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Fast Kinetics of Acetylcholine at Synaptic Membranes

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Abstract

The following theses are to be proven: activity of acetylcholinesterase is evident at cholinergic membranes during nerve excitation; activity of acetylcholinesterase is sufficient to induce permeability increase in elementary membranes; acetylcholinesterase wins the kinetic competition with the α-bungaro-toxin receptor for acetylcholine.

Introduction

From a thermodynamic point of view [1], there is no need for a teleologically specific receptor mechanism to induce nerve† excitation, because the latter only requires *passive* [2] flow—such as of ACh^+, Na^+, K^+—from compartments of high toward low concentration, i.e., toward equilibrium; many mechanisms may therefore be able to excite* the membrane.

The actual *in vivo* processes, however, are specific due to enzymatic control. Though being active during excitation of any cholinergic neuron *in vivo*, the specific enzyme acetylcholinesterase [5-7] (AChase E.C.3.1.1.7) has been ruled out [2, 8] as an acetylcholine (ACh) receptor protein even though this creates a kinetic paradox: *How can any other protein receive the ACh during physiological excitation while ACh is hydrolyzed by AChase much faster?*

Pharmacological observations apparently (1) exclude any role of AChase and (2) suggest α-bungaro-toxin receptor protein (αR) as the ACh receptor protein during nerve membrane excitation:

(a) When incubating cholinergic end-plates, specific inhibitors block the observable AChase activity, but in general not the postsynaptic potentials (PSPs) [2, 5, 9, 10].

(b) Correspondingly, collagenase dissolves AChase out of the membranes, but PSPs are still observed [11].

(c) When iontophoretically applying carbachol, an ACh analog which is not hydrolyzed by the AChase, PSPs do arise [12].

(d) α-bungaro-toxin and antibodies, which bind specifically to αR but not to AChase, do block the PSP [13-15].

For reviews see Refs. [5, 8, 16-18].

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* The cause of *excitability* is very different from that of excitation, maintaining [3] a nonequilibrium concentration gradient at rest, by necessity not understood within thermodynamic equilibrium terminology [4].

† The term "nerve" is not just used for axons. Biochemical data on nerve membranes are from synaptic end-plates in general.

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Though satisfactorily describable by a sequential interaction of ACh with αR—inducing Na^+ permeability increase—and, later, with AChase—preventing further excitation [2, 19]—these observations are inconclusive for the *in vivo* situation: The membrane function M and its unknown composition

$$M = M_1 \& M_2 \& M_3 \& \cdots \& M_N$$

cannot be by logical calculus* determined from the comparison of observations (statements) on again unknown membranes

$$M, \quad M \& A_1, \quad M \& A_2, \quad \ldots$$

even if the supplemented pharmacological agent A_i interacts specifically with component M_i:

"antagonist"	"agonist"
$A_i \rightarrow \overline{M}_i$	$A_i \rightarrow M_i$

Proof by means of one example: consider, with $i > N$ and $j \leqq N$,

$$\overline{M}_i \rightarrow \overline{M}_j \qquad M_i \rightarrow M_j$$

Examples are given in the text, e.g., in the Conclusion. Then, respectively,

$M \& A_i \rightarrow \overline{M}$	$M \& A_i \rightarrow M$
but not $\overline{M} \rightarrow \overline{M}_i$	but not $M \rightarrow \overline{M}_i$

Therefore, the action of the agents A_i, but not the functional composition M of the membrane, can be pharmacologically determined.†

Other interpretations of these data are easy to construct, but are logically inconclusive as well.

A unique analysis of a system requires the decomposition into its logical parts.

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* Definitions of the logical calculus [20]:

(1) M, M_i, A_i are statements; respective examples are "ACh produces an excitation at cholinergic membranes *in vivo*"; "ACh produces an excitation of microsacs containing αR"; "α-bungaro-toxin blocks the depolarization due to ACh."

(2) $\overline{X}$ (read "not X") denotes the statement contradictory to X. $\overline{X}$ is true if X is false, $\overline{X}$ is false if X is true.

(3) $X \& Y$ (read "X and Y") denotes the statement which is true if and only if both X and Y are true.

(4) $X \vee Y$ (read "X or Y") denotes the statement which is true if and only if at least one of the statements X, Y is true.

(5) $X \rightarrow Y$ (read "if X, then Y") denotes the statement which is false if and only if X is true and Y is false.

(6) $X \rightleftarrows Y$ denotes the statement which is true if and only if X and Y are both true or X and Y are both false. $X \rightleftarrows Y$ hence means that X and Y both possess the same truth value.

† "Avant d'aborder l'examin de ces théories, je dois vous faire remarquer que les actions toxiques ou médicamenteuses sont excessivement variées dans leur promptitude, dans leur intensité, dans l'expression symptomatique de leurs effets et dans leur mode d'action. . . . Les effets peuvent être bien distincts pour deux substances que agissent sur le même système. Cette localisation des actions toxiques nous permettra d'*en* suivre le mécanisme jusque dans les organes; elle met aux mains du physiologiste expérimentateur de véritables *réactifs de la vie*." Claude Bernard (1857), *Leçons sur les Effets des Substances Toxiques et Médicamenteuses*.

Molecularly, the function of the membrane, M, is therefore only known if reconstituted from the corresponding subsystems M_1, M_2, . . . , e.g., from lipid bilayer, AChase, αR, ...

The analysis is complete, if an evident subsystem (thesis I) is sufficient (thesis II) for the described function. The analysis is unique, if competing subsystems can be excluded (thesis III).

Thesis I: *Acetylcholinesterase activity is evident during excitation of cholinergic nerve membranes*

Macroscopically in *space,* Nachmansohn proved that any nervous tissue possesses AChase activity, the latter being maximal within the electric organ of the electric fish *Electrophorus electricus* or *Torpedo marmorata* [5]. Microscopically, activity and active site of AChase are histochemically localized at the nerve membrane mainly [21]. Diffusion artifacts can be excluded by the use of photon resonance energy transfer [22, 23], which allows a direct observation of spatial association between the lecithin lipid membrane and the tailed 18 S AChase [24] (Fig. 1). Temporally, the slow kinetics of ACh [25, 26] correlates the modifications of the PSPs to the rate of ACh hydrolysis, determined from the kinetic cycle of ACh during stimulation [27]. The fast kinetics of ACh during excitation at the AChase exceeds by orders of magnitude the speed of the physiological excitation and of potential noise kinetics [28]:

$$5 \times 10^{-5}\ \text{sec} \simeq \tau_{\text{hydrolysis}} \ll \tau_{\text{excitation}} \simeq 10^{-3}\ \text{sec}$$

By consequence, AChase activity is evident at the site and during the time of cholinergic excitation *in vivo,* and any consequence of ACh hydrolysis on any membrane component has by necessity to be part of the events during nerve excitation.

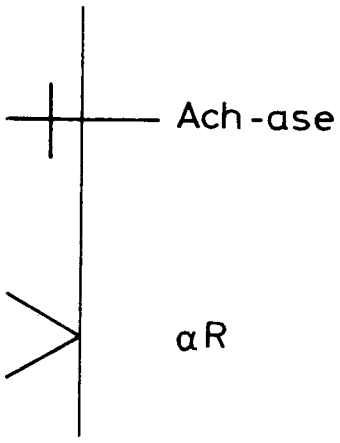


Figure 1. Elementary cholinergic nerve membrane, constituted of lipid phase ——, acetylcholinesterase +, and α-bungaro-toxin receptor protein V, according to Refs. [18, 21].

Thesis II: ***Acetylcholinesterase activity is sufficient to increase the permeability of the lipid component of nerve membranes***

It has been reproducibly demonstrated [29, 30] that AChase activity does increase the passive permeability of black-film lipid bilayer membranes (Fig. 2). Beyond any reasonable doubt, ACh will have the same consequence at an equivalent elementary membrane "lipid bilayer & AChase" *in vivo*.

Why intact membranes possess permeabilities larger by several orders of magnitude than the pure lipid membranes is not yet known [31]; carrier [32] or pore [33] mechanisms so far require molecules which are not present at nerve membranes. Thesis II, therefore, cannot be tested at present for the total nerve membrane permeability.

Other proteins are as capable as AChase to produce this "receptor" interaction with ACh; indeed, quite generally, protein–ligand or enzyme–substrate interactions at lipid membranes do increase their passive, inspecific permeability for monovalent cations [34], as required to induce a nerve membrane excitation. These other interactions, however, are not evident *in vivo*.

Thesis III: ***At nerve membranes in vivo, the enzyme acetylcholinesterase wins the kinetic competition with the α-bungaro-toxin receptor protein for the acetylcholine***

There exist at least two kinetic compartments for ACh evident at the postsynaptic membrane of cholinergic end-plates: one (E) containing AChase and one (R) containing a second cholinergic protein, e.g., the toxin receptor αR. It is not necessary to specify the spatial arrangement at this stage [35] (Fig. 3).

At time $t = 0$, the end-plate is stimulated and ACh is released from the synthesizing compartment [27] into these two compartments with mole numbers $n_E(0)$ and $n_\alpha(0)$, respectively. The initial condition $n_\alpha(0) = n_E(0)$ may be chosen for a molecular ratio 1:1 between these cholinergic binding sites [14].

For $t > 0$ this kinetic system obeys the following set of equations which is solvable

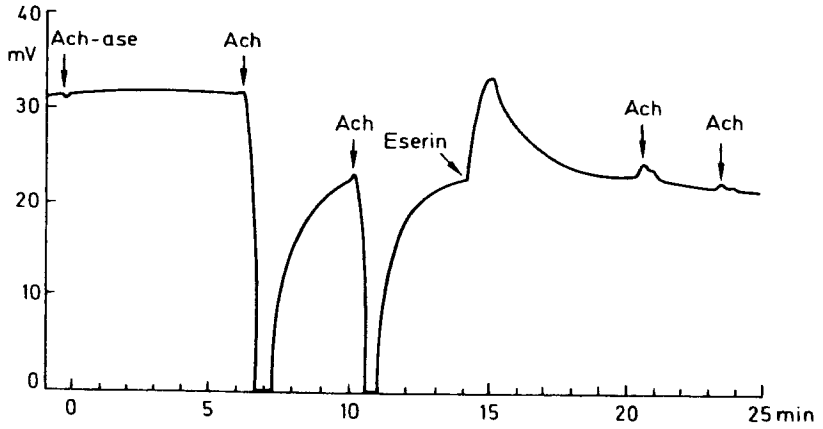


Figure 2. Records of potential difference across lipid bilayers subjected to acetylcholinesterase activity. From Ref. [29].

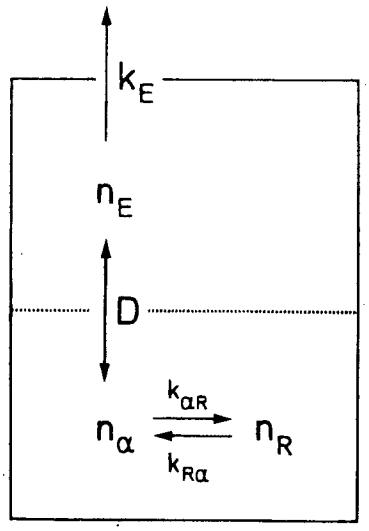


Figure 3. Acetylcholine released during nerve stimulation from the synthesizing compartment [27] with mole number $n_E(0) + n_\alpha(0)$ is either (n_E) accessible to the acetylcholinesterase, AChase, or to a second cholinergic protein (n_α), say, the α-bungarotoxin receptor protein αR, with kinetic constants comparable to *in vitro* systems containing only AChase or αR. Surface diffusion parallel to the membrane (or, *in vitro*, free diffusion between these proteins) is described by constant D. The macroscopically irreversible hydrolysis (rate $k_E n_E$) creates a kinetic asymmetry: all initial acetylcholine is finally hydrolyzed while only a part is received by αR.

in the linear case analytically, and for other cases numerically. (For derivation and limits see the Appendix.)

$$\frac{dn_E}{dt} = -k_E n_E + D(n_\alpha - n_E)$$

$$\frac{dn_\alpha}{dt} = -D(n_\alpha - n_E) - k_{\alpha R} n_\alpha + k_{R\alpha} n_R$$

$$\frac{dn_R}{dt} = +k_{\alpha R} n_\alpha - k_{R\alpha} n_R$$

All parameters are observables: $k_{\alpha R}$ and $k_{R\alpha}$ are the pseudo-first-order association and the dissociation constants of ACh onto and from purified αR [16–18]; $k_E n_E$ is the total activity of AChase [6–7] in the hydrolyzing subcompartment; D is the constant of diffusion.

A still unsolved problem arises from the fact that the membrane system is rather two dimensional, while the kinetic systems are usually determined in three dimensions, thereby being slower by orders of magnitude [36]. To avoid this and related problems, an analysis is designed which does not require quantitative knowledge of the kinetic constants. The basic idea is that, in general, the kinetic constants are *different* from each other. Then, all possible general cases are covered if all permutations of the in-

equality $D \gg k_E \gg k_{\alpha R} \gg k_{R\alpha}$ are solved. Numerical solutions demonstrate that the results thus obtained are a fair approximation even for weak inequalities (Fig. 4).

In Table I, the linear solutions for the most interesting case of short times are listed, assuming small $k_{R\alpha}$ in order to allow a large ACh-to-αR binding constant $k_{\alpha R}^{(2)}/k_{R\alpha}$ as reported [16–18, 37].

It is now essential to note that, for each permutation, the analytical solution is specifically different. Therefore, fast kinetic studies in the presence of both proteins allow one to diagnose which of these permutations is the realistic one without quantitative knowledge of all kinetic parameters.

By consequence, the observations [2, 14, 16–18, 37, 38] of hydrolysis-controlled decay of all free ACh, i.e., of $n_E(t) + n_\alpha(t) \sim e^{-k_E t}$, is then sufficient to *conclude* from Table I that $D \gg k_E \gg k_{\alpha R} \gg k_{R\alpha}$ and that the relative binding of ACh to αR is very small:

$$\int_0^\infty k_{\alpha R} n_\alpha dt \Big/ \int_0^\infty k_E n_E dt = k_{\alpha R}/2k_E \ll 1$$

This result is congruent with the fact that there is no binding of ACh onto a second

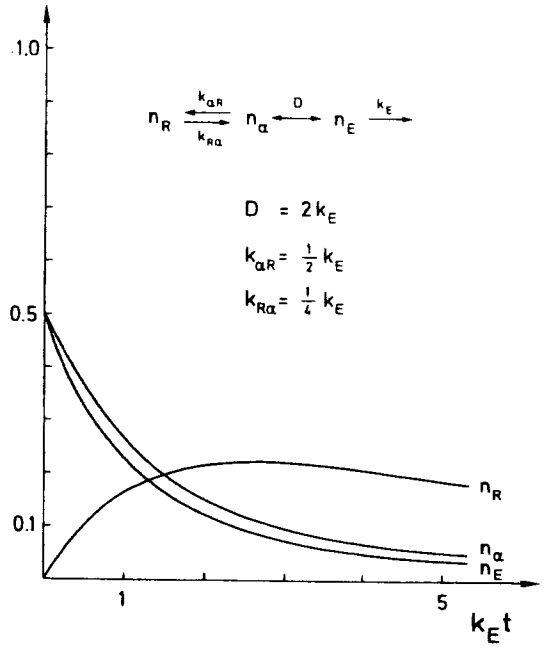


Figure 4. The fate of acetylcholine at a membrane containing the two proteins of Figure 1 is illustrated for a linear example with $D > k_E > k_{\alpha R} > k_{R\alpha}$ (see Fig. 3). These characteristics are much more pronounced for strong inequality $D \gg k_E \gg k_{\alpha R} \gg k_{R\alpha}$, concluded below from the observation *in vitro* and *in vivo*. The fastest initial kinetics is that of hydrolysis of n_E, and the fall of n_α is in part due to diffusion and hydrolysis without reception by αR. The part n_R of acetylcholine received by αR is smaller by a factor on the order of $k_{\alpha R}/2k_E$ (see Table I) as compared to that received by the enzyme.

TABLE I. Linear solution for short times after application of acetylcholine to a cholinergic nerve membrane or an *in vitro* system containing α-bungaro-toxin receptor protein and acetylcholinesterase. It follows from the general solution that all ACh is irreversibly hydrolyzed: $\int_0^\infty k_E n_E dt = n_E(0) + n_\alpha(0) + n_R(0)$, while only a part of it is bound reversibly to the second cholinergic protein. The relative binding of ACh to the toxin receptor is given in the last column; it accounts for multiple reception, too.*

$n_R \underset{k_{R\alpha}}{\overset{k_{\alpha R}}{\rightleftharpoons}} n_\alpha \xrightarrow{D} n_E \xrightarrow{k_E}$	$n_E(t)$	$n_\alpha(t)$	$n_R(t)$	$\dfrac{\int_0^\infty k_{\alpha R} n_\alpha(t)dt}{\int_0^\infty k_E n_E(t)dt}$
$D \gg k_E \gg k_{\alpha R} \gg k_{R\alpha}$	$\frac{1}{2} e^{-k_E t}$	$\frac{1}{2} e^{-k_E t}$	$\frac{k_{\alpha R}}{2k_E}(e^{-k_{R\alpha} t} - e^{-k_E t})$	$\frac{k_{\alpha R}}{2k_E}$
$D \gg k_{\alpha R} \gg k_E \gg k_{R\alpha}$	$\frac{1}{2} e^{-k_{\alpha R} t}$	$\frac{1}{2} e^{-k_{\alpha R} t}$	$\frac{1}{2}(e^{-k_{R\alpha} t} - e^{-k_{\alpha R} t})$	$\frac{1}{2}$
$k_E \gg D \gg k_{\alpha R} \gg k_{R\alpha}$	$\frac{1}{2} e^{-k_E t}$	$\frac{1}{2} e^{-Dt}$	$\frac{k_{\alpha R}}{2D}(e^{-k_{R\alpha} t} - e^{-Dt})$	$\frac{k_{\alpha R}}{2D}$
$k_E \gg k_{\alpha R} \gg D \gg k_{R\alpha}$	$\frac{1}{2} e^{-k_E t}$	$\frac{1}{2} e^{-k_{\alpha R} t}$	$\frac{1}{2}(e^{-k_{R\alpha} t} - e^{-k_{\alpha R} t})$	$\frac{1}{2}$
$k_{\alpha R} \gg D \gg k_E \gg k_{R\alpha}$	$\frac{1}{2} e^{-Dt}$	$\frac{1}{2} e^{-k_{\alpha R} t}$	$\frac{1}{2}(e^{-k_{R\alpha} t} - e^{-k_{\alpha R} t})$	$\frac{1}{2}$
$k_{\alpha R} \gg k_E \gg D \gg k_{R\alpha}$	$\frac{1}{2} e^{-k_E t}$	$\frac{1}{2} e^{-k_{\alpha R} t}$	$\frac{1}{2}(e^{-k_{R\alpha} t} - e^{-k_{\alpha R} t})$	$\frac{1}{2}$
$k_{\alpha R} \gg D \gg k_{R\alpha} \gg k_E$	$\frac{1}{2} e^{-Dt}$	$\frac{1}{2} e^{-k_{\alpha R} t}$	$\frac{1}{2}(e^{-k_{R\alpha} t} - e^{-k_{\alpha R} t})$	$\frac{1}{2}$

* In case only hydrolysis, i.e. only k_E, can be observed in the presence of *in vivo* amounts of AChase, the first-row permutation has to be concluded within the limits of linear competition; in consequence, the association of ACh onto αR is very small as compared to the association onto AChase, $k_{\alpha R}/2k_E \ll 1$, no matter whether equilibrium binding is strong, $k_{\alpha R} \gg k_{R\alpha}$.

cholinergic membrane protein observed in the presence of AChase [16–18], i.e., *in vivo*.

Conclusion

In the presence of acetylcholinesterase activity, the hypothesis of an acetylcholine receptor independent of the enzyme requires one to postulate a supplementary mechanism which—during excitation—blocks the observed hydrolysis of acetylcholine, but—some microseconds later—unblocks the enzyme in order to account for the observed rapid and complete hydrolysis. Such a postulate cannot be excluded, but it is not based as yet on observables.

Cholinergic receptor proteins independent of the enzyme do, however, fulfill other, observed functions. For example, in contrast to the esterase activity, the α-bungaro-

toxin receptor does regulate the level of Ca^{2+} ions [39] required for the synthesis of adenosin-tri-phosphate and acetylcholine and hence for nerve excitability.

Not any binding site at any protein is capable *per se* of reproducing the physiological and pharmacological responses of nerve membranes. Recent demonstrations may be found in Refs. [40, 41].

Hydrolysis is still the fastest process of acetylcholine evident at cholinergic membranes. Establishment of its quantum chemical mechanisms (e.g., possibility of hydrogen-bridge formation [42] between enzyme and substrate) and determination of the precise surface kinetics (e.g., rate enhancement by nonspecific association of the positively charged substrate onto the negatively charged lipid membrane; e.g. local pH and pAc decrease during hydrolysis) should quantitatively contribute to the understanding of the role in nerve excitation of the remarkable enzyme acetylcholinesterase.

Appendix: Derivation of the Kinetic Equations

1. First-Order Kinetic Parameters

For independent binding sites P holds:

$$P + A \underset{k_{AP}}{\overset{k_{PA}}{\rightleftharpoons}} AP$$

(A = ACh, P = binding site at αR or AChase, respectively)

and

$$\frac{d[A]}{dt} = -k_{PA}^{(2)}[P][A] + k_{AP}^{(1)}[AP]$$

with $[P] + [AP] = [P_0] = \text{const.}$

For concentrations of ACh not too high, $[A] \ll [P_0]$, i.e., $[P_0] - [P] = [AP] \leqq [A_0] \ll [P_0]$, hence

$$\frac{d[A]}{dt} = -k_{PA}^{(1)}[A] + k_{AP}^{(1)}[AP]$$

with $k_{PA}^{(1)} = k_{PA}^{(2)}[P] \approx k_{PA}^{(2)}[P_0] = \text{const.}$

These are the kinetic constants of first order:

$$k_{\alpha R} = k_{\alpha R}^{(2)}[\alpha R]; \qquad k_E = k_E^{(2)}[\text{AChase}]$$

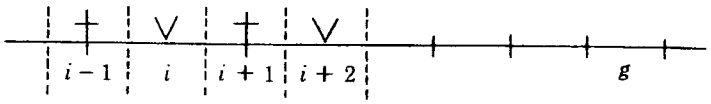
For $[A] \lesssim [P_0]$, the result deviates accordingly, and the parameters of the equations are no longer constant; in addition, they do depend on pH and other nonconstant factors. More than one rate-limiting association or dissociation step would also alter the equations in their quantitative, but not their qualitative, feature.

2. Diffusion

Fick's law reads

$$\frac{\partial c}{\partial t} = +D_F \nabla^2 c$$

In the one-dimensional case, for discrete compartments,



of "lattice" constant g, the gradient of concentration c

$$\nabla c\Big|_i = \frac{c_{i+1} - c_i}{g}$$

leads to

$$\nabla^2 c\Big|_i = \frac{c_{i+1} + c_{i-1} - 2c_i}{g^2}$$

For indistinguishable kinetics in corresponding membrane fragments it follows that $c_{i+1} = c_{i-1}$, hence

$$\nabla^2 c\Big|_i = \frac{c_{i+1} - c_i}{g^2/2}$$

and

$$\frac{dc_i}{dt} = \frac{2D_F}{g^2}(c_{i+1} - c_i)$$

or, macroscopically,

$$-\frac{dn_\alpha}{dt}\Big|_{\text{diff}} = \frac{dn_E}{dt}\Big|_{\text{diff}} = D(n_\alpha - n_E)$$

The "diffusion constant" D corresponds, in the one-dimensional protein crystal, to Fick's constant:

$$D = 2D_F/g^2$$

In two-dimensional and in noncrystalline cases the derivation is much the same, D is proportional to D_F; the proportionality factor, however, is more complicated according to the geometry. A quantitative treatment should include in addition an analysis of the surface kinetics and surface diffusion.

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