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# Dynamic properties of isolated acetylcholine receptor protein: Kinetics of the binding of acetylcholine and Ca ions*

(relaxation kinetics/temperature jump/relaxation time analysis/conformational change)

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**ABSTRACT** **Kinetic and thermodynamic constants for elementary steps associated with the interaction of acetylcholine (AcCh) and Ca with isolated AcCh receptor from *Torpedo californica* have been determined by chemical relaxation spectrometry. Murexide is used as a Ca indicator to monitor changes in Ca bound to the AcCh receptor. In the presence of AcCh this technique permits an indirect monitoring of AcCh binding, because the AcCh and the Ca binding reactions are competitively coupled. A temperature-jump perturbation in the Ca-murexide-AcCh receptor system induces a spectrum of relaxation processes characterized by at least three relaxation times: $\tau^1 = 5\ (\pm 1)$ msec; $\tau^2 = 35\ (\pm 5)$ msec; and $\tau^3 = 300\ (\pm 30)$ msec. In the presence of AcCh, the Ca relaxation spectrum is altered in a characteristic way. A formalism is developed to describe the normal mode relaxation times of the coupled reaction system in terms of total concentrations of both AcCh and receptor binding sites. The analysis also allows one to determine the stoichiometry of the reactions involved or to estimate a molecular weight of the AcCh receptor. The kinetic data suggest that the reaction of AcCh with receptor proceeds in at least two steps. The rate constant of the association of AcCh with receptor was found to be $2.4(\pm 0.5) \times 10^7\ M^{-1}\ sec^{-1}$ at 23.5°, 0.1 M NaCl, 50 mM Tris·HCl, pH 8.5. Reaction schemes consistent with the present kinetic data are discussed in terms of a physicochemical model that accounts for the rapid transient conductivity changes in excitable membranes during nerve and muscle excitation.**

The conductance changes in excitable membrane during nerve and muscle excitation are controlled by physicochemical membrane processes. At present, however, the molecular mechanism that underlies such changes is unknown. One may anticipate that several reaction steps are involved that are coupled with each other, possibly in a sequential form (1). Consequently, the time constants of the measured electrical changes reflect normal mode time constants of the whole regulatory system.

An experimental approach toward an understanding of the molecular mechanism of excitability is a study of elementary reactions involving components thought to be involved in the excitation process. One such component is the acetylcholine receptor protein. Association of the neural activator acetylcholine (AcCh) with receptor appears to initiate a sequence of reactions which ultimately result in local permeability changes in excitable membranes (for review see refs. 1 and 2).

Recent experiments with highly purified AcCh receptor isolated from electric organs of electric fish indicate that the binding of AcCh (3) or other neural activators (4) induces release of Ca bound to AcCh receptor. In contrast, binding of α-bungarotoxin, an inhibitor, causes an uptake of Ca ions on the receptor (3). These observations suggest that AcCh and α-bungarotoxin cause different conformational shifts in the AcCh receptor.

Study of the elementary reactions involving AcCh and AcCh receptor is complicated by the lack of a suitable reaction indicator. In this study these reactions are monitored indirectly through their coupling to Ca-AcCh receptor binding equilibria. With the use of murexide as an indicator of Ca ions, a spectrophotometric method has been developed to measure changes in the concentration of Ca ions bound to AcCh rceptor (3). To the extent that AcCh reactions are coupled to the Ca binding, changes in bound Ca can be used to indicate changes caused by AcCh in presumed AcCh receptor conformational states. The reaction equilibria involving receptor, Ca, and AcCh are investigated here by the temperature-jump relaxation method, and kinetic and thermodynamic constants associated with elementary reaction equilibria are reported. The preliminary results of these studies have been briefly referred to in previous publications (1, 3, 5, 6).

## MATERIALS AND METHODS

**AcCh Receptor.** The preparation of AcCh receptor used in the present study was freshly purified from electric tissue of *Torpedo californica* that had been frozen in liquid nitrogen, as described (3). The purified AcCh receptor samples had a binding capacity of 7.6–10 nM $^{125}$I-labeled α-bungarotoxin per mg of protein. The protein preparations were characterized by a major AcCh binding component with an equilibrium dissociation constant $K_A = 1.5\ (\pm 0.2) \times 10^{-6}$ M and a maximum of 2 AcCh molecules bound to 260,000 molecular weight (3). Immediately before the experiments, solutions of AcCh receptor in 0.1% Brij, 15 mM sodium phosphate buffer, 0.1 M NaCl, pH 7.0 were concentrated by vacuum dialysis in a collodion bag (Schleicher & Schuell) at 0° against three changes of buffer, 50 mM Tris·HCl, 0.1 M NaCl, pH 8.5 at 20° (buffer I) and centrifuged to remove particulate matter. The temperature-jump samples, 0.8 ml of solution, in buffer I, contained 2.4 mg of AcCh receptor and 80 μM murexide. The stock solution of murexide (0.4 mM) in buffer I had been twice filtered through Millipore filters (0.45 μm) before it was mixed with the AcCh receptor solutions. $CaCl_2$ and AcCh were added where appropriate from stock 50 mM and 5 mM solutions in buffer I, respectively, in Aglar microsyringes.

**Kinetic Method.** A Joule-heating temperature-jump spectrophotometer apparatus (7, 8) was used to measure the chemical relaxation spectrum of AcCh receptor. The apparatus, a modified version of a previously reported instrument (8), was further developed by C. R. Rabl, Göttingen, and was equipped with an electronic cooling correction device. The tempera-

Abbreviation: AcCh, acetylcholine.
* This paper is part II of a series. Paper I is ref. 3.

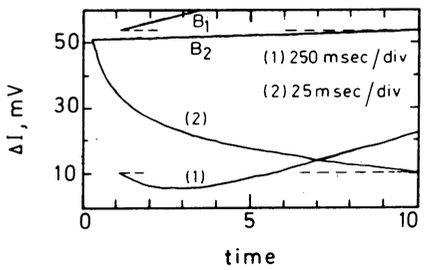


FIG. 1. Chemical relaxation spectrum (section $t \geq 5$ msec) of AcCh receptor from *T. californica*, at 1 mM Ca, 23.5°, after a temperature jump of 3.5°. $\Delta I$ measures light intensity changes at 480 nm. 10 mV correspond to an absorbance change of 0.0005 per 0.7 cm. Double-sweep oscilloscope record at two time scales; $B_1$ and $B_2$ are reference tracings (no temperature jump). The pre-amplitude of 578 mV ($t \leq 5$ msec) was electronically suppressed, and cooling correction was applied.

ture-jump cell contained 0.7 ml of carefully degassed temperature-jump samples described above. The electrical resistance, $R$, of the filled cell was 220 Ω. The capacitance, $C$, of the high voltage discharge capacitor was $5 \times 10^{-8}$ F. The temperature increase per jump was between 3 and 5°. Under the given experimental conditions, the heating time $\tau_h \simeq 0.5\,RC$ was 6 μsec. The relaxation of the Ca-AcCh receptor system in the presence of murexide was monitored at 480 nm and 23.5° (see ref. 3).

**Analysis.** A relaxation time simulator was used to estimate relaxation amplitudes and time constants of the measured relaxation spectrum (10). A method was developed to analyze the estimated relaxation time constants in terms of the total concentrations of the reaction partners (11).

## RESULTS

### Ca-AcCh receptor relaxations

The concentrations of the reaction partners were chosen so that the equilibration of the indicator system Ca plus murexide is very rapid (relaxation time $\tau_{CaMu} = 10$ μsec) and practically falls in the time range of the Joule heating of the measuring cell ($\tau_h = 6$ μsec). The indicator system can then be used to monitor any slower relaxations involving Ca ions (7, 9). Typical relaxation curves of isolated AcCh receptor from *Torpedo californica* in 1 mM Ca are shown in Fig. 1. Due to photoreaction of murexide and photomultiplier drift at the extremely high sensitivity of the recording device, the base lines are inclined as shown. For the time range $t \geq 0.1$ msec in 1 mM Ca, it was found that the Ca relaxation spectrum is characterized by at least three major relaxation times: $\tau^1 = 5$ (±1) msec, $\tau^2 = 35$ (±5) msec, and $\tau^3 = 300$ (±30) msec (mean deviation in parentheses).

The relaxation spectrum of Ca-AcCh receptor varies with changes in the Ca concentration. However, the Ca concentration dependence of both time constants and relaxation amplitudes is small and rather complicated. The present accuracy of the data is not sufficient to differentiate between alternative mechanisms for the Ca–AcCh receptor interactions. It appears unlikely, however, that the two larger relaxation times in particular reflect simple bimolecular equilibria between Ca ions and AcCh receptor binding sites.

### Changes caused by AcCh

In the presence of AcCh, the relaxation spectrum of the Ca–AcCh receptor system changes. As shown in Fig. 2, the relaxation times $\tau^1$ and $\tau^2$ pass through a maximum as a function of

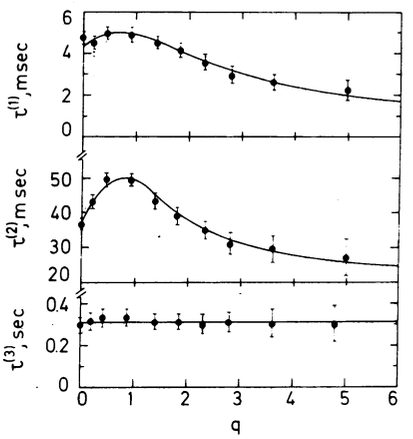


FIG. 2. Kinetic titration with AcCh of AcCh receptor from *T. californica*. Plot of time constants $\tau^i$ of the Ca–AcCh receptor relaxation at 1 mM Ca and 23.5° as a function of $q = [A^0]/[R^0]$, where $[A^0]$ is the (variable) total concentration of AcCh and $[R^0]$ is the (practically constant) total concentration of AcCh binding sites of AcCh receptor ($5 \times 10^{-6}$ M). See *text*.

AcCh concentration, whereas the large time constant $\tau^3$ practically remains constant.

## DISCUSSION

**Ca Binding to AcCh Receptor.** The chemical relaxation spectrum obtained for the Ca–AcCh receptor system reflects multiple interactions between Ca ions and receptor. This observation is consistent with the results of thermodynamic Ca binding studies, which indicate multiple Ca binding sites on the AcCh receptor (3). The kinetics of metal ion binding to many reaction partners is usually close to diffusion controlled, and the rate constant of association of Ca ions with simple ligands at 0.1 M ionic strength and 20° is usually in the order of $10^8$–$10^9$ $(M\ sec)^{-1}$ (7). As seen in the next section, for [Ca] = 1 mM, bimolecular elementary reactions involving Ca relax in the 20 μsec time range. Therefore, the values of $\tau^2$ and $\tau^3$ indicate that slower intramolecular reactions, such as conformational changes, are reflected in the Ca–AcCh receptor relaxation.

**Kinetics of AcCh Binding to Receptor.** An analysis of the relaxation times represented in Fig. 2 suggests the presence of conformational equilibria of AcCh receptor. The isolated AcCh receptor equilibrates between at least two conformational states $R \rightleftharpoons R''$, having different apparent equilibrium constants for AcCh and Ca ions.

As shown in our previous report (3), the binding sites for Ca and AcCh may be treated as indistinguishable and independent. Furthermore, the dependence of the AcCh binding constants on the Ca concentration suggests competition between AcCh and Ca for the AcCh receptor macromolecule (3). In the simplest case, if such competition involves binding sites in AcCh receptor, R, to which both Ca and AcCh ions can bind, then a minimum reaction scheme that accounts for the observed dependence on AcCh, A, of the observed relaxations must consist of at least three elementary steps:

$$(0) \qquad Ca + R \underset{k_{10}}{\overset{k_{01}}{\rightleftharpoons}} CaR \qquad [1]$$

$$(I) \qquad A + R \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} AR \qquad [2]$$

$$(II) \qquad AR \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} AR'' \qquad [3]$$

The experimental data show that [R″] is very small, so that the bimolecular step A + R″ $\rightleftharpoons$ AR″ is not visible.

In the above scheme, the $k$ terms are the rate constants of the elementary steps associated with the equilibrium constants $K_{Ca} = k_{10}/k_{01}$, $K_1 = k_{21}/k_{12}$, and $K_2 = k_{23}/k_{32}$, respectively. The kinetic data suggest that the observed equilibrium constant $K_A$ for the interaction of AcCh with receptor, determined by thermodynamic methods (equilibrium binding of [$^3$H]AcCh), is actually an overall equilibrium constant. As given in the reaction scheme, the reaction partners for AcCh are R and R″. Therefore,

$$K_A = \frac{[A]([R] + [CaR])}{[AR] + [AR'']} = (K_1 \cdot \phi^{-1})/(1 + K_2) \quad [4]$$

where $\phi = (1 + K_{Ca}^{-1}\cdot[Ca])^{-1}$. As shown previously (3), the dependence of $K_A$ on [Ca] is associated with $K_{Ca} = 3.1\ (\pm 0.2) \times 10^{-3}$ M. Thus, at [Ca] = 1 mM, $\phi = 0.76$. It is now noted that Eq. **6** in ref. 3 must be specified to $K_A = K_1\cdot(1 + K_2)^{-1}\cdot(1 + K_{Ca}^{-1}\cdot[Ca])$.

Due to the coupling of the elementary reactions, the measured relaxation times are normal mode time constants of the reaction scheme given above. As has already been discussed, if $k_{01} = 10^8$ (M sec)$^{-1}$, then for the present conditions the intrinsic relaxation time for Eq. **1**, given by $\tau_0 = \{k_{01}([Ca] + [R] + K_{Ca})\}^{-1}$ is calculated to be about 2.5 μsec. The bimolecular Ca–AcCh receptor relaxation can thus safely be considered to be very rapid compared to the measured (and kinetically resolvable) time constants (see Fig. 2). It will be seen that the dependence of $\tau^1$ on AcCh concentration suggests that this time constant is the normal mode relaxation time, $\tau_I$, associated with the coupled reaction in Eq. **2**. Since $\tau_0 \ll \tau_I$, the relaxation time $\tau^1 = \tau_I$ includes the equilibration factor $\{1 + [Ca]\cdot(K_{Ca} + [R])^{-1}\}^{-1}$ which, for the present experimental condition of $K_{Ca} \gg [R]$, can be approximated to $\phi$ (see Eq. **4**).

For the conditions of a rapid equilibration in Eq. [**1**],

$$1/\tau_I = k_{12}\cdot\phi([A] + [R]\cdot\phi^{-1}) + k_{21} = k_{12}' + k_{21} \quad [5]$$

Applying a procedure recently suggested by Eigen and Winkler-Oswatitsch (11), it is possible to express $\tau_I$ in terms of total concentrations of AcCh, [A$^0$], and of total binding sites of AcCh receptor, [R$^0$]. Using the definitions $q = [A^0]/[R^0]$ and $p = K_A/[R^0]$ and introducing $[A] = [A^0]-([AR] + [AR''])$ and $([R] + [CaR]) = [R]\cdot(1 + K_{Ca}^{-1}\cdot[Ca]) = [R^0] - ([AR] + [AR''])$ into Eq. **4**, it can be written that $2([AR] + [AR'']) = [R^0]\{(1 + p + q) - \sqrt{(1 + p + q)^2 - 4q}\}$. Now, Eq. **5** can be rewritten to:

$$1/\tau_I = k_{12}\cdot\phi[R^0]\left(\sqrt{(1 + p + q)^2 - 4q} + p\cdot K_2\right) \quad [6]$$

If $p < 1$, i.e., $[R^0] > K_A$, $\tau_I$ passes through a maximum as a function of $q$, at $q^m = 1 - q$.

The dependence of $\tau^2$ on $q$ suggests that this relaxation is associated with the intramolecular reaction in Eq. **3**. With $\tau_{II} = \tau^2$ and since $\tau_{II} > \tau_I$, the reaction represented in Eq. **2** may be considered to be equilibrated during the slower relaxation of the intramolecular step. Introducing the equilibration factor $k_{12}'/(k_{12}' + k_{21})$, the relaxation time of the slow mode is given by

$$1/\tau_{II} = k_{23}\{k_{12}'/(k_{12}' + k_{21})\} + k_{32} \quad [7]$$

See refs. 7 and 11. With the definitions of $p$ and $q$, Eq. **7** is rewritten as:

$$\tau_{II} - \tau_2 = \tau_2 \cdot \frac{p\cdot K_2}{\sqrt{(1 + p + q)^2 - 4q}} \quad [8]$$

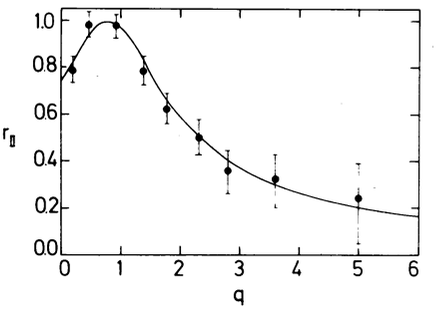


FIG. 3. The ratio $r_{II} = (\tau_{II} - \tau_2)/(\tau_{II}^m - \tau_2)$ as a function of $q$, plotted according to Eq. **11**. See *text*.

where

$$\tau_2 = (k_{23} + k_{32})^{-1} = \{k_{32}(1 - K_2^{-1})\}^{-1} \quad [9]$$

is the intrinsic time constant of the uncoupled reaction represented by Eq. **3**.

If $p < 1$, $\tau_{II}$ passes through a maximum as a function of $q$ at $q^m = 1 - p$. The time constant $\tau_{II}^m$ of the maximum is given by

$$\tau_{II}^m - \tau_2 = (1/2)\,\tau_2\cdot K_2\cdot\sqrt{p} \quad [10]$$

The combination of Eqs. **8** and **10** results in an expression for the ratio, $r_{II}$,

$$r_{II} = \frac{\tau_{II} - \tau_2}{\tau_{II}^m - \tau_2} = \frac{2\sqrt{p}}{\sqrt{(1 + p + q)^2 - 4q}} \quad [11]$$

where the measurable relaxation times $\tau_{II}$ and $\tau_2$ (which equals $\tau_{II}$ at $q \gg 1$) are only correlated with the variables $q$ and $p$. The shape of the plot of $r_{II}$ against $q$ gives the value of $p$ and thereby of $K_A$.

Recall that $\tau_I$ and $\tau_{II}$ pass through a maximum as a function of $q$. In some preparations of AcCh receptor the position of the maximum corresponded to $q$ values lower than $1 - p$; in these cases only a fraction of the protein appeared to bind AcCh.

In Fig. 3 the measured relaxation times $\tau_{II}$ are replotted according to Eq. **11** and compared with the calculated function $r_{II} = f(q,p)$ using $p = 0.2$ and $\tau_2 = 20$ msec. With $[R^0] = 5 \times 10^{-6}$ M on the basis of a molecular weight of 260,000 (see ref. 3), the kinetic method gives $K_A = p\cdot[R^0] = 1.0\ (\pm 0.5) \times 10^{-6}$ M, in good agreement with $K_A = 1.5\ (\pm 0.3) \times 10^{-6}$ M derived from equilibrium dialysis binding data with this AcCh receptor preparation. When the maximum value $\tau_{II}^m = 50$ msec is inserted in Eq. **10**, $K_2 = 6.7$; i.e., about 90% of complexed AcCh receptor is in the conformation AR″, under the present experimental conditions. Inserting the $K_2$ value in Eq. **4**, the calculation yields $K_1 = 6 \times 10^{-6}$ M. Thus, the kinetic method provides the individual equilibrium constants for the single steps whereas thermodynamic procedures only give the overall distribution constant, $K_A$. With Eq. **9** and $K_2 = 6.7$, the rate constants for the conformational change are $k_{23} = 43.5$ sec$^{-1}$ and $k_{32} = 6.5$ sec$^{-1}$.

The value of $K_2$ is now used to analyze the dependence on $q$ of the relaxation time $\tau_I = \tau^1$ (see Fig. 2). It is found that, for $k_{12} = 2.4\ (\pm 0.5) \times 10^7$ (M sec)$^{-1}$, the measured time constants agree within experimental accuracy with the curve calculated according to Eq. **6**. Since $k_{21} = K_1\cdot k_{12}$, we find $k_{21} = 144\ (\pm 30)$ sec$^{-1}$. Thus, the individual rate coefficients and equilibrium constants of the interaction between AcCh and receptor are obtained from relaxation data of Ca-receptor interactions.

The analysis of relaxation times in terms of total concentrations of the reaction partners can be extended to a determination of (unknown) reaction stoichiometries and molecular weights. If, as in our case, the concentrations are chosen so that $[R^0] > K_A$, the position of the maximum of the relaxation times is at $q^m = 1 - p$. The concentration of the (kinetically independent) AcCh binding sites, $[R^0]$, is calculated from $[R^0] = n_A \cdot [AcChR]$ where [AcChR] is the macromolecular receptor concentration and $n_A$ is the (maximum) number of AcCh molecules combining with AcCh receptor. Denoting by $[A^0]_m$ the total AcCh concentration at $q^m$, we obtain

$$n_A \cdot [AcChR] = [A^0]_m / q^m \quad [12]$$

Thus, at known molecular weight of AcCh receptor, $n_A$ can be calculated and vice versa; at known $n_A$ the molecular weight can be determined, using Eq. 12. For the example shown in Fig. 2, the data are consistent with $n_A = 2$ and an AcCh receptor molecular weight of 260,000 (±30,000).

The kinetic analysis of the relaxation times provided the second order rate constant $k_{12} = 2.4\ (\pm 0.5) \times 10^7\ (M\ sec)^{-1}$ (see Eq. 2). The association rate constants of comparable reactions, for instance, of diffusion controlled substrate enzyme reactions, are between $10^7$ and $10^8\ (M\ sec)^{-1}$ (see, e.g., ref. 12). The association rate of the AcCh binding to receptor may therefore be considered as close to diffusion controlled.

**Relationship of Kinetic Data to Physiological Mechanisms.** According to electrophysiological data (13), the life time of AcCh receptor occupation by AcCh may limit the permeability change induced by AcCh in excitable membranes. The decay rate constant of the measured ionic currents, controlled by the rate of the permeability change, is about $10^3\ sec^{-1}$. This value is about 10 times larger than the value found for the dissociation rate $k_{21}$ of AcCh receptor in the framework of the reaction scheme represented by Eqs. 1–3. It is, however, possible that the membrane permeability change is controlled by a reaction step AR ⇌ AR′ associated with the rate coefficients $k_{24}$ and $k_{42}$, respectively. If this reaction is rapidly coupled to the slower second order step (according to

$$A + R \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} AR \underset{k_{42}}{\overset{k_{24}}{\rightleftharpoons}} AR')$$

the measured value of $144\ sec^{-1}$ would reflect the value of $k_{21} \cdot k_{42}/(k_{42} + k_{24})$. Thus, as a consequence of this coupling, the elementary rate constant $k_{21}$ will always be larger than $144\ sec^{-1}$. The present kinetic data, however, are not sufficient to decide on the existence of such a scheme. The measured relaxations do indicate slower reactions characterized by $\tau_{II}$ and $\tau^3$. As a purely speculative remark, these slow reactions are possible candidates that could account for the time range of physiological desensitization (14).

The purified AcCh receptor from *Torpedo californica* so far used in this kinetic study was characterized by a major AcCh-binding component with a $K_A$ of about $10^{-6}$ M (3), whereas the membrane-bound AcCh receptor and 1% Triton extract of crude AcCh receptor exhibited AcCh equilibrium constants of only high affinity of about $2 \times 10^{-8}$ M (3, 15, 16). If the high affinity form of AcCh receptor also equilibrates between at least two different conformations, similar to the low affinity AcCh receptor, then only a large value of $K_2$ in the relationship $K_A = K_1(1 + K_2)^{-1}$ can result in a physiologically relevant value for the AcCh dissociation rate constant $k_{21}$.

It should also be mentioned that a recently developed physicochemical model for the permeability control by AcCh receptor systems in excitable membranes (1, 5; P. K. Rawlings and E. Neumann, submitted for publication) can readily account for the experimentally observed low AcCh dissociation constant. In this model the permeability change is regulated by the conformational transition AR ⇌ AR′. This reaction is the central part of a sequence of steps in which AcCh is translocated from a microreaction space, where the only reaction partner for AcCh ($A_1$) is the R form of AcCh receptor, to another microreaction space where AcCh ($A_2$) becomes available also for the enzymatic decomposition by acetylcholinesterase. If, in addition, physiological desensitization, as far as the receptor is concerned, is taken into account (by AR″), the following reaction scheme results:

$$A_{(1)} + R \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} AR \underset{k_{42}}{\overset{k_{24}}{\rightleftharpoons}} AR' \rightleftharpoons R' + A_{(2)} \quad [13]$$

$$AR \underset{k_{23}}{\overset{k_{32}}{\rightleftharpoons}} AR''$$

In this scheme, R and AR (and also AR″) represent low permeability configurations of AcCh receptor, whereas AR′ is the high permeability form. The transition of R′ to R represents the control step for the closing of the permeation zones for ionic currents. Now, for efficiency reasons, the dissociation rate constant $k_{21}$ should be small so that the main reaction flow of AcCh ($A_1$) is toward AR and AR′. This efficiency criterion is matched if the equilibrium constant $K_1 = k_{21}/k_{12}$ is small and the influx of AcCh ($k_{12}$) is rapid, i.e., practically diffusion controlled. Since $K_1$ is proportional to the overall constant $K_A$, a low value of $K_A$ would guarantee an efficient net AcCh influx into the AcCh receptor control system. Thus, our dissipative nonequilibrium model for the control of ion flows in excitable membranes is consistent with a high affinity binding constant of AcCh to AcCh receptor.

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