ACTA MEDICA-IRANICA
Vol.25.1983.p.-17

THE NATURE OF ACETYLCHOLINE RECEPTOR

M.E. TASHAYOD

Receptors are known as especial molecular structures in the cell membrane suited to interact with the appropr-iate drug molecule and produce an especial pharmacologi-cal action. Acetylcholine (ACh) nicotinic receptor is of paramount importance because of its many duties in vital nervous functions such as neuro-muscular transmission. In the past 3 decades many scientists including bioche-mists, pharmcologists, histopathologists and electrophy-siologists have attempted to unveil the nature of this

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* Professor, Dept. of Anesthesiology, Tehran Med. School. Teheran University.

receptor. But inspite of the extensive studies even by the use of affinity labeling reagents, radiographic and electron microscopic investigation the nature of ACh receptor still remains a mystery. Recent work has served as much to complicate the picture as to simplify it.This is due to the scattered pieces of information, mostly gained from animals in different species,which now form a kind of Jigsaw puzzle.

The aim of this study is to reconcile in vitro findings with in vivo experiments and actual clinical practice. This is an approach to the recognition of ACh receptor. In this respect it seems necessary to design a series of clinical experiments to elucidate The important principles governing the reign of neuromuscular agonist-antagonist interaction with in-situ receptor in man.

Experiments

Experiment No.1 (Tashayod-Feldman experiment):0.5 mg of neostigmine diluted in 40 ml of saline was injected intravenously into an arm(Fig.1, bottom trance), isolated for 2 min, from the general circulation by an arterial tourniquet. One min after the release of the cuff 10 mg of d-tubocurarine (dtc)was given systematically.The neostigmine arm was protected from the blocking action of dtc for 22 min.

Experiment No.2:(Tashayod - Feldman experiment):1.5.mg of neostigmine was injected into an arm isolated for 2 min from the general circulation by an arterial tourniquet

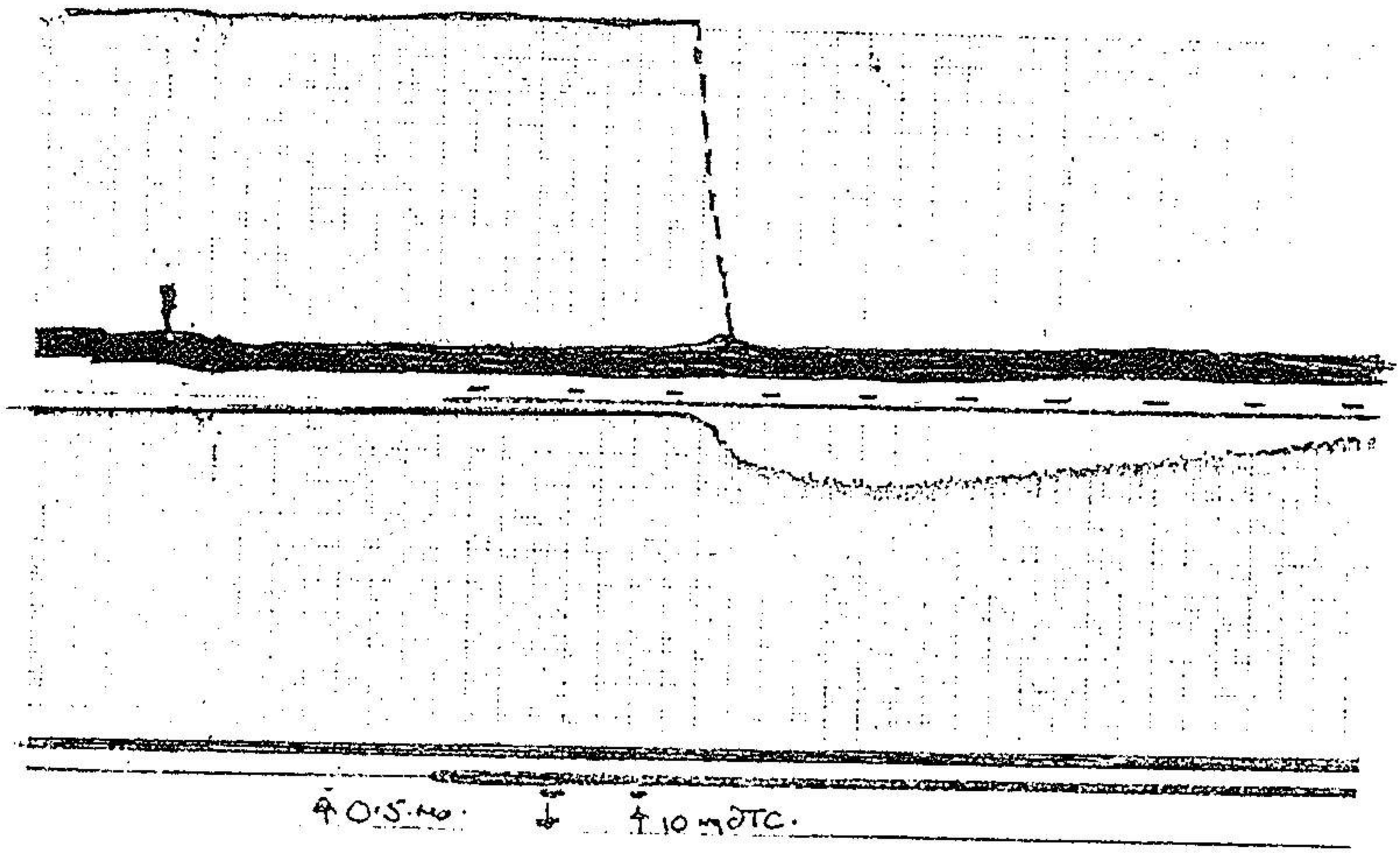

Fig. 1: Neuromuscular block produced by the systemic use of 10 mg dtc. top trace-left arm, no tourniquet bottom trance-Rt.arm which received neostigmine prior to dtc. while isolated by an arterial tourniquet.

(Fig.2). This arm was completely paralised. Systemic use of small dose of dtc produced some recovery at first but showed additive effect by giving 20 mg.

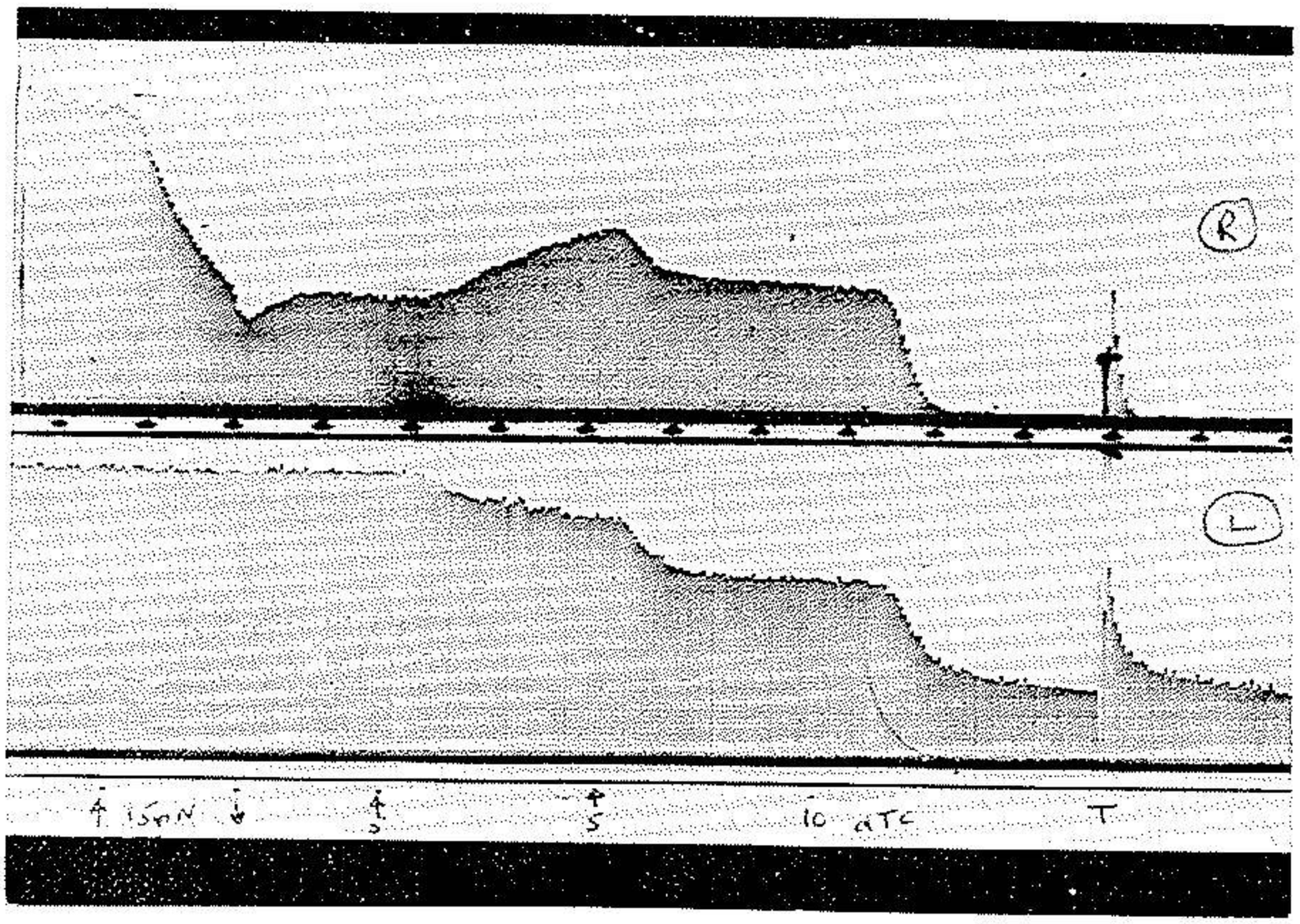

Fig. 2: Neuromuscular block produced by the use of 1.5 mg neostigmine in right arm(top trace)followed by the use of 5+5+10 mg of dtc systemically. Notice the initial recovery followed by additive blocking effect resulted from dtc administration.

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Experiment No. 3: 0.5 mg of neostigmine diluted in 40 ml of saline was injected into an arm, isolated for 2 min from the general circulation by an arterial tourniquet. One min after the release of the cuff,it was blown again and 1 mg of neostigmine was injected into the isolated arm.In this experiment 1 mg neostigmine did not produce any blokade of transmission.

Experiment No. 4: 0.5 mg of neostigmine diluted in 40 ml of saline was injected into an arm, isolated for 2 min from the general circulation by an arterial tourniquet. Three min after the release of the cuff,it was blown again and 6 mg of suxamethonium was injected into this isolated arm which produced a prolonged block preceded by intense fasciculation which persisted for 15 min after the release of the cuff.

Experiment No. 5: In a group of 180 anesthetised patients receiving 0.5 mg/Kg dtc, neostigmine 0.5-1 mg was given. The blocking action of dtc was greatly modified, manifested by maintainance of spontaneous respiration and restoration of normal blood pressure.

Experiment No. 6: In two groups of 50 patients the prophylactic action of 3 mg dtc has been compared to the action of prior IV injection of 4 mg suxamethonium given 10 min before induction. Fasciculation was prevented effectively in both cases.

RESULTS

1- Neostigmine possesses high affinity for curare receptor preventing dtc binding(Exp.1). But it shows addi-

tive paralytic effect with dtc,when injected in the isolated arm. This clearly shows the dual action of neostigmine; that is in low dose it stimulates the release of ACh from nerve ending preventing or reversing dtc block (Exp.5), but when it is used in high dose, it causes blockade indicating its receptor binding.

2- There is no direct competition in dissociation from the receptor in the isolated arm,between dtc and neostigmine,untill the blood circulation is restored. We know that ACh release is reduced enormously in the isolated arm so, it appear that an excess of ACh is necessary for the facilitation action in reciprocal dissociation between dtc and neostigmine from the receptor (Exp.1,3,5). When curarization is below 100 percent, neostigmine reverses the block effectively. On the other hand, Gallamine or dtc bring about a rapid recovery in case of neostigmine blockade(14,15)

3- Injection of 1 mg of neostigmine in isolated arm (Exp.2), and more than 5 mg in systemic use blocks neuromuscular transmission. The injection of 0.6 mg/Kg dtc also produces complete muscular paralysis.

Result 1 and 2 suggest a common receptor for dtc and neostigmine and result 3 indicates that the same receptor is involved in ACh chemical transmission.

4- Some of the suxamethonium molecules possess high affinity for binding to curare receptor (Exp.6.).

DISCUSSION

Miledi et al.(1971)[1] have separated a protein from the electric organ of "torpedo" fish. Kato(1968)[2] prepared a

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non-homogeneous extract of skeletal muscle whose activity he studied by means of nuclear magnetic resonance. His extract indicated separate sites for the anionic and esteratic groups (Fig.3). On the other hand, from the studies of Wilson and Bergman (1950)[3] and extended by Wilson and others, it has been firmly established that the active surface of ACh E enzyme unit consists of two sites, an anionic and an esteratic site. (Fig.4).

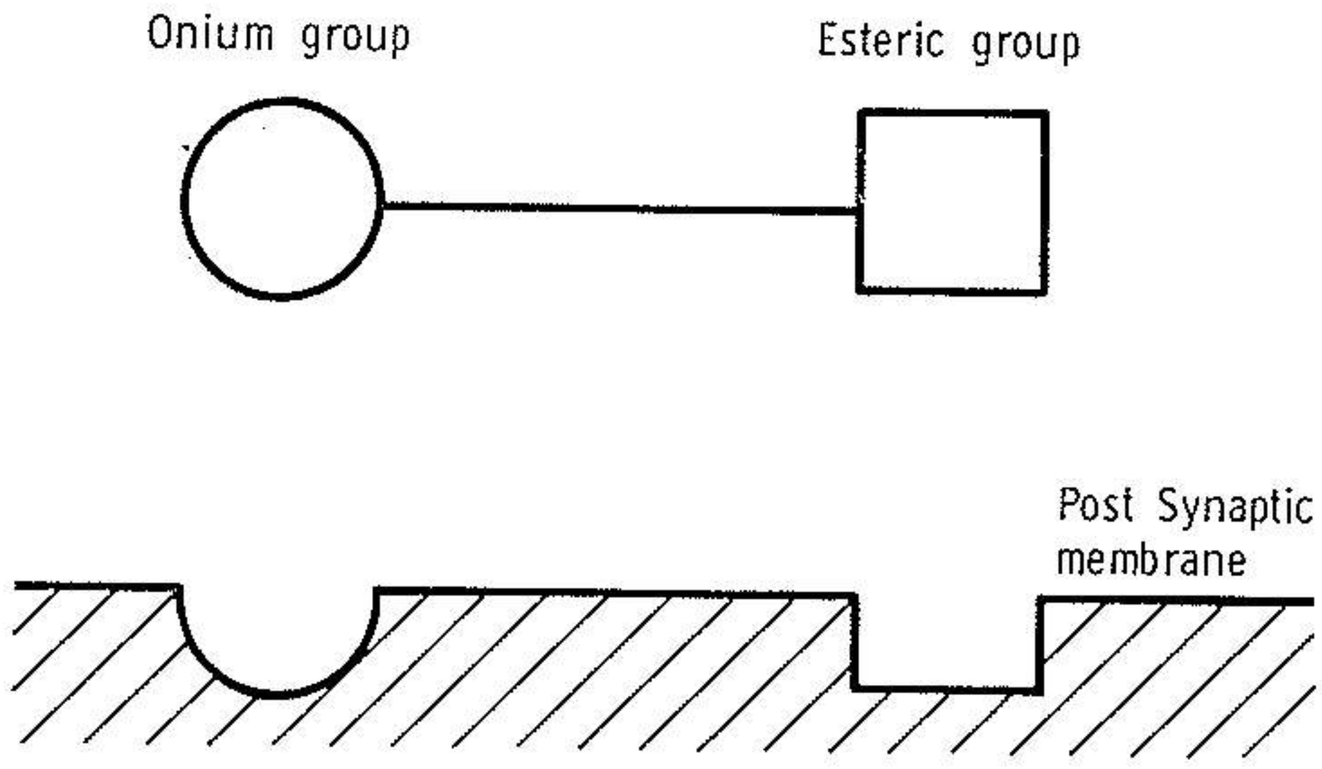

Fig. 3: Schematic representation of a cholinergic receptor, illustrating concept of a separate anionic receptor site and an esteric binding site on the postsynaptic membrane. after Kato(1965).

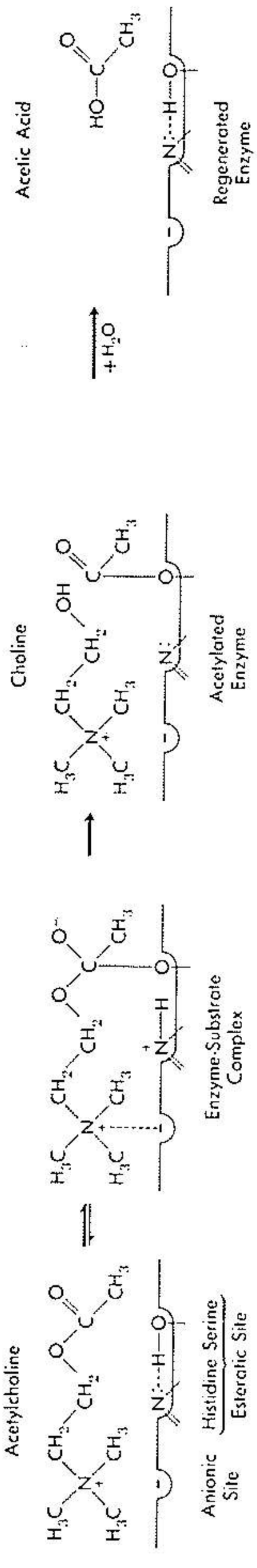

Fig. 4: The two active sites of an AChE molecule in interaction with a molecule of ACh till the production of acetylated enzyme with the esteratic site(after Wilson 1971).

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Proposed mechanism of Cholinergic receptor

The log-dose relationship resulted from in vitro studies(Jenkinson, 1960[7] ;Lu, 1970; Wand et al, 1973[8]) have demonstrated a consistent antagonism of neuromuscular block with larger dose of agonist. This finding substantiates the mass of clinical experiments which have shown similarity between dtc and neostigmine in association to the cholinergic receptor and in dissociation from the receptor. (Exp.1,2,3,5).Both of these agents can block effectively the physiological function of ACh. So, it appears that cholinergic receptor is composed from the combination of 1-2 molecules of AChE, to the gate of a Na^+ channel, at MEP(Fig.12A).

AChE molecules are assigned to control the passage of ACh at the gate, and molecular structure of Na^+ channel consisting of polypeptid chains containing mucopolysaccharids and probably lipid molecules, controls the free ionic flux, The close cooperation of this unit in the presence of ACh will result in muscle contraction. This hypothesis will be further substantiated by study of the actual mechanism of different forms of neuromuscular block produced by various relaxant agents.

I - CURARE BLOCK

The marvellous autoradiographs of mouse diaphragm (Waser, 1967, 1970)[4,5] have demonstrated a quantity of 4 millions curare-receptors in a unit of motor end-plate (MEP) of mouse diaphragm which suited exactly with the distribution and localization of AChE molecules in MEP

This localization closely fits with the distribution of AChE molecules which can be easily visualized by Koelle's staining technique(Fig.6)[9]. This evidence suggests that AChE molecules have definitely an essential duty in neuromuscular transmission.

(Fig.6This fixed quantity of curare receptor is in agreement with clinical experiments which indicate that the prior use of neostigmine modifies the curare block and the prior use of sufficient dose of dtc prevents the reversing action of neostigmine (Baraka 1977)[6]. By the evidence from autoradiographs, Waser suggests that curare molecules stick to AChE molecules but because of peculiar action of depolarizing drugs and dtc-Anti-AChE mixture, he was not able to identify the nature of cholinergic receptor.

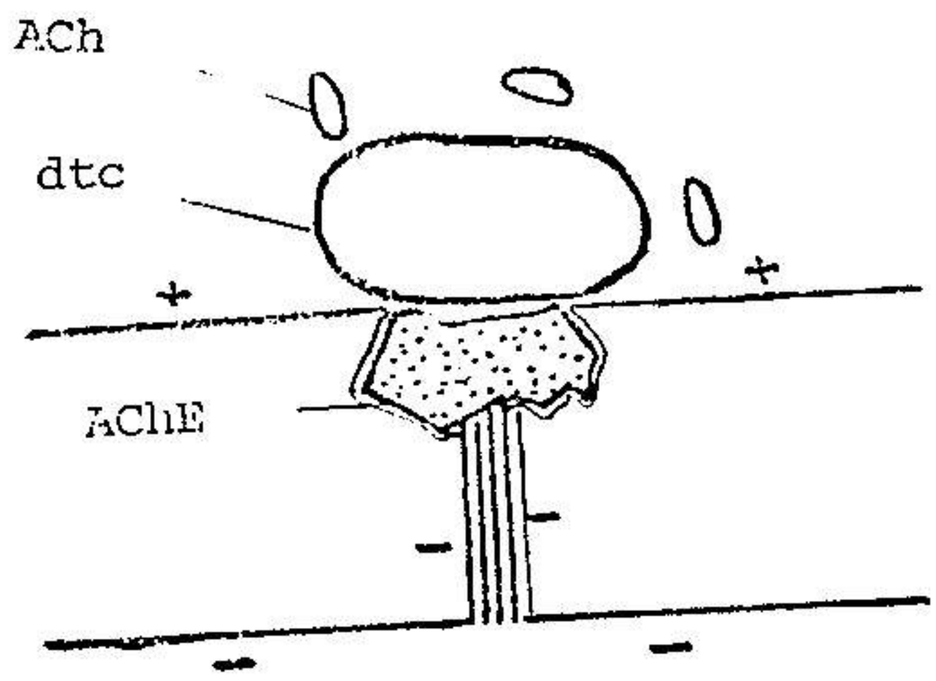

Fig.5: M.-Scjematic cross-section of receptor - The gate and the Na^+ channel are both obstructed by curare interaction(After waser 1967).

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Fig. 6: Mouse diaphragm as seen from above. Left:Cholinesterase in end plates stained by Koelle's method. Right: Autoradiograph with (C^{14})toxiferine specifically localized in the endplates after intravenous injection. (After Waser 1967).

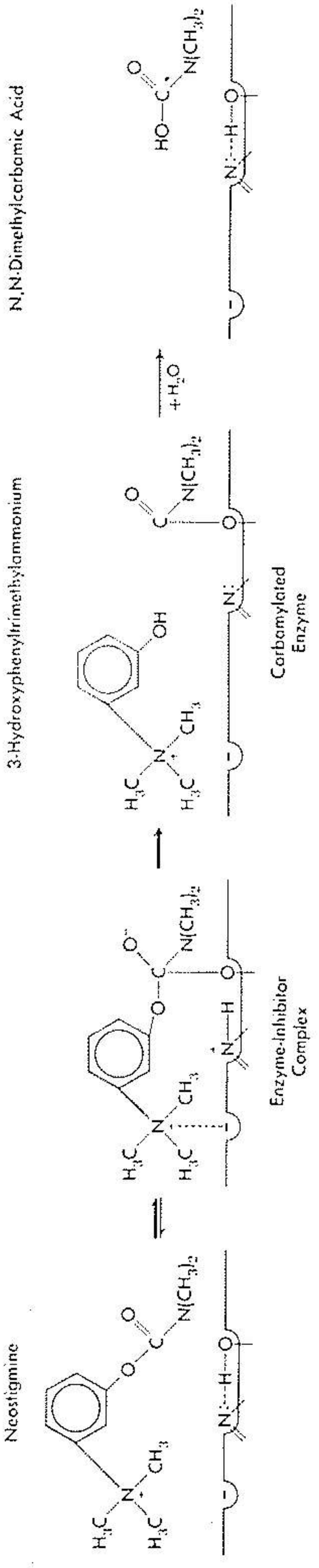

Fig.7. Steps involved in the inhibition of AChE by reversible carbamyl ester and organophosphorus agents(After Wilson 1971).

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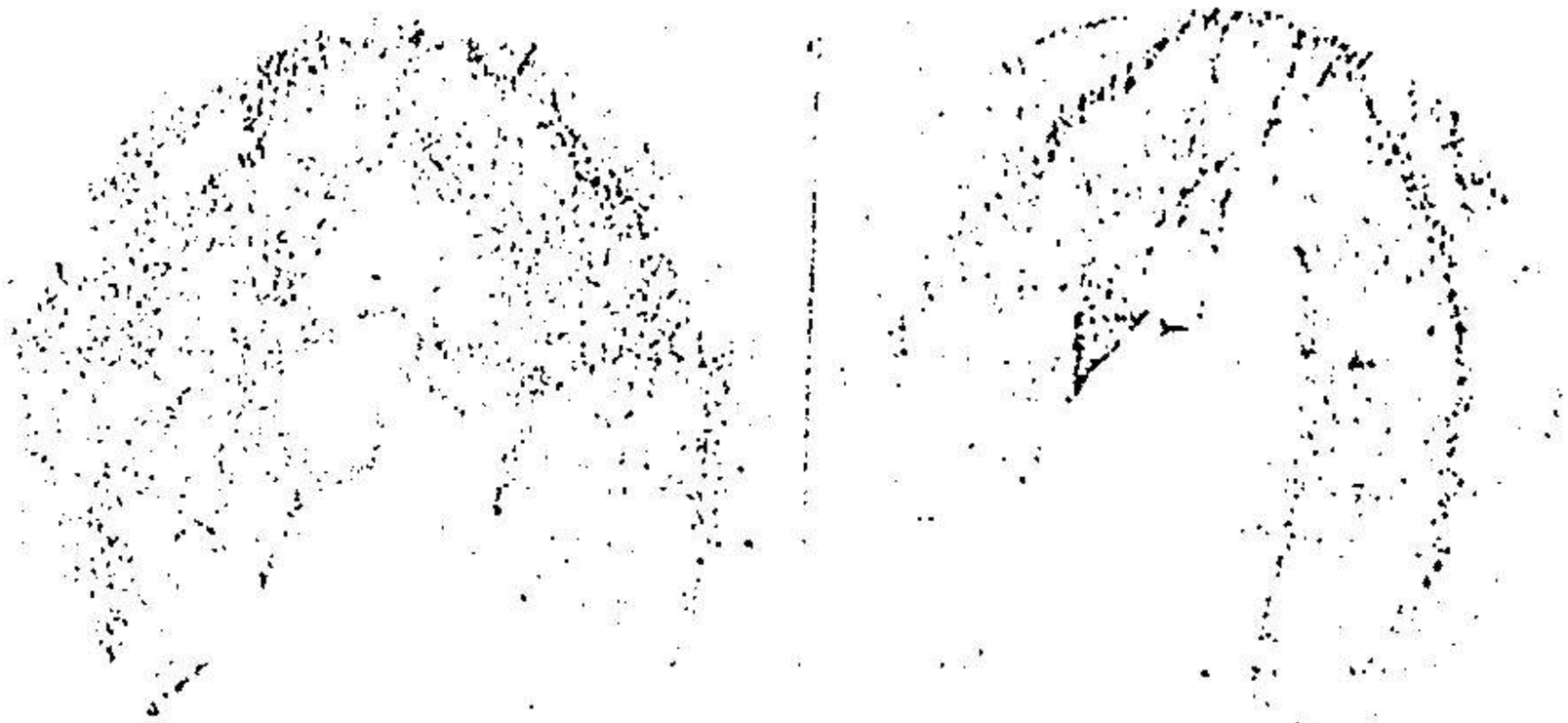

^{32}P-DFP 10^{-5}m

^{14}C-DFP 10^{-5}m

Fig. 8: Autoradiographs of diaphragms incubated with 10^{-1}m 32p-DFP(Left)and 14C-DFP(right).

II Neostigmine block

AchE inhibitors or inactivators agents are eventually bound to the esteratic site of AChE molecule although they might be attracted primarily by the anionic site(Wilson and Harrison 1961)[10]. This can either be a long-standing reversible or an irreversible acid transferring interaction producing carbomulated or alkylphosphorylated enzyme(Fig.7). The AChE molecule bearing significant change in conformation, finding a particularly fixed configuration. This possibility which was advocated by Wilson

(1967)[11] will be substantiated by clinical finding (Exp No.4) which will demonstrate the free passage of ACh into the Na^+ channel, potentiating the action of depolarizing componuds. The autoradiograph and densitometer using labelled Di-isopropyl fluorophosphate(DFP)(Fig 8) have demonstrated twice binding sites comparing with dtc, at the level of AChE localization in motor end-plate(Waser, 1967). In clinic,the use of neostigmine below 2.5 mg produces spasm of striated muscles, potentiation of depolarisation and reversion of non depolarisation blockade. However, the repeated use of neostigmine in excess of 5 mg will produce an intermediary block characterized by tetanic fade combined with potentiated twitch response (Payne 1980). The appearance of tetanic fade indicates that more than 75% of AChE molecules are stabilized by neostigmine binding. The persistance of twitch response suggests that the gate of the channel remains penetrable by ACh molecule (Fig.9). Thus,Na^+,ACh, and probably neostigmine molecule will pass into the Na^+ channel. This assumption is confirmed by potentiation of this block by the use of suxamethonium, by the preventive effect of dtc on the changes of EPP by physostigmine and other anti-ChE agents(Eccles et al 1942[12]; Kuffer 1942[13];)and also by reversing effect exerted by dtc and gallamine in neostigmine block(Briscoe 1938;[14] Payne et al, 1980[15]).

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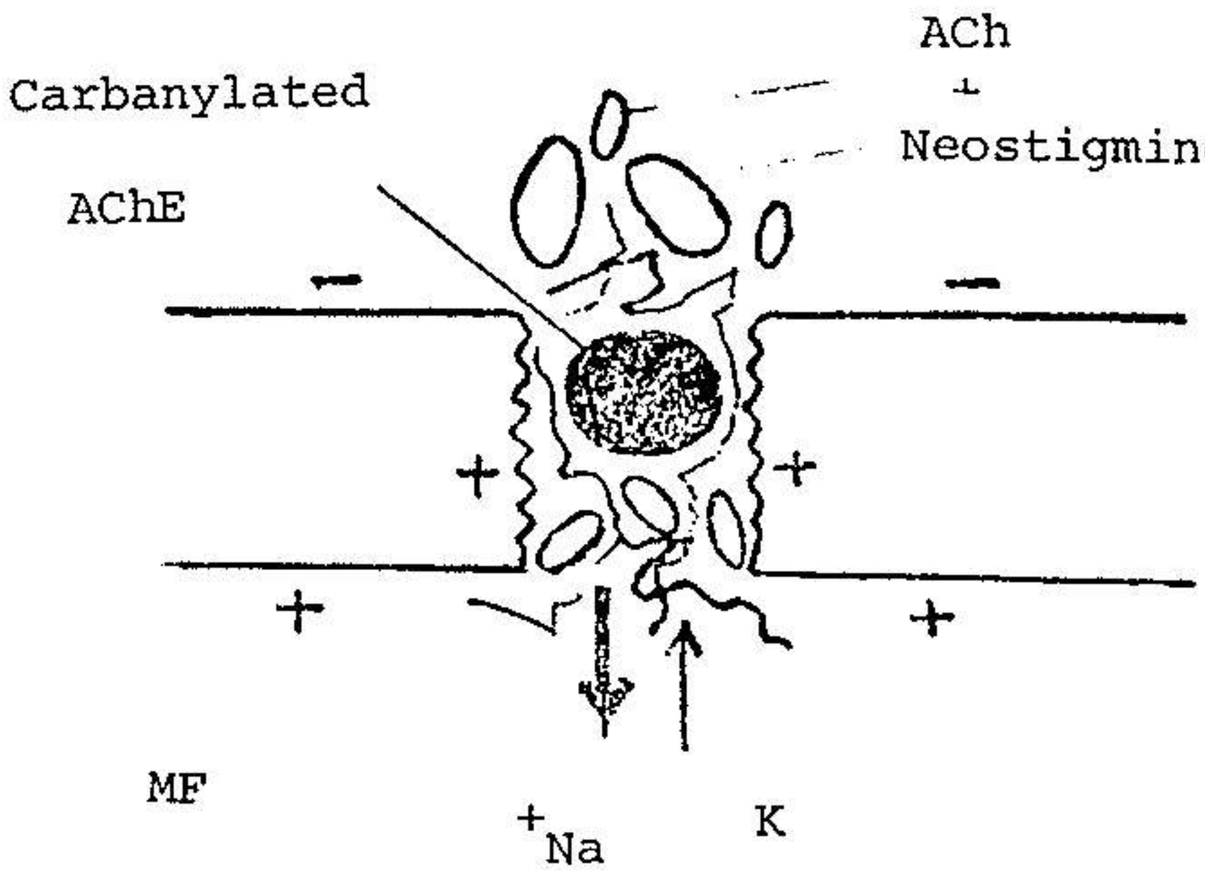

Fig. 9: Schematic cross section of receptor area. The gate and the Na channel are both patent by neostigmine interaction.

The dual action of neostigmine, as mentioned above, explains the mechanism of action of nicotinic agents which activate the receptor at the beginning, later followed by depression.

III Suxamethonium block

Stage I - (depolarizing block):

The slender molecules of depolarizing agents produce a completely different picture (Fig.10) in autoradiographs(Waser 1967).They are distributed diffusely in and around the end plate. The production of fasciculation succeeded by sustained depolarization,suggests that the Na^+ channel has become fully patent for ionic flux.

Since the prior use of dtc prevents this depolarization, it is deduced that suxamethonium molecules stick to the same receptor as curare. So, the AChE molecule is subject of a marked change in conformation and succinated enzyme will hold a particularly retracted configuration.

By the opening of the gate, suxamethonium molecules are attracted by the lipo-polypeptide structure of Na channel containing mucopolysaccharide, as shown in autoradiographs. From this interaction the dynamic state of these molecules, is altered resulting in opening of the channel (Fig. 11-A).

The Na^+ conductance which was previously low, is suddenly increased causing a rapid inflow of Na into the cell producing a sustained depolarization of the end-plate. This change in Na^+ conductance will be also observable by the use of Suxamethonium after denervation. This fact indicates that even after disappearance of AChE molecules, due to denervation, the channel especial structure for some period still hinders the free transfer of ions. Thus, in case one uses suxamethonium for relaxation in paraplegies, a lethal rise in serum potassium concentration ensues.

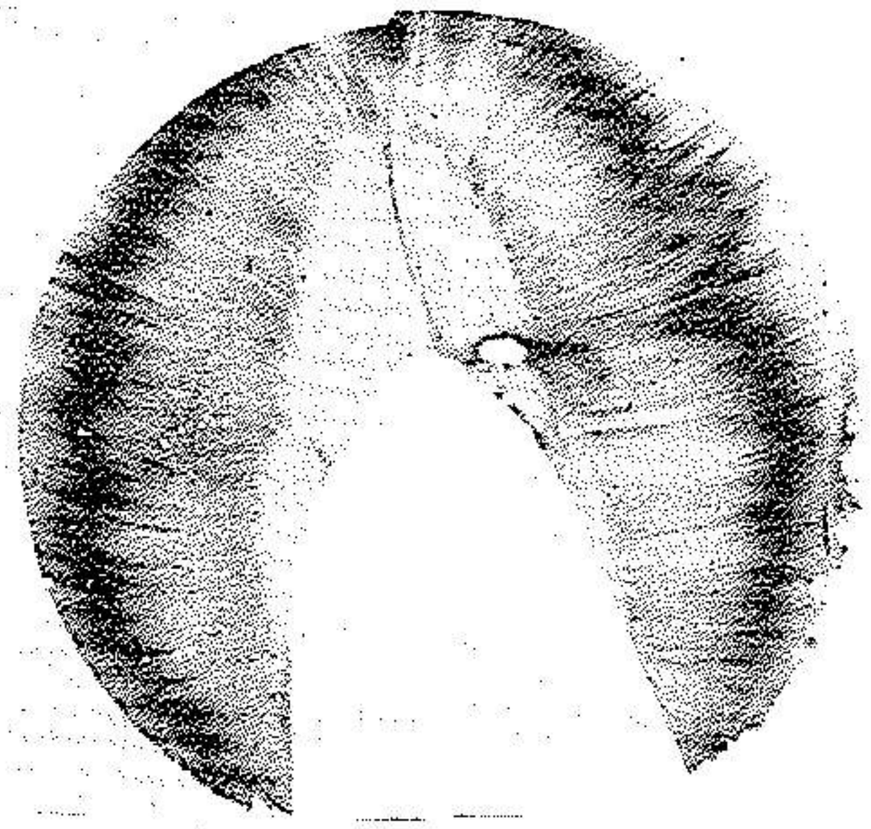

Fig.10- Effect of C^{14} decamethonium on the mouse diaphragm.Note the difuse fixation of the drug in the end-plate region as contrasted with the localisation of toxiferine.

Stage II(Intermediary block): When suxamethonium administration is continued, the pattern of block will change gradually. It has been proposed that due to the non-rigid molecule of depolarizing agent they may exist in various steric configuration. These atypical molecules will bind to the receptor in similar manner as curare molecule and will form high affinity bonds(Goat and Fledman 1972).[16] They can only be removed from the receptor by blood, circulating a low concentration of the drug, but whenever an excessive dose of suxamethonium has been used, they can not be completely evacuated from receptor site. In clinic, this paradoxical effect is an inevitable accompaniment of the use of depolarizing agent but as a subclinical entity. It is detectable even by the use of 4 mg of suxametonium(Exp.6), but it will not be noticeable untill the depolarizing effect has been completely worn away.Thus,the administration of suxamethonium 20 min before parcuronium or curare, enhances the amount of paralysis produced by these agents(Katz,1971)[17]

The high affinity bonds are first established at the esteratic active site of the enzyme. This phenomenon is manifested initially by tachyphylaxis, terminating to a stage of complete enzyme inhibition (Fig.11-B)manifested clinically by the appearance of tetanic fade with persistance of twitch response(Wednesky inhibition)which resembles the clinical feature of neostigmine block(Fig.8,9). In practice, up to this time the use of neostigmine markedly potentiate the suxamethonium block(Churchill Davidson et,al., 1960)[18].

Stage III(non-depolarizing block): When the overdosing of suxamethonium is further continued their molecules,

not being broken at the esteratic site now, will attach to the anionic active site of the enzyme and will produce total obstruction of the gate (Fig.11-C). In clinic, now a complete picture of curare block will be observed with both tetanic and twitch fade and also exhibiting post-tetanic potentiation of twitch response. In this state,the use of edrophonium will cuase an initial improvement in the neuromuscular paralysis,probably by liberating the molecular jam at the anionic site, but will potentiate the depolarizing activity at the esteratic site of the enzyme which will also be present. So, it appears that neuromuscular transmission depends mainly upon the state of affair prevailing at the end-plate,irrespective of the relaxant molecules still present in the interior of the fibre at this stage(Taylor [19] 1965). This indicates that there is other exit for the extrusion of depolarizing molecule. Provided the blood concentration of the drug is low, the channel will clear up soon and the esteratic site of the enzyme recovers its activity. Thus, the administration of Anti-ChE will produce a dramatic improvement as a result of liberation of the anionic site which opens up the gate.

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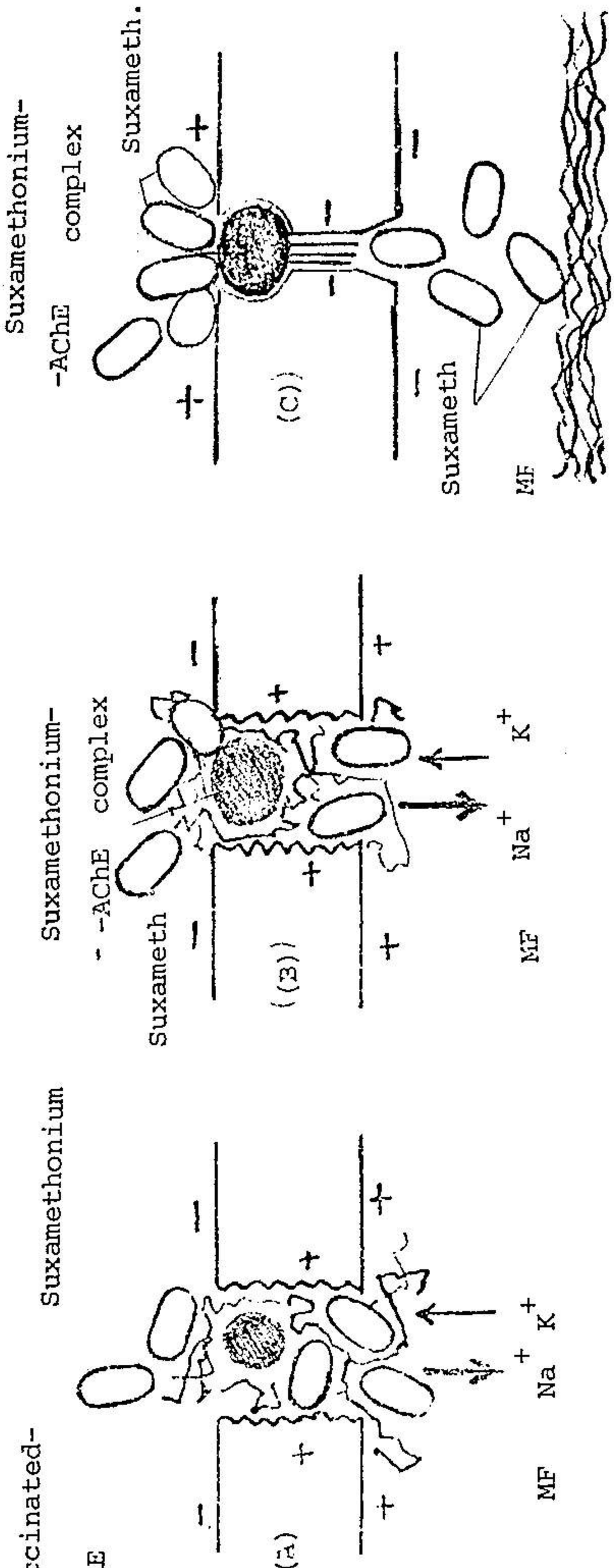

Fig. 11: Schematic cross section of receptor area and its interaction with increasing overdose of suxamethonium.

Cholinergic Receptor

The foregoing account on the mechanism of depolarizing agent also clearly indicates the mechanism of ACh function, as follows:

On the arrival of nervous discharge ACh molecule is liberated from the nerve terminal into the synaptic cleft. It will be attracted to the active sites of AChE situated at the surface of post synaptic membrance. The quaternary N atom of choline moiety of ACh is attracted by the anionic site of the enzyme (Fig.4) which results in exact alignment of the ester function of the substrate in especific position for union with the esteratic site of the enzyme which will hold it for 42 micro seconds. (Wilson 1967).

This occupation of the esteratic active site of the enzyme by ACh is substantiated by competitive interaction between substrate and DFP. It has been shown in vitro(Koelle 1949)[20] and in vivo(Leopold and McDonald,1948)[21] that the presence of sufficient concentration of ACh prevents alkylphosphorialation of AChE by DFP. Therefore, distinct changes can be brought about in molecular properties of AChE by its acetylation resulting in change in its conformation, opening the gate of Na channel to ACh action(Fig. 12. B). It has been possible to apply ACh solution directly to the motor end-plate to reach the surface of the receptor areas and under these condition as little as 10^{-16} M equivalants of ACh gives rise to an effective localized depolarization (EPP)which triggers the propagated muscle action potential(AP) and the latter in turn leads to muscle contraction. The application of a similar

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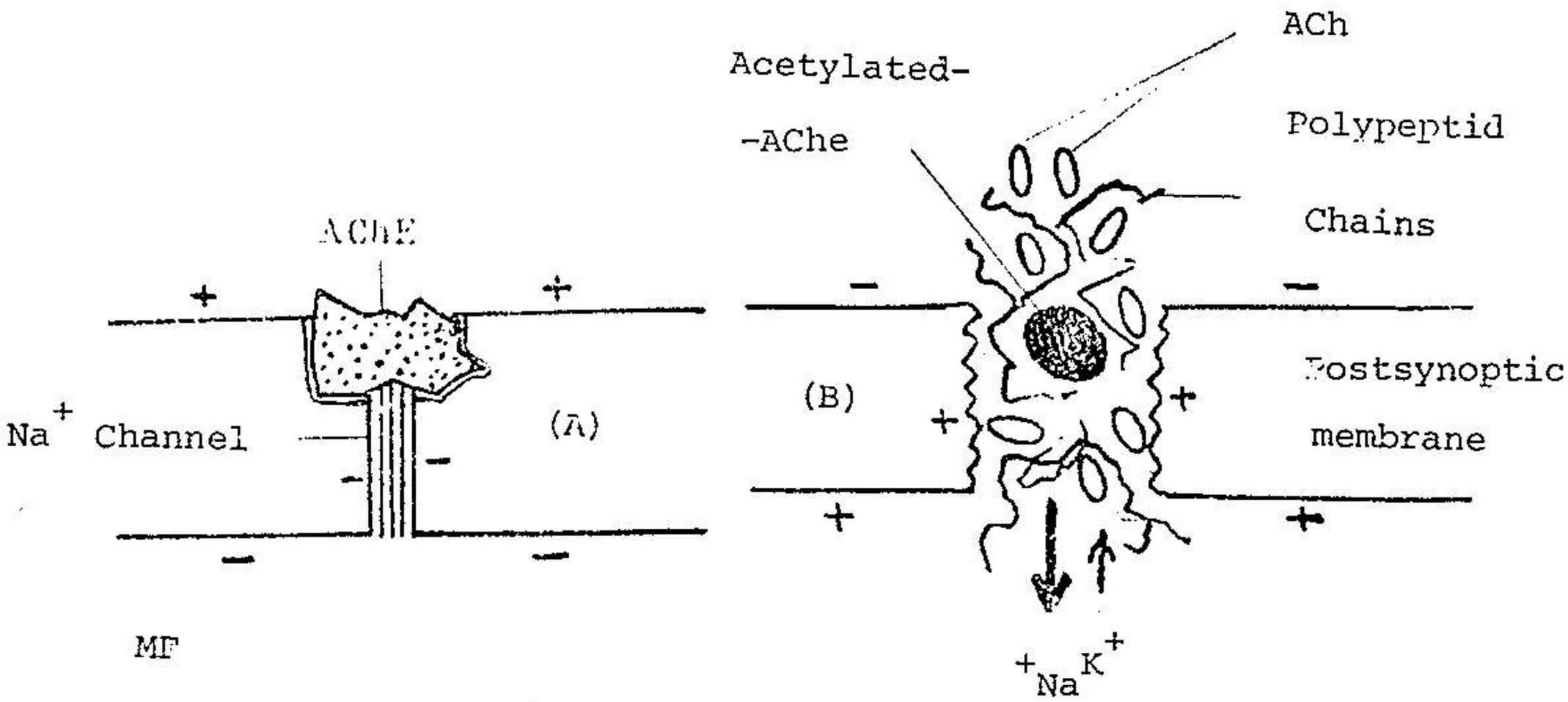

Fig. 12: Schematic cross section of cholinergic receptor composed of an AChE molecule situated at the gate of a Na^+ channel.
A-At rest: Gate and Channel both closed-no free ionic flux.
B-In action: Gate and channel both patent-free ionic flux.

concentration of ACh inside the muscle fibre has no effect(Zacks,1944[22]; del Castillo and Katz 1967[23]). This evidence indicates the importance of AChE molecules in controlling the passage of ions across the membrane. In contrast, when the gate of the channel is left unprotected by the use of DFP or denervation, which alter the efficiency of AChE enzyme in collecting ACh molecules, a supersensitivity will be observed(Cannon and Rosenblueth 1949)[24]

with marked reduction in the treshold doses of the transmitter and of certain other drug to elicit a response. These evidences argues in favour of AChE to be the principal part of ACh receptor--strategically situated at the surface and infolding of the post-junctional membrane (Davis and Koelle 1946;[25] Couteaux, 1972)[26]. After denervation all the functional AChE molecules gradually disappear(Koelle et al; 1975)[27] probably due in-part to the loss of an unknown trophic factor released normally from nerve terminals (Albuquerque et al; 1972)[28].

AChF a large molecular weight with four subunits is presumed to have an esteratic and anionic active sites with a 5 A^{o} distance between them(Wilson and Harrison 1961)[10]. This charge characteristic resembles exactly the active sites found in the nicotinic receptor of skeletal muscle (Kato, 1965)[2]. So, it follows that as a result of ACh interaction, the AChE molecules undergo marked change in conformation allowing the passage of ACh molecule into the Na^{+} channel. On the arrival of ACh the dynamic state of the channel constituants is suddenly changed giving rise to Na^{+} and K^{+} increased conductance.A vesicular membrane system reconstituted from the purified ACh receptor of electroplax and lipid of the same organism responds to ACh with increased[22] Na conductance (Michaelson and Raftery 1974)[24]. Takeuch and Takeuchi (1960)[20] using the voltage clamps technique at the motor and-plate of skeletal muscle have indicated that the EPP produced by ACh is due to marked increase in permeability of Na^{+} and K^{+} not cl^{-}. It has been estimated that for each molecule of ACh that combines, there is a flow of 50.000 cation across the post-junctional membrane (Katz and Miled, 1972)[31]. Nevertheless, the molecular structure

of the channel cannot be presumed as the sole site of ACh action because it has already been stated that the application of ACh inside of the muscle fibre has no effect. It has been known that denervation is accompanied by a spread of cholinoceptive sites from the end-plate region to the adjacent area(Axelsson and Tesleff, 1959)[32].Thus, in the absence of AChE function the entirety of Sarcopasmic membrane will find ability of translocating ions across the membrane producing a twitch response, but this differs greatly from the normal muscle contraction which is the result of a high rate stimulation and always demands the presence of AChE function.

After achieving its physiological function,ACh probably diffuses from the channel into the interior of the muscle fibre and from there into interstitial space being hydrolized by plasma AChE which so far has been known to have no physiological function. Evidence for this diffusion process is based on the finding that ACh molecules can be collected from the perfusion fluid of a nerve muscle preparation that has been protected from hydrolysis by the use of eserine(Brown et, al 1936).[33]

ACKNOWLEDGEMENT

The autor is indebted to Dr. S.A. Feldman from Westminister hospital university of London for his kind production of tracing No. 1,2.

SUMMARY

The present work with consideration to the autoradiographic pictures, suggests that cholinergic receptors are located at the gate of a channel originating from synaptic cleft coming to lie within the muscle fibre. AChE molecules stand at the gate of this channel, controlling the entrance of different cholinergic agents. It was reported previously that dtc molecules stabilize the AChE molecules and will obstruct the gate. This blocks the access of ionic flux within the channel thus producing a non-depolarizing neuromuscular paralysis. The presented experiments imply that depolarizing agent will bring a considerable change in conformation of AChE mole cule and this causes the opening of the gate allowing ioni flux and depolarization. In case of ACh this process is repeated in a fraction of milli second, due to rapid regeneration of AChE while in case of suxamethonium and neostigmine (given in high dose), the regeneration of AChE takes much longer time thus will produce a depolarizing blockade.

In this hypothesis the main responsability of AChE is confined to identification of cholinergic agents and Cooperation in their function so, it can be accepted as Cholinergic receptor.

In regard to clinic, this work suggests that only the use of minimum effective dose of neostigmine is advisable, in reversing curarisation. In contrast to general

belief, the dose of neostigmine should be selected in relation to receptor dtc occupation and not depending on patient's weight. As it was demonstrated, the early use of high dose of neostigmine may also potentiate curarisation.

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