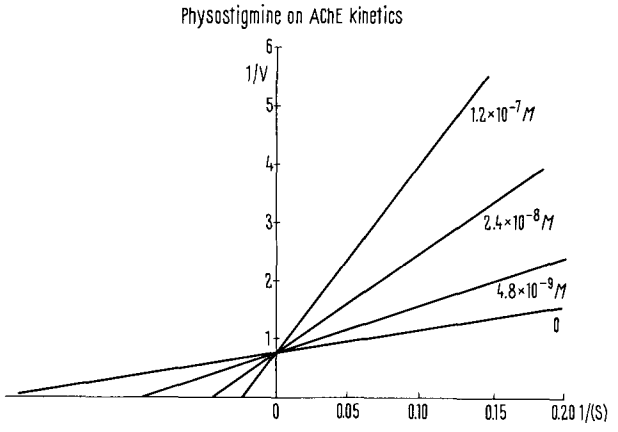

Fig. 3. Double reciprocal plot of 1/v versus 1/(S) at various concentrations of physostigmine.

lines becomes increasingly steeper than the control. That intercepts at the 1/v axis of the control and experimental lines meet at the same spot indicates that TTX is a specific competitive inhibitor for the AChE in situ. For comparison, Figure 3 shows a similar Lineweaver and Burk plot at various concentrations of physostigmine. The striking resemblance between Figure 2 and Figure 3 infers that TTX and physostigmine act on the same enzyme. The apparent K_m for TTX is $2.6\times10^{-6}M$ and that for physostigmine is $4.2\times10^{-6}M$. The K_i for TTX is 1.8×10^{-11} and that for physostigmine is 1.7×10^{-8}. Thus it indicates that TTX binds AChE stronger than physostigmine. Consequently, TTX is a very effective blocking agent for AChE at very low concentration.

A plausible explanation of this remarkable affinity of TTX for AChE may be due to the several characteristic features of TTX molecule. It is generally held that the usual biological active molecules are bound to the active portion of the membrane by 3 forces, namely van der Waals forces, dipole-dipole interactions and electrostatic bindings. With a highly reactive carbonyl group at C_{10} in the TTX molecule (Figure 4), the electron cloud at oxygen atom is sufficiently dense to act as an electron donor capable of forming hydrogen bonds with the esteric sites of AChE molecules of the membrane. The quanidyl group is relatively positive in comparison with the rest of the TTX molecule and fit into the anionic site of the AChE molecule. Since TTX molecule protracts itself as a bird-cage configuration, it is quite conceivable that TTX blocks the 2 active sites more effectively than other AChE inhibitors[12].

Zusammenfassung. Am isolierten Vagus-Herzen von *Rana pipiens* wird die Wirkung der Vagusreizung gemessen: Konzentrationen unter 4×10^{-10} g/ml Tetrodotoxin erhöhen die vagale Reizungswirkung, nicht aber die Herzkontraktionen.

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[12] This work was supported by U.S. Atomic Energy Commission.

Effect of EDTA on the Distribution Pattern of Acetate-^{14}C in Rats

It has been shown previously[1,2] that following injection of ^{14}C-labelled ethylenediaminetetraacetic and diethylenetriaminepentaacetic acids (EDTA and DTPA) the accumulation of ^{14}C in the liver and kidneys is markedly higher with lower dose of the chelate. This might suggest a dose-dependent deposition of chelating agents and/or of their metabolites and/or of low molecular radioactive contaminants present in the ^{14}C-stock solution[3,4]. Since EDTA and DTPA are broadly used for enhancing excretion of incorporated metals, the present investigation was undertaken in order to elucidate the mechanisms responsible for the effect observed.

Adult female albino rats (weighing 185–200 g, 5 per group) were injected i.v. with 2 μCi of sodium acetate-2-C-14 (20–50 mCi/mmol) alone or with varying amounts of disodium calcium chelate of EDTA. The animals were sacrificed 2 h later and distribution of ^{14}C was determined by methods described previously[2].

As shown in Table I, there are no differences in the concentrations of ^{14}C in the plasma. Furthermore, with