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Ions and excitable membrane

Ion flow gating by the acetylcholine system : Kinetics of isolated receptor and esterase and of receptor-mediated ion flux*

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SUMMARY :

1° Basic features of the elementary bioelectric signals such as miniature-endplate-potentials are molecularly interpreted on the basis of relaxation kinetic data of isolated acetylcholine receptor and acetylcholinesterase. Electrophysiological and molecular data suggest an essentially sequential processing of acetylcholine by receptor and esterase.

2° Flux measurements with sealed biomembrane fragments containing acetylcholine receptor show that the ion-transporting conformation of the receptor-channel is a short-lived metastable state. In the presence of neuroactivators the receptors inactivate. The description of the flux-inactivation requires a cyclic reaction scheme similar to the desensitization scheme of KATZ and THESLEFF (1957).

3° The recently introduced concept of integrated flux rate coefficients permits us to derive gating mechanisms from flux data under well-defined experimental conditions : sealed biomembrane vesicles, activator concentration, type of transported ion.

4° With respect to activation and inactivation and the metastability of the ion-conducting conformation, there are fundamental similarities between the axonal Na^+ ion channel and the acetylcholine receptor-channel.

Keys-words : Acetylcholine system. Acetylcholine receptor. Acetylcholinesterase. Ion flux.

I. — INTRODUCTION

Bioelectric signal transmission in nerve cells and across synapses is undoubtedly based on membrane permeability changes to Na^+, K^+, Ca^{2+}, or Cl^- ions ; the resulting local transmembrane ion flows are conformationally controlled by gating proteins. The gating reactions responsible for the opening and closing of the ion pathways of channels in excitable biomembranes appear to generally include *bimolecular* reaction steps (DOROGI and NEUMANN, 1980 *a, b* ; NEUMANN, 1980).

The molecularly most thoroughly studied ion flow « gating-apparatus » is the acetylcholine (AcCh) system, regulating transmembrane ion flux by a direct interaction of the neuroactivator AcCh with two membrane-associated proteins : acetylcholine receptor (AcChR) and acetylcholinesterase (AcChE), E.C. 3.1.1.7. The intrinsic function of AcChR is to gate Na^+-K^+ flows via AcCh-induced conformational changes leading to open and closed channel states. The esterase limits the availability of AcCh by extremely rapid hydrolytic decomposition of the neuroactivator.

The most direct way to study ion flow gating is to measure the ion flux itself which is rate-limited by the gating reactions of the control system. Historically, ion movements in excitable cellular systems have been extensively measured electrically and information on the gating system has been derived from electrophysiological data. In the nicotinic acetylcholine system, essential details on the relationship between receptor and esterase can be deduced from the electrical epiphenomena ; particularly instructive is a comparison with recent relaxation kinetic data of the isolated proteins. Further insight into gating processes is gained from flux data. We report on recent progress in theory and experiment of gated ion flow in general, and on the application of a new analytical flux technique to AcChR in sealed biomembrane fragments. It is rather illuminating to see that, in general, so-called half-response constants and flux rate constants are rather complicated quantities containing rate and equilibrium parameters of various gating steps. Among the physiologically relevant results which can be directly derived from the flux curves is that the ion-transporting receptor conformation is a rather short-lived *metastable* state. In the presence of activator the AcChR system completely inactivates (desensitizes) ; the description of flux inactivation requires a cyclic reaction scheme. With respect to activation-inactivation and metastability of the open ion-pathways, the AcChR system shows close similarities to the behaviour of the Na^+-channel of axonal membranes. Finally, it is indicated that the flux method and the theoretical analysis are not only complementary to but in many respects an alternative, to electrophysiological techniques.

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II. — ELEMENTARY ELECTRIC SIGNALS AND AcCh GATING CONCEPT

Probably, the most elementary epiphenomena resulting from the AcCh action are the ionic current « blips » due to single-channel fluctuations. When the concentration of AcCh increases, both the number of channels and the frequency of fluctuations increase. The spontaneous miniature endplate currents (m.e.p.c.) and the other more macroscopic electric signals represent a larger collection of temporarily open single channels. The time course of a m.e.p.c. is suited for a comparison with the kinetic results of isolated AcCh proteins because the kinetic data of isolated molecules always reflect ensemble averages. In Fig. 1, such a m.e.p.c. is redrawn from a review by GAGE (1976) : a rapid growth phase (reflecting the rapid increase in the number of AcChR-channels) is followed by a slower decay phase reflecting the decrease in the number of open receptor-channels. A particular important observation is expressed by GAGE and MCBURNEY (1975) : the « decay is exponential from the peak, *no rounding* is observed ». The growth phase probably reflects the rate of AcCh-induced conformational change in the AcChR. The decay phase is determined by esterase activity, but *not in a direct rate-limiting manner.*

It was found that the average life time, $\bar{t}_0$, of the open AcChR-channel does not depend on the concentration of the neuroactivator (see, *e.g.*, STEVENS, 1976 ; GAGE, 1976). At given temperature and membrane voltage, $\bar{t}_0$ is equal to the decay time constant τ_d of the m.e.p.c. ; in addition, $\bar{t}_0$ and τ_d both have the same voltage and temperature dependence. It therefore appears that the closure phase of a m.e.p.c. is independent of AcCh (see, *e.g.*, STEVENS, 1976). Furthermore, since practically complete inhibition of AcChE with neostigmine causes an increase in the peak amplitude of m.e.p.c. by a factor of 1.4 and a prolongation of the decay phase by a factor of about 2 (see Fig. 3 ; GAGE and MCBURNEY, 1975), the receptors in a « normal m.e.p.c. » appear not to be saturated (ROSENBERRY, 1979). The independence of AcCh in the m.e.p.c. decay phase requires that during the decay phase the neuroactivator is removed much faster than it can return to AcChR (R). The effective binding of AcCh to AcChE (and hydrolysis) should therefore be faster than to AcChR, or in terms of the effective association rate constants :

$$k_{ass}(R) < k_{ass}(E) \qquad (1)$$

where $k_{ass}(R)$ reflects the overall reaction leading to the conducting conformation with a life-time of about 5 to 10 ms.

According to current knowledge the simplest reaction scheme describing main features of *in vivo* AcChR gating is given by :

$$\begin{array}{l} 2\,A + R \rightleftharpoons \cdots (K_1) \rightleftharpoons A_2\,R \\ \qquad\qquad\qquad\qquad\quad k_c \,\downuparrows\, k_0 \\ \qquad\qquad\qquad\qquad\quad A_2\,R' \end{array} \qquad (2)$$

(See also SAKMANN and ADAMS, 1979.)

Assuming rapid binding of AcCh (A) to form the complex $A_2\,R$, coupled to a slower conformational transition of the occupied receptor $A_2\,R$ to the open channel configuration $A_2\,R'$, normal mode analysis of the reaction equations (2) yields the relaxation time τ_{II} of the slow mode :

$$\frac{1}{\tau_{II}} = k_c + k_0 \cdot \frac{[A]^2 + [R]}{[A]^2 + [R] + K_1} \qquad (3)$$

where k_0 and k_c are the rate constants for the opening and closing steps, respectively, and K_1 is the overall dissociation constant in terms of assumed Hill-type cooperativity for the AcCh binding.

In equation (3) it is readily seen that τ_{II} can be independent of the activator concentration [A], if [A] → 0. According to the gating concept the decay phase of a m.e.p.c. is determined by the slow receptor mode or

$$\tau_d = \tau_{II} = f(T, V) . \qquad (4)$$

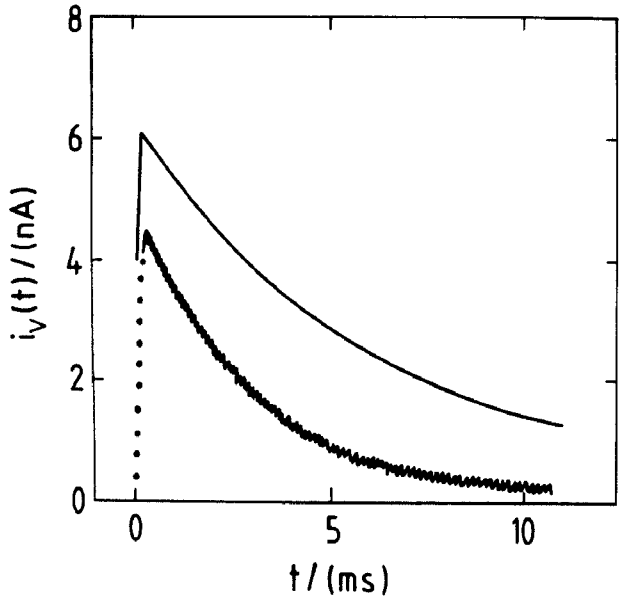

FIG. 1. — *Miniature endplate current, m.e.p.c., in toad neuromuscular junction.*

Redrawn from Fig. 8 of GAGE and MCBURNEY, 1975 : and Fig. 2 of GAGE, 1976. Clamp potential — 70 mV, 293 K, in standard Ringer solution. Upper trace recorded after 30 min exposure to 1 mg/l neostigmine in Ringer solution, a condition assumed to completely inhibit esterase activity : the slow decay reflects slow diffusion of acetylcholine in the junction (and probably also further receptor desensitization).

Because for [A] → 0, τ_{II} is determined only by rate and equilibrium constants, the dependence of τ_d solely on temperature (T) and voltage (V) is obvious.

The essential condition [A] → 0 during the decay phase of a m.e.p.c. may be fulfilled when the inequality (1) holds. In this context it is instructive to recall that under *in vivo* conditions not a trace of free AcCh is detectable, either at synaptic or at axonal parts of excitable membranes (NACHMANSOHN, 1959, 1975). These observations suggest that a very efficient, not purely diffusive but enzymatic, removal mechanism exists for AcCh. Indeed, it has been shown that the AcChE macromolecule has particular electrostatic properties such that already the diffusive association of cationic ligands like the AcCh to the enzyme surface is drastically accelerated (see, *e.g.*, NOLTE *et al.*, 1980).

In order to model AcChE inhibitor effects on m.e.p.c. consistent with the inequality (1), the elementary conductance increase must involve *at least two AcCh* molecules (NEUMANN *et al.*, 1978 ; ROSENBERRY, 1979). This conclusion is supported by experimental data (see, *e.g.*, RANG, 1975 ; LESTER *et al.*, 1975 ; SHERIDAN and LESTER, 1977 ; ADAMS, 1975 ; KUFFLER and YOSHIKAMI, 1975). Using several assumptions on receptor density in the synaptic cleft and applying the AcCh reactions in a competitive model, ROSENBERRY (1979) simulated main features of the time course of a m.e.p.c. ; however, the sharp peak of m.e.p.c. was not reproduced, and it cannot be modelled by any simple competitive model. The time course of the AcCh concentration, [A], appears to be a delta-function during the growth phase. The particular shape of m.e.p.c. indicates an essentially sequential processing of AcCh : neurally evoked AcCh binds first with AcChR in a reaction phase 1. Because of the inequality (1) it appears that initially in the growth phase of a m.e.p.c. the AcCh concentration $[A]_1$, close to activatable receptors is larger than the AcCh concentration $[A]_2$ in a second microreaction phase where AcChE fully competes with AcChR for activator. To fulfill the initial m.e.p.c. condition

$$[A]_1 \gg [A]_2 \qquad (5)$$

a partial separation of receptor and esterase sites appears to be necessary ; permitting, say, 90 % of the available AcCh to reach the receptors and to bind ; the rest of AcCh and all AcCh that dissociate from AcChR is rapidly removed by AcChE.

Previously, different microreaction spaces had been suggested ; this assumption appears not to be necessary (NEUMANN, 1980). These conclusions from purely electrophysiological data have been summarized in a flow scheme (NEUMANN and BERNHARDT, 1977 ; NEUMANN *et al.*, 1978). The AcChR-gating cycle for neurally triggered AcCh is shown in Fig. 2 ; the desensitization processes are not included. It is readily realized that, once AcCh is more rapidly removed, *i.e.* [A] → 0, closure of a channel can occur solely along intramolecular pathways, *via* $R' \rightleftharpoons R$ and/or $A_2 R \rightleftharpoons A_2 R'$, where, however, the step $R' \rightleftharpoons R$ could be rate-limited by the preceding dissociation step $AR' \rightleftharpoons R' + A$; both intramolecular pathways are consistent with first order decay of a m.e.p.c.

When AcCh is of non-neural origin, for instance applied artificially using a micropipette and in the presence of esterase inhibitors, AcChRs are multiply activated (KATZ and MILEDI, 1973) and the decay phase of a m.e.p.c. is prolonged. The inequality (5) may no longer hold for bath application of AcCh and the opening-closure kinetics is mainly determined by the kinetics of the $A_2 R \rightleftharpoons A_2 R'$ step, as suggested by experiments (see NEHER and SAKMANN, 1975 ; STEVENS, 1976 ; SHERIDAN and LESTER, 1977 ; STEVENS, 1980).

We shall return to further details after the discussion of some kinetic properties of isolated AcChR and AcChE, because the rate constants of the effective binding of AcCh to the two proteins are of fundamental importance ; see the inequality (1).

Desensitization.

Prolonged exposure to AcCh indicated additional reaction pathways for the closure of AcCh-activated channels. Longer bath application of activators causes inactivation (pharmacological desensitization) of AcChRs, according to a cyclic scheme first proposed by KATZ and THESLEFF (1957) :

$$\begin{array}{ccc} A + R & \rightleftharpoons & \cdots AR' \\ \downarrow\uparrow & & \downarrow\uparrow \\ A + R'' & & \cdots AR'' \end{array} \qquad (6)$$

where R″ represents a set of desensitized receptor states.

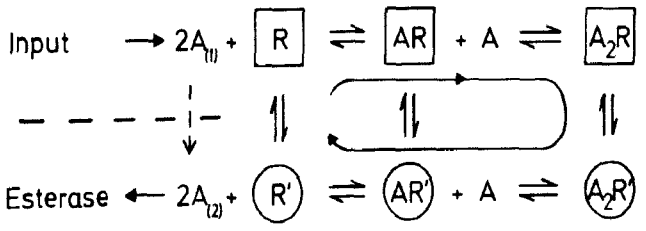

FIG. 2. — *Flow scheme of the AcCh gating.*

Neurally triggered AcCh (input) flows toward the closed receptor (R) conformation, binds and induces the structural change to the ion transporting state $A_2 R'$ (reaction phase 1). In the second phase, AcCh which dissociates from $A_2 R'$ is rapidly hydrolyzed by the esterase (E). The receptors return to their activatable structure R. In phase 1, the concentration of free AcCh, $[A]_1$, is initially much larger than in phase 2, where [A] → 0 ; see text.

A direct consequence of the electrophysiological data that can be described by scheme (6) is that, *even in the absence of AcCh,* a certain fraction of AcChR exists *a priori* in the inactivated conformations R″, characterized by a higher affinity to AcCh than the (activatable) R-state (KATZ and THESLEFF, 1957). The « diffusion barrier » suggested by DE MOTTA and DEL CASTILLO (1977) appears to be receptors in the high AcCh-affinity desensitized R″-forms. Furthermore, AcCh-induced inactivation following the activation phase suggests that the activated conformation of the AcChR-channel is *metastable* ; the inactivated states AR″ are thermodynamically the most stable states in the presence of activator.

Ca^{2+}-ions.

The permeability changes induced by AcCh in many biomembranes are cation selective. Corresponding to the high concentrations of external Na^+ ions and internal K^+ ions the alkali metal ions predominantly contribute to the ion flows. Since Ca^{2+} appears to selectively inhibit the Na^+-flow there appears competition for sites and thus transient, *short-lived binding* of Na^+ and Ca^{2+} ions in the Na^+ pathway (TAKEUCHI and TAKEUCHI, 1972 ; see also GAGE and VAN HELDEN, 1979).

The detailed role of Ca^{2+} ions in activation and inactivation is not yet molecularly understood. In frog muscle increased external $[Ca^{2+}]$ slightly decreases the m.e.p.c. decay time constants and life times of open channels (see Table 3 of MAGLEBY and WEINSTOCK, 1980). Thus Ca^{2+} ions appear to facilitate the closure reaction and to stabilize the R-conformation of AcChR.

AcCh-induced rapid activation eventually releasing Ca^{2+} ions followed by slower inactivation processes accompanied by uptake of Ca^{2+} ions, appear to be a fundamental characteristic of functionally intact AcChRs.

III. — KINETICS OF ISOLATED AcChR AND AcChE

As discussed, the rate constants $k_{ass}(R)$ and $k_{ass}(E)$ of the effective binding of AcCh to the two proteins AcChR and AcChE are of fundamental importance. The aim is to quantify the inequality (1), probably in terms of rate constants estimated from kinetic studies, preferably from relaxation kinetic data of the isolated proteins.

1) ACETYLCHOLINESTERASE.

A minimum estimate for $k_{ass}(E)$ may be derived from catalytic parameters of the enzyme. In the minimum reaction scheme for the enzyme (E) acylation by AcCh (A) according to

$$A + E \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} E{\cdot}A \overset{k_2}{\rightarrow} E{\cdot}acyl + Ch\cdots \qquad (7)$$

$k_{12} \geq k_{cat}/K_{app}$ is the bimolecular association rate constant for which a minimum value of $2.10^8\ M^{-1}\ s^{-1}$ (at 0.1 M ionic strength at 298 K and pH 8) can be estimated from the catalytic turnover rate constant k_{cat} and the apparent Michaelis constant K_{app} ; see, *e.g.*, ROSENBERRY and NEUMANN, 1977. Since $k_2 \approx 10^5\ s^{-1}$ and $k_{21} \approx 10^4\ s^{-1}$, thus $k_2 > k_{21}$, we may safely set

$$k_{12} \approx k_{ass}(E) \geq 2 \times 10^8\ M^{-1}\ s^{-1} \qquad (8)$$

and obtain an estimate which is adequate for comparison with $k_{ass}(R)$ of the receptor.

Recent relaxation kinetic investigations demonstrated that cationic ligands bind extremely rapidly to the enzyme active site. The observed bimolecular rate constants k_{12} between 10^{10} and $10^9\ M^{-1}\ s^{-1}$ are unusually high for enzyme ligand interactions. In addition, the association rate constants k_{12} are very strongly dependent on the ionic strength, I_c, of the solution. Virtually the same strong I_c-dependence has been observed for a catalytic parameter proportional to k_{12} of acetylthiocholine, k_{cat}/K_{app}, a substrate whose structure and kinetic properties are very similar to those of acetylcholine (NOLTE *et al.*, 1980).

The analysis of the I_c-dependencies shows that *at least six to seven monovalent anionic groups* contribute to the comparatively large values k_{12} for both the non-substrates and acetylthiocholine

$$(k^0_{12} = 0.42 \times 10^{10}\ M^{-1}\ s^{-1}),$$

suggesting that an enzyme surface area larger than the ligand binding site itself is effective in trapping a ligand in encounter complexes. This larger surface area might include peripheral anionic sites from which ligand would move to the active site by surface diffusion. Thus due to a dominantly multi-anionic surface structure, this polyionic enzyme appears to be a powerful electrostatic sink for trapping and decomposing the acetylcholine cation.

2) ACETYLCHOLINE RECEPTOR.

The smallest, functionally intact acetylcholine binding protein which can be isolated under gentle experimental conditions from Torpedo fish, minimizing chemical modifications, is the so called heavy (H) form of receptor rich membrane fragments (see, *e.g.*, CHANG and BOCK, 1977). This H-form of a molecular weight of roughly 500,000 d *(Torp. cal.)* is a dimer of two,

possibly not identical, monomers : the L- (and the L'-) forms which are linked by intersubunit disulfide bonds. Provided that endogenous lipids remain attached to the isolated receptor-lipid complex, the H-form exhibits essentially the same positive-cooperative acetylcholine binding isotherms as biomembrane fragments (CHANG and BOCK, 1979, 1980).

A) *Ca^{2+} and $AcCh^{+}$ binding.*

The isolated AcChR in detergent solution (probably largely in the L-forms) was found to bind large amounts of Ca^{2+} ions associated with high (μM) and low (mM) affinity sites (ELDEFRAWI *et al.*, 1975 ; CHANG and NEUMANN, 1976 ; RÜBSAMEN *et al.*, 1976).

There is competition of Ca^{2+} binding with other cations (see also RAFTERY *et al.*, 1976). This competition has been used to estimate some thermodynamic and kinetic constants of acetylcholine binding to AcChR. It was found that upon binding of one AcCh-ion about 2 to 3 Ca^{2+} ions are released (CHANG and NEUMANN, 1976 ; see also RÜBSAMEN *et al.*, 1976).

The increase in the concentration of free Ca, $\Delta[Ca] \approx 10^{-5}$ M occurs in a solution of $\approx 10^{-3}$ M ; the change of ≈ 1 percent is rather small and may be indicated with the help of metallochromic indicators and special spectrophotometers.

Relaxation kinetic studies have shown that an overall reaction step (among others),

$$A + RCa \underset{(k_{-1}(R))}{\overset{(k_1(R))}{\rightleftharpoons}} AR\,(+\,Ca) \qquad (9)$$

exists, with the rate coefficients

$k_1(R) = 2.4 \times 10^7\ M^{-1}\ s^{-1}$ and $k_{-1}(R) = 140\ s^{-1}$;

both kinetic parameters refer to the AcCh-binding part assuming rapid equilibration for the Ca-binding step (NEUMANN and CHANG, 1976) ; see also Table I.

As found by the Ca-relaxation,

$$k_1(R) = 2.4 \times 10^7\ M^{-1}\ s^{-1}$$

for AcCh suggests that the measured association rate constant is probably an overall, complex rate parameter involving several more rapid elementary steps, see Fig. 3. In the simple case of very rapid pre-equilibria, the effective association rate constant $k_1(R)$ for the reaction model :

$$A + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} A{\cdot}R \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} AR \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} AR' \qquad (10)$$

is given by $k_1(R) = k_3(k_1/k_{-1}).(k_2/k_{-2})$ where the rate constants of diffusion controlled ion pairing may be estimated to be $k_1 \approx 10^8\ M^{-1}\ s^{-1}$ and $k_{-1} \approx 10^7\ s^{-1}$. The life time $t_0(R)$ associated with the overall process characterized by $k_1(R)$, is given by

$$t_0(R) = (k_{-1}(R))^{-1} = 7\ ms\ .$$

The kinetic constants for acetylcholine so far characterize a receptor preparation (partially delipidated) with overall dissociation equilibrium constant $\overline{K}_A \approx 10^{-6}$ M (at 296 K, pH 8.5 and 0.1 M NaCl). This value is close to the acetylcholine concentration which causes the « electrical half-response ». On the other hand, K_A for crude extracts, membrane fragments and recent re-

TABLE I. — *Interaction parameters for the isolated acetylcholine receptor from* Torpedo californica *and acetylcholine and Ca^{2+} ions in terms of a direct competition of both ligands, for the main reaction path $A + R \rightleftharpoons \ldots AR \rightleftharpoons AR^*$* (NEUMANN and CHANG, 1976 ; NEUMANN *et al.*, 1978).

Data refer to 0.1 M NaCl, 0.1 % Brij, 1 mM Ca^{2+}, 0.05 M Tris · HCl, pH 8.5 at 296 K. The overall equilibrium constant for acetylcholine is given by $K_A = K_1(1 + K_0^{-1}.c_{Ca})\,(1 + K_2)^{-1} = 10^{-6}$ M, where $K_1 = k_{-1}/k_1$, $K_2 = k_2/k_{-2}$ and for the Ca^{2+}-binding $K_0 = k_{-0}/k_0$.

$A + R \rightleftharpoons AR$	$AR \rightleftharpoons AR^*$	$Ca + R \rightleftharpoons CaR$
$k_1 = 2.4\ (\pm 0.5) \times 10^7\ M^{-1}s^{-1}$	$k_2 = 43.5\ s^{-1}$	$k_0 \cong 10^8\ M^{-1}s^{-1}$
$k_{-1} = 140\ s^{-1}$	$k_{-2} = 6.5\ s^{-1}$	$k_{-0} \cong 10^5\ s^{-1}$
$K_1 = 0.6 \times 10^{-5}$ M	$K_2 = 6.7$	$K_0 \cong 10^{-3}$ M

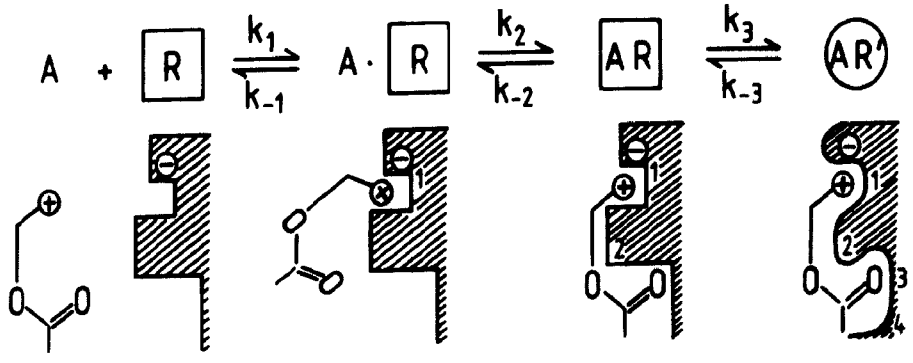

FIG. 3. — *Chemical model for the induced conformational change in AcChR.*

Step 1, encounter ion pairing with $K_1 = k_{-1}/k_1 \approx 0.1$ M, $k_1 \approx 10^8\ M^{-1}\ s^{-1}$ and $k_{-1} \approx 10^7\ s^{-1}$; step 2, contact with a second site in the lifetime of the ion-pairing ; step 3, conformational change to the permeable state induced by steps 1 and 2. The state AR' may involve a « distorted » AcCh.

ceptor preparations where chemical modification could be largely reduced (CHANG and BOCK, 1979, 1980) is between 10^{-8} M and 10^{-9} M, most likely representing the acetylcholine affinity to the inactivated receptor. Moreover, the rate constants for the bimolecular overall reaction, $k_1(R)$ and $k_{-1}(R)$, compare well with data from electric current relaxation (SHERIDAN and LESTER, 1977) : $k_{on} = 10^7\ M^{-1}\ s^{-1}$ and $k_{off} = 10^2$ to $10^3\ s^{-1}$, depending on membrane potential. The electrophysiological data further indicate that at least two acetylcholine ions must bind in order to open a single permeation site. This may be related to the fact that the H-form of the (freshly) isolated receptor protein has (at least) two binding sites for AcCh.

Among the various points of comparison, in particular the similarity between $k_{on} = 10^7\ M^{-1}\ s^{-1}$ and $k_{off} = 10^2$-$10^3\ s^{-1}$ from current relaxations on the one hand, and $k_1(R) = 2.4 \times 10^7\ M^{-1}\ s^{-1}$ and $k_{-1}(R) = 1.4 \times 10^2\ s^{-1}$ from studies on isolated receptors on the other hand suggests that the rate of coupled Ca^{2+} release (upon binding of AcCh) may not only reflect the overall rate of effective acetylcholine binding associated with a life time of 7 ms, to isolated (partially delipidated) receptor, but may also be characteristic for the rate-limiting step in the conductivity increase of the membrane. Thus, stoichiometry of acetylcholine binding, equilibrium and rate constants suggest the low affinity receptor (with $K_A \approx 10^{-6}$ M) as a candidate for the *in vivo metastable,* conducting receptor conformation, which by chemical modification (sulfhydryl-disulfide redox reactions) during isolation may be stabilized in detergent solution.

In contrast to the relatively low value for acetylcholine, the measured association rate constant for the binding of the dicationic inhibitor bis(3-aminopyridinium)-1,10-decane (DAP) to isolated *Torp. marm.* AcChR is $k_{12} = 1.2 \times 10^8\ M^{-1}\ s^{-1}$ in 0.1 M ionic strength, pH 7.0 and 293 K. The ionic strength dependence of the association rate of k_{12} (DAP) suggests an effective charge of $-3(\pm 1)$ on the binding site of the protein (NEUMANN *et al.,* 1978 ; CHANG and NEUMANN, 1980, unpublished results). This value is somewhat less negative than that indicated for the esterase. But it seems that in both proteins of the permeability control system there are larger electrostatic contributions to the rate with which cationic ligands like acetylcholine are bound.

The estimates for the bimolecular rate constants k_{12} of AcChR-ligand binding appear to depend strongly on the type of ligand used ; for decamethonium $k_{12} = 2 \times 10^8\ M^{-1}\ s^{-1}$ and for carbamylcholine and acetylcholine $k_{12} \cong 10^7\ M^{-1}\ s^{-1}$ (SHERIDAN and LESTER, 1977), for suberyldicholine $k_{12} = 0.98 \times 10^7\ M^{-1}\ s^{-1}$ (BARRANTES, 1978), for NBD-5-acylcholine $k_{12} \approx 10^8\ M^{-1}\ s^{-1}$ (JÜRSS *et al.,* 1979), for Dns-C_6-Cho $k_{12} \cong 9.5 \times 10^7\ M^{-1}\ s^{-1}$ (HEIDMANN and CHANGEUX, 1979).

B) *AcChR-lipid complexes.*

Recent studies with the isolated AcChR-lipid complexes from *Torp. cal.* confirm that the Ca^{2+}-binding isotherm is essentially two-phasic with equilibrium constants in the μM and mM range (DOROGI *et al.,* 1981), suggestive of intracellular and extracellular Ca^{2+} sites in AcChR. Ca^{2+} ions may be involved to preserve stability of the protein-lipid complex (CHANG and BOCK, 1980) and may also bind to the anionic groups of the channel subunits. A large number of anionic, probably carboxylate, groups are suggested by the large Ca^{2+} ion binding capacity of AcChR. Provided that the density of the anionic charges exceeds a certain value, divalent ions like Ca^{2+} are preferentially bound. If AcCh induces a structural change which increases the average distance between the charged groups, this polyelectrolyte preference for Ca^{2+} ions would be lost and ion exchange with, say, Na^+ ions could occur (NEUMANN *et al.,* 1973). The « indicator » of « effective binding » of AcCh to the AcChR-gating macromolecule would therefore be the (allosteric) release of bound Ca^{2+}. However, prolonged exposure to receptor activators leads to definitely allosteric uptake of Ca^{2+}-ions ; see also SUGIYAMA and CHANGEUX (1975).

Transient release of Ca^{2+} ions followed by uptake of Ca^{2+} upon addition of activators to AcChR (SPILLECKE *et al.,* unpublished results) suggest at least one metastable state *in vitro,* parallel to the metastability for the conducting channel configuration electrophysiologically indicated *in vivo* and parallel to the metastability suggested for the permeable state in sealed biomembrane vesicles (BERNHARDT and NEUMANN, 1978 ; NEUMANN, 1979).

C) *Asymmetry in AcChR.*

BULGER and HESS found two types of α-bungarotoxin binding sites in membrane-bound AcChR ; this nonequivalence appears to be induced by α-bungarotoxin and leads to interconversion of the sites (BULGER *et al.,* 1977), which in unbound state must not necessarily be of different types. Binding studies with DAP and α-bungarotoxin have also been interpreted in terms of two classes of binding sites (RAFTERY *et al.,* 1976). Site heterogeneity has been reported from various laboratories and is extensively discussed by CHANGEUX (1981).

The data suggestive of intrinsic asymmetry of AcChR structure and function invite speculation on a possible functional role. If the « monomers » of the dimeric H-form have different cyclic sequences for the subunit positions, *i.e.,* an L-form with $\alpha_2\beta\gamma\delta$ and an

L'-form with $\alpha_2\ \gamma\beta\delta$ sequence, a molecular weight of about 250,000 d for each monomer and of about 500,000 d for the asymmetric H-form would result, a value not inconsistent with recent estimates (RAFTERY *et al.*, 1976 ; REYNOLDS and KARLIN, 1978). Functionally, the L and L'-forms are *candidates* for two possibly *separate* channels for Na^+-inflow and K^+-outflow, both, however, cooperatively controlled by the binding of at least two acetylcholine ions. Recent reconstitution experiments into planar lipid bilayers indeed indicate that the H-form leads to a « double channel », whereas the conductivity contributions of the monomers are only half of those from the H-form (SCHINDLER, SPILLECKE and NEUMANN, 1982, unpublished results).

These characteristic features of the dimeric AcChR gating protein are only covered by more complicated reaction schemes. In the light of the previous arguments of a dimeric channel structure, it appears appropriate to formally express the experimental complexity in terms of the AcChR dimer, see Fig. 4 ; the scheme is analogous to a general scheme developed by EIGEN (1967) for a tetrameric subunit system.

IV. — MOLECULAR INTERPRETATION OF M.E.P.C.

To the extent to which data on isolated proteins can be used to extrapolate to the cellular level, it is very tempting to compare the rate constant $k_1(R) = 2.4(\pm 0.5) \times 10^7\ M^{-1}\ s^{-1}$ associated with a life time of $(k_{-1}(R))^{-1} = 7$ ms, with that of the enzyme $k_1(E) \geq 2 \times 10^8\ M^{-1}\ s^{-1}$, probably $10^9\ M^{-1}\ s^{-1}$.

With respect to the inequality (1) suggested by the electrophysiological data of the m.e.p.c., the result $k_1(R) < k_1(E)$ of the isolated proteins indicates that there is a receptor reaction (involving AcCh and Ca^{2+} ions) which meets the conditions for $k_{ass}(R)$ and $\bar{t}_0 \approx 5$ ms. If, as is consistent with the data, indeed $k_1(R) = k_{ass}(R)$ and $k_1(E) = k_{ass}(E)$, then the result $k_1(R) < k_1(E)$ parallels and quantifies the inequality (1).

The sequence of events underlying the time course of a m.e.p.c. (Fig. 1) may be viewed as schematically shown in Fig. 5. Neuronal activity releases a pulse of AcCh (part 5.2) which diffuses to the receptors causing a conformational change (part 5.3) with an average life time of a few ms (phase 1). Esterase activity rapidly hydrolyzes the remaining AcCh, $[A] \rightarrow 0$, causing the sharp peak in the m.e.p.c. When the AcCh ions which dissociate from AR'-sites of AcChR appear again in the reaction space (part 5.4), they are more rapidly attracted by the esterase than they can return to the receptors : the receptor channels close (phase 2).

In conclusion, we may describe the particularly characteristic time course of an AcCh-induced elementary electric signal (the m.e.p.c.) in terms of appropriate kinetic constants which are observed for the isolated proteins AcChR and AcChE, and in terms of a microspatial separation of esterase activity and receptor reactions. Full esterase activity is necessary for both reaction phases discussed above, the growth and the decay phase of the spontaneous miniature end plate currents. The molecular properties of the two AcCh processing proteins appear to be the essential reason for the particular form of the electric signal.

V. — GATING MECHANISMS FROM FLUX DATA

The most direct approach to ion flow gating is to measure ion transport as such, using for instance tracer ions. The application of this method to cellular systems encounters difficulties similar to those of electro-

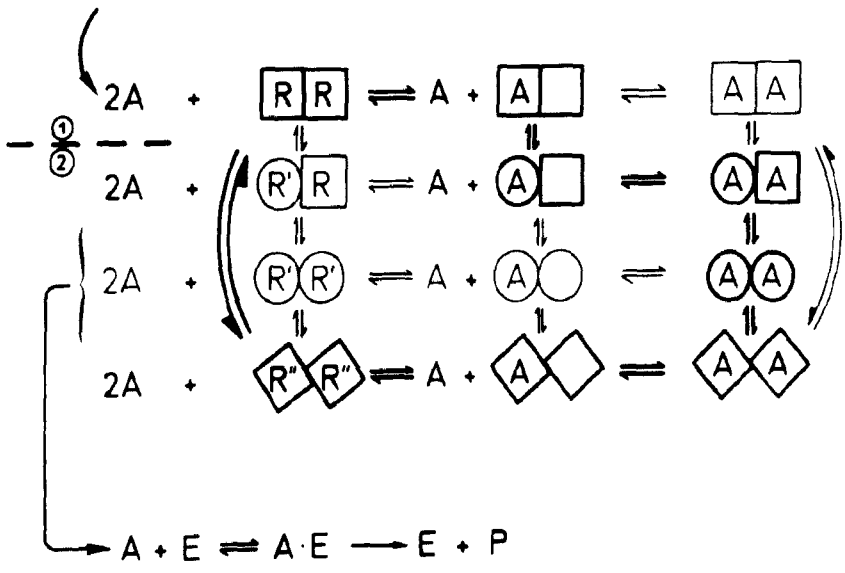

FIG. 4. — *Chemical representation of acetylcholine (AcCh)-induced activation and inactivation of acetylcholine receptors (AcChR) in terms of a basically sequential processing of AcCh by receptor and acetylcholinesterase (AcChE).*

The receptor states, in terms of activator binding sites, correspond to R_2 (closed), R_2' (open) and R_2'' (desensitized). The main reaction pathways are drawn in thick symbols. (For neurally evoked AcCh the changes along the desensitization pathways $R_2 \rightleftharpoons R_2''$ and $(AR')_2 \rightleftharpoons (AR'')_2$ appear to be uninvolved.)

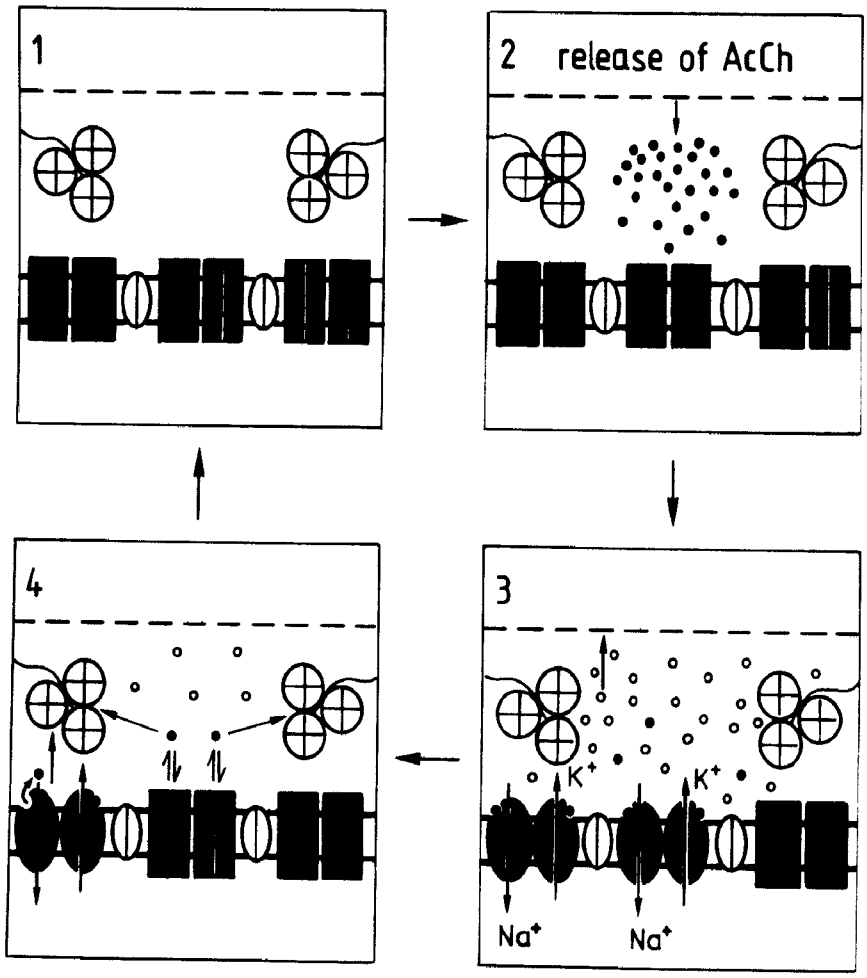

FIG. 5. — *Model for the sequence of events causing the generation of a spontaneous postsynaptic miniature end-plate current (see Fig. 3) by acetylcholine (AcCh).*

(5.1) resting state, [AcCh] → 0, the receptor double channel (HH; H-form, binding ≥ 2 AcCh) is in the closed state ;

(5.2) appearance of a pulse of AcCh (●) ;

(5.3) binding of AcCh to the receptors and channel opening, causing K^+-outflux and Na^+-influx; the remaining AcCh is rapidly hydrolized by esterase activity (⊕, ◫) ; predominantly presynaptic uptake of choline (○) ;

(5.4) dissociation of AcCh from the receptors and rapid hydrolysis by esterase activity ; return of the receptors to the closed (resting state ; 5.1).

physiological techniques. The local concentration of applied activator can only be estimated, pH and ionic strength, type and concentration of transported ions can only be varied to a limited extent. Compared to these limitations there is an experimental level of less complexity, where ion flux can be measured under well-defined conditions, for instance, of activator and inhibitor concentration, various ion types, etc. This approach is based on a technique introduced by KASAI and CHANGEUX (1971) into AcChR studies : vesicularly closed membrane fragments (microsacs) prepared from electric tissue are first filled with tracer ions and then upon addition of activators, efflux of these tracers from the vesicles is measured.

In the mean time the tracer flux technique has been developed to permit measurements in the millisecond time range (HESS *et al.*, 1980 ; NEUBIG and COHEN, 1980). Analytically, a new concept comprising integrated flux coefficients has been introduced and developed as an analytical method for the study of gating mechanisms (BERNHARDT and NEUMANN, 1978, 1980*a*, *b*, 1981). The full power of the method is apparent when flux can be measured in a time range where the gating system is not equilibrated, the number of channels either increases or decreases. In this case, the flux coefficient is time-dependent thus offering the chance to obtain kinetic gating constants. But even if the gating system is equilibrated before the first flux data can be measured, the new method may yield mechanistic details of the gating system. This was first demonstrated in 1978 for the AcChR in sealed biomembrane fragments, at a time when the time range of conventional flux measurements was 10 sec. Under these conditions biovesicles from *Torpedo marmorata* and *Torpedo californica* electric tissue both yield parallel (and slanted) base lines already before the first flux measurement can be performed, *i.e.* 10 sec after addition of the activator. The resulting flux « curves » are flux lines as seen in Fig. 6.

From the observed parallelism of the (slanted) base lines it could be immediately concluded that not only preincubation with activator causes inactivation of receptor-channels but that the flux lines themselves reflect inactivation as a function of activator concentration. Furthermore, the rate constant k_i for the rate-limiting step of the gating mode of inactivation is much larger than that for the backward step, k_{-i}. Because of this observation ($k_i \gg k_{-i}$) the active receptor conformation associated with the open channel can only be a short-lived metastable state.

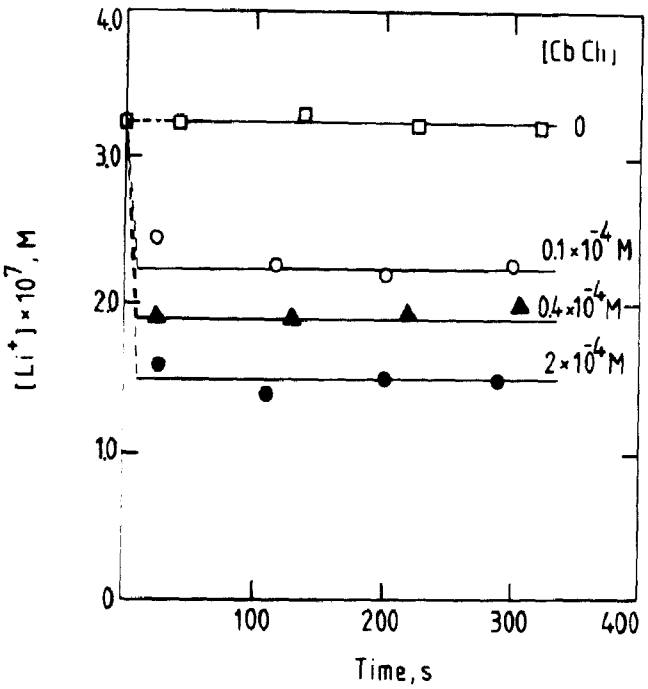

FIG. 6. — *Carbamoylcholine (CbCh)-induced efflux of Li^+-ions from sealed membrane fragments (vesicles)* of Torpedo marmorata.

The acetylcholine receptor containing biomembrane vesicles were purified in self-generating Percoll gradients, filled with Li^+ by AcCh-« pulses » and the Li^+ content was determined after filter-assaying by flame emission spectroscopy.

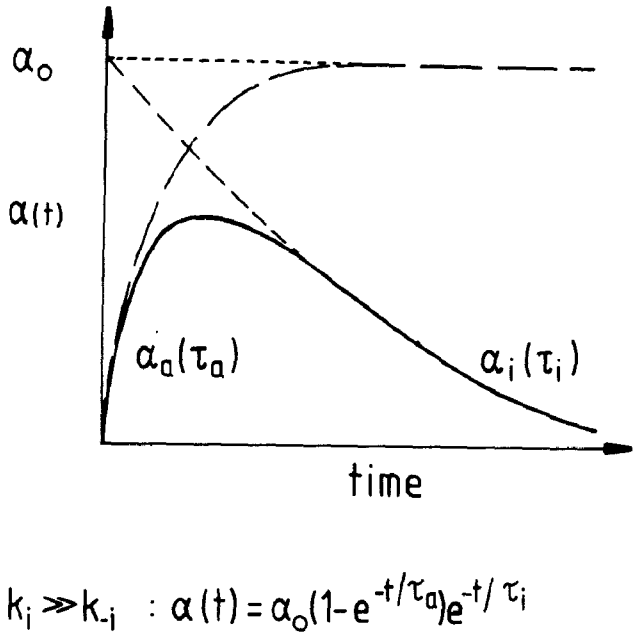

FIG. 7. — *The fraction of open channels, α(t), for rapid activation of the gating system with the time constant τ_a, followed by slower inactivation (τ_i) as a function of time t.*

α_0 is the amplitude of both the activation, and the inactivation mode.

The general feature for the activation-inactivation sequence of AcChR is shown in Fig. 7 where the rate limiting gating modes are expressed as a rapid increase in the fraction α(t) of open receptor-channels followed by a slower decrease. It should be stressed that this picture can be directly deduced from the flux lines ; see Fig. 6.

Finally, as shown for the activator substance β-pyridine methyl-trimethylammonium, the description of the flux data requires a cyclic reaction scheme as given in equations (6) ; see BERNHARDT and NEUMANN, 1978.

The AcChR in biomembrane vesicles shows the same basic characteristics as revealed by electrophysiological techniques : rapid activation followed by inactivation with $k_i \gg k_{-i}$, short-lived metastability for the ion-transporting gating state, and a cyclic inactivation reaction scheme (BERNHARDT and NEUMANN, 1978 ; NEUMANN, 1979 ; NEUBIG and COHEN, 1980). Recently, the preparation techniques for intact biomembrane vesicles were appreciably improved (BERNHARDT *et al.*, 1981). Functionally intact vesicles can be separated from crude mixtures in self-generating Percoll gradients ; these vesicles can be filled with, *e.g.*, Li^+-ions by applying short pulses of AcCh to prevent premature inactivation of AcChR (minute amounts of AcChE activity is always present to hydrolyse AcCh) and, after filter assay, the vesicular content of Li^+ can be conveniently determined by flame emission spectroscopy (BERNHARDT *et al.*, 1981).

Flux data analysis.

The nicotinic cholinergic gating system controls the flow of two types of ions, influx of Na^+-ions into the cell and coupled to it an outflux of K^+-ions. Matching the two types of non-equivalent binding sites for activators of the macromolecular receptor system, it is probable that the functional unit for ion transport is a two-channel system (Na^+-influx, K^+-outflux) cooperatively gated by (at least) two acetylcholine molecules. Whereas *in vivo* AcChR gates an overall exchange flux of Na^+ and K^+ ions, the *in vitro* flux experiments with vesicle-internal Li^+ probably involve an overall *exchange flux* with external alkali metal ions. Because of this exchange condition, the flux equation of linear nonequilibrium thermodynamics, with time-dependent flux rate constants, may be applied (BERNHARDT and NEUMANN, 1978). The flow of internal ions X into a large external volume of solution may thus be described by

$$X(t) = \sum_n X_n(0) \exp[-n\kappa(t)] \qquad (11)$$

where the amplitude factor κ(t) is given by

$$\kappa(t) = k \int_0^t \alpha(t')\, dt' \qquad (12)$$

with t' as a dummy integration variable.

These expressions reflect the grouping of vesicles into subspecies having a total of n activatable channels and containing initially at time zero, $X_n(0)$ ions. The integrated flux coefficient $\kappa(t)$ is a function of the intrinsic single-channel flux coefficient k (ions per sec) and the time-dependent fraction $\alpha(t)$ of open channels. In the AcChR system the concentration dependence of the integrated flux coefficient $\kappa(t)$ leads to the establishment of a minimum reaction mechanism for AcChR inactivation (BERNHARDT and NEUMANN, 1978).

In general, the kinetics of $\alpha(t)$, experimentally derivable from X(t), contains the gating reaction modes of activation $\alpha_a(t)$ and of inactivation $\alpha_i(t)$, if present. Thus,

$$\alpha(t) = \sum_a \alpha_a(t) + \sum_i \alpha_i(t) \qquad (13)$$

where for the activation modes we have

$$\partial \sum_a \alpha_a(t)/\partial t \geqslant 0$$

and for the inactivation modes

$$\partial \sum_i \alpha_i(t)/\partial t \leqslant 0.$$

When an activation mode is well separated from an inactivation mode on the time scale we may write

$$\alpha_a(t) = \alpha_a(\infty) + [\alpha_a(0) - \alpha_a(\infty)]\, e^{-t/\tau_a} \qquad (14)$$

for the gating process from $\alpha_a(0)$ at $t = 0$ to $\alpha_a(\infty)$ at $t \to \infty$ (equilibrated or stationary), and

$$\alpha_i(t) = \alpha_i(\infty) + [\alpha_i(0) - \alpha_i(\infty)]\, e^{-t/\tau_i} \qquad (15)$$

for the inactivation mode from $\alpha_i(0)$ to $\alpha_i(\infty)$.

The time constants τ_a and τ_i are normal mode constants (EIGEN, 1967) which for a two-state gating model for activation and inactivation, respectively, are given by

$$\begin{aligned} \tau_a &= (k_a + k_{-a})^{-1} \\ \tau_i &= (k_i + k_{-i})^{-1} \end{aligned} \qquad (16)$$

where the k-values are the overall rate coefficients for the forward and the backward step, respectively.

The explicit expressions for the k-terms depend on the mechanism of the reaction model ; examples are given by BERNHARDT and NEUMANN (1978) and NEUMANN (1979).

Recently, it has been shown that the flux equation (11) can be expressed in terms of an average single-channel flux contribution $< e^{-kt} >$ where k is the single channel flux rate constant (BERNHARDT and NEUMANN, 1981). In brief, a rigorous physical-chemical framework, based on first principles, has been developed for the study of gating mechanisms. One main result is that, in analogy to equation (11), one can write for efflux,

$$X(t) = \sum_n X_n(0)\, [< e^{-kt} >^n]_v \qquad (17)$$

where $[< e^{-kt} >^n]_v$ denotes the average over a distribution in vesicle volumes, v, of the mean single-channel flux contribution $< e^{-kt} >$ to the power of n ; n is the number of channels per vesicle or microsac. It is noted that, *prior to any analysis of the flux kinetics,* it is necessary to first determine the distribution of vesicle size (or volume) and of content of gating units.

When in a flux experiment a given mode of the gating reaction reaches steady state or equilibrium, $< e^{-kt} >$ will approach the equilibrium value $< e^{-kt} >_{eq}$ of the mean single-channel flux contribution :

$$\langle e^{-kt} \rangle_{eq} = e^{-k_{eq} \cdot t} \qquad (18)$$

where the effective (equilibrium) flux rate constant is given by

$$\begin{aligned} k_{eq} &= \frac{k_0}{k_0 + k_c} \cdot \frac{k_c}{k_c + k} \cdot k \\ &= \left[(1 + K_{0,c}) \left(\frac{1}{k} + \frac{1}{k_c} \right) \right]^{-1} \end{aligned} \qquad (19)$$

In equation (19), k_0 and k_c are the apparent rate coefficients of the particular gating mode, respectively, and $K_{0,c} = k_c/k_0$ is the respective equilibrium constant.

When transitions of state of the gating units are much more rapid than flux, *i.e.,* $k^{-1} \gg k_0^{-1}$, and $k^{-1} \gg k_c^{-1}$, then equation (18) reduces to

$$k_{eq} = k \prod_s \alpha_{eq,s}^{(0)} \qquad (20)$$

where $\alpha_{eq,s}^{(0)}$ is the equilibrium fraction of open channels of the gating mode s ; the product is over all modes s that have reached equilibrium or a steady state (see BERNHARDT and NEUMANN, 1982).

To summarize this section, we can state that recent theoretical and experimental progress in flux methods show that this approach is not only complementary to, but perhaps an alternative to electrophysiological-cellular techniques.

VI. — AXONAL GATING

The existence of basic similarities between synaptic and axonal parts of excitable membranes has been frequently discussed. In particular, the conducting conformation of the Na^+ ion channel is clearly a metastable, short-lived state as appears to be the case with the permeable AcChR conformation (NEUMANN, 1979). In a recent study it was found that kinetic models which can successfully simulate the ion-permeability features of axonal Na^+ channels, suggest the presence of bimolecular reaction steps in the activation of channels (DOROGI and NEUMANN, 1980 *a*). The implied chemical formalism is highly suggestive of an activator-controlled gating system with strong similarities to the acetylcholine regulated ion transport system. It is suggested that conformational changes which underlie the ion conductance changes possess a greater sensitivity to the membrane field in axonal than at synaptic parts of excitable membranes. This allows axonal permeability changes to be regulated energetically more conservatively ; axonal K^+ channels with delayed activation kinetics would serve to reverse the increase in membrane permeability to Na^+ ions with a minimum of chemical dissipation (DOROGI and NEUMANN, 1980 *a*). Thus, rapid bioelectrical signals based on transient permeability changes in axonal and synaptic parts of excitable biomembranes appear to be specialized cases of a more general chemically-dissipative control principle involving activator-receptor interactions and structural metastability for the activated, ion conducting state.

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