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SINGLE CHANNEL GATING EVENTS IN TRACER FLUX EXPERIMENTS

II. FLUX AMPLITUDE ANALYSIS

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Measurement of tracer ion flux from or into a collection of closed membrane structures (CMS) constitutes a broadly applicable technique for studying ion channel gating by specialized gating molecules in biological membranes. The amplitudes for the flux process reflect the overall change in tracer content due to flux during a period in which channels on at least some of the CMS were open. In practice, the attainment of a time-invariant, finite overall tracer content, indicating a cessation of flux, need not imply that flux has reached completion, i.e., that the CMS internal and external tracer concentrations have fully reached equilibrium. Less than maximum flux amplitudes arise when binding of control ligands leads to an inhibition or inactivation of the channel gating molecules prior to a complete equilibration of tracer. Analysis of the dependence of the flux amplitudes on control ligand concentration permits determination of characteristic parameters of the CMS that may vary with the methods of preparation (e.g., the distributions of CMS size and CMS content of gating units). Knowledge of these parameters in turn permits evaluation of the mean single channel flux amplitude contribution, which is functionally dependent on the rate constant ratio (k'_{eff}/k_i), where k'_{eff} and k_i are, respectively, the effective rate constants for tracer flux and for gating unit inactivation.

1. Introduction

Tracer ion flux measurements are a commonly used method for studying ion transport through gated channels of closed membrane structures (CMS) such as sealed membrane fragments (microsacs), reconstituted vesicles or entire cells. In a general mathematical treatment of tracer flux, explicit expressions for the overall tracer content $X(t)$ of the CMS at time t were derived for efflux from CMS into a large bath, and for influx from a large bath into CMS [1]. When flux through transmembrane channels on the CMS is controlled by specialized gating molecules, the time course of $X(t)$ will depend on the gating reaction that regulates channel opening and closing. Analysis of the flux data based on previously derived schemes [1–4] will then yield information about the kinetics of the gating process.

When efflux into a large bath initially containing no tracer is able to reach completion, the overall change in tracer content of the CMS is given by $X(0)$ — the value of $X(t)$ at $t = 0$, i.e., a complete emptying of tracer content takes place. Similarly, when influx of tracer from a large bath into CMS initially containing no tracer is able to reach equilibrium, the overall change in tracer content is given by $X(\infty)$ — the value of $X(t)$ when $t \to \infty$. $X(0)$ and $X(\infty)$ therefore represent the maximum overall flux amplitudes for flux carried out under respective conditions.

When inhibition or inactivation of the channel gating molecules leads to a net closing of channels prior to complete equilibration of tracer, the overall change in tracer content will be less than these maximum values. Less than maximum flux amplitudes have been observed in tracer flux experiments with CMS containing acetylcholine recep-

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tors (AcChR) when (a) binding of activator ligands leads to inactivation (desensitization) of receptors [2,5] and (b) binding of snake toxins leads to inhibition of receptors [6,7]. The flux amplitudes were found to depend on the respective concentration of activator or inhibitor ligand that induces channel closing.

In practice, it is considerably more simple to accurately measure flux amplitudes than time-dependent flux processes. In this article, it will be shown that analysis of the ligand concentration-dependent flux amplitudes permits determination of several important parameters. In section 2, general expressions for the overall relative flux amplitudes R_∞ are presented. Fundamental empirical parameters occurring in these expressions are shown to depend on the distribution of CMS size, and content of gating units. In section 3, general expressions for the mean single channel flux amplitude contributions $\langle e^{-kt}\rangle_\infty$ resulting from inhibition and inactivation of gating units are presented. In section 4, explicit equations for the quantities $\langle e^{-kt}\rangle_\infty$ and R_∞, for the special case of AcChR-regulated flux are derived.

2. Overall flux amplitudes

2.1. Fundamental relationships

A collection of ν CMS can be divided into subfractions, where a given subfraction consists of all ν_n CMS having exactly n gating units per CMS. As shown previously [1], for CMS containing monomeric, independent gating units, the time-dependent overall tracer content $X(t)$ is then given by

$$X(t)=\begin{cases}\sum_n X_n(0)[\langle e^{-kt}\rangle^n]_v & \text{(efflux)} \quad (1)\\ \sum_n X_n(\infty)\{1-[\langle e^{-kt}\rangle^n]_v\} & \text{(influx)} \quad (2)\end{cases}$$

where $X_n(0)$ and $X_n(\infty)$ are the component maximum flux amplitudes for the subfraction of CMS having n gating units per CMS, and $[\langle e^{kt}\rangle^n]_v$ denotes the average over a distribution in CMS volumes of the mean single channel flux contribution $\langle e^{-kt}\rangle$ to the nth power.

$\langle e^{-kt}\rangle$ represents the mean factor by which the tracer content of a single CMS is changed due to flux through a single channel in the period of measurement from 0 to t. Expressions for $\langle e^{-kt}\rangle$ in the case of activation and inactivation modes of a channel gating reaction were derived using the relationship

$$\langle e^{-kt}\rangle=\int_0^t p(\tau,t)e^{-k\tau}\,d\tau \quad (3)$$

where $p(\tau,t)$ expresses the normalized probability that a single channel was open for a period τ during the interval from 0 to t, and k is the intrinsic rate constant for tracer flux through a single channel. When the mechanism whereby tracer is transported through channels involves (transient) binding to channel sites, k is given by

$$k=k'/v \quad (4)$$

where v is the internal volume of the CMS on which the channels are located, and k' the volume-independent flux rate constant in units of volume per unit time [1]. Analysis of the measured flux data representing $X(t)$ using eq. 1 or 2 permits determination of $\langle e^{-kt}\rangle$, which in turn can be analysed using equations derived from eq. 3, to determine kinetic parameters for gating processes.

The quantities $X_n(0)$ and $X_n(\infty)$ in eqs. 1 and 2, respectively, represent the total amount of tracer that flows from or into the subfraction of CMS having n gating units per CMS, when flux is able to reach completion. In general, if the rates of channel opening and channel closing remain finite during a gating reaction, $\langle e^{-kt}\rangle$ will always approach the limiting value $\lim_{t\to\infty}\langle e^{-kt}\rangle=0$ at long times. This implies that the overall flux amplitudes are $X(0)=\sum_n X_n(0)$ for efflux, and $X(\infty)=\sum_n X_n(\infty)$ for influx. The initial CMS internal concentration of tracer C_0 in efflux experiments, and the final CMS internal concentration C_∞ in influx experiments, are the same for all CMS. This implies that $X_n(0)=C_0V_n$ (efflux) and $X_n(\infty)=C_\infty V_n$ (influx), where V_n is the sum of the internal volumes of all ν_n CMS having n gating units per CMS. Using these relationships it is possible to recast eqs. 1 and 2 in a form more suitable for practical applications. Introducing the time-depen-

dent relative tracer content

$$R(t)=\begin{cases} X(t)/X(0) & \text{(efflux)} \quad (5') \\ X(t)/X(\infty) & \text{(influx)} \quad (6') \end{cases}$$

one obtains

$$R(t)=\begin{cases} \sum_n P_n[\langle e^{-kt}\rangle^n]_v & \text{(efflux)} \quad (5) \\ \sum_n P_n\{1-[\langle e^{-kt}\rangle^n]_v\} & \text{(influx)} \quad (6) \end{cases}$$

where the fractional internal volume P_n is given by

$$P_n = V_n/V \tag{7}$$

with $V=\Sigma_n V_n$, so that $\Sigma_n P_n = 1$. In contrast to eqs. 1 and 2, eqs. 5 and 6 do not depend on the concentration or amount of tracer.

When flux through gated channels is able to reach completion, $\langle e^{-kt}\rangle$ varies from 1 to 0, therefore $R(t)$ varies from 1 to 0 (efflux), or from 0 to 1 (influx). If inactivation or inhibition of gating units leads to effectively irreversible channel closing prior to completion of flux, $\langle e^{-kt}\rangle$ will approach a finite, time-independent limiting value $\langle e^{-kt}\rangle_\infty$ — the mean single channel flux amplitude contribution — defined as

$$\langle e^{-kt}\rangle_\infty = \lim_{t\to\infty}\langle e^{-kt}\rangle. \tag{8}$$

Substitution of $\langle e^{-kt}\rangle_\infty$ for $\langle e^{-kt}\rangle$ in eqs. 5 and 6 leads to the finite, time-independent relative flux amplitudes R_∞ defined as

$$R_\infty = \lim_{t\to\infty} R(t). \tag{9}$$

2.2. Fundamental parameters

Eqs. 5 and 6 imply that the relative overall tracer content $R(t)$ is a weighted sum of terms $[\langle e^{-kt}\rangle^n]_v$, expressing the component relative tracer content of the ν_n CMS having n gating units per CMS. The quantities $\langle e^{-kt}\rangle$ express the dependence of flux on the gating process. In order to determine $\langle e^{-kt}\rangle$ from the primary flux data expressed as $R(t)$, it is first necessary to account for the weight factors P_n, and the volume average implicit in the terms $[\langle e^{-kt}\rangle^n]_v$.

Each CMS is uniquely characterized by the number n of gating units it contains, by its internal volume v, and by its surface area s. For spherical CMS, v is given by the general surface-to-volume relationship

$$v=\frac{s^{3/2}}{6\pi^{1/2}} \tag{10}$$

A collection of ν CMS can be characterized by its distribution in n given by the fractions $\xi_n = \nu_n/\nu$, and by the normalized volume distribution function $Q(v)$. ξ_n and $Q(v)$, respectively, express the probability that a given CMS has n gating units, and an internal volume v. The mean number of gating units per CMS $\bar{n}$, and the mean CMS internal volume $\bar{v}$, are then given by

$$\bar{n} = \sum_n \xi_n n \tag{11}$$

$$\bar{v} = \int_0^\infty v Q(v)\,dv. \tag{12}$$

The mean surface area of a CMS, $\bar{s}$, can be determined from $\bar{v}$ using the relationship eq. 10. Each subfraction of ν_n CMS having n gating units per CMS is characterized by a normalized component distribution function $Q_n(v)$, expressing the probability that a CMS with n gating units has an internal volume v. The mean internal volume $\bar{v}_n$ of a CMS with n gating units is then given by

$$\bar{v}_n = \int_0^\infty v Q_n(v)\,dv. \tag{13}$$

Using eq. 10 it is then possible to determine the mean surface area $\bar{s}_n$, of a CMS with n gating units. Averaging over component contributions one obtains the relationships

$$Q(v)=\sum_n \xi_n Q_n(v) \tag{14}$$

$$\bar{v}=\sum_n \xi_n \bar{v}_n \tag{15}$$

$$\bar{\rho}=\sum_n \xi_n\left(\frac{n}{\bar{s}_n}\right) \tag{16}$$

where $\bar{\rho}$ is the mean surface density of gating units.

The total internal volume V_n of all CMS in a subfraction is given by

$$V_n = \nu \xi_n \bar{v}_n. \tag{17}$$

Substitution of this result into eq. 7 yields

$$P_n = \xi_n\left(\frac{\bar{v}_n}{\bar{v}}\right). \tag{18}$$

The volume average occurring in the terms $[\langle e^{-kt}\rangle^n]_v$ can be expressed as

$$[\langle e^{-kt}\rangle^n]_v = \int_0^\infty \langle e^{-kt}\rangle^n Q_n(v)\,dv. \tag{19}$$

2.3. Limiting cases

In part III of this series [8], experimental techniques for determining the parameters P_n and $Q(v)$ will be presented. Unfortunately, knowledge of P_n and $Q(v)$ alone does not permit evaluation of quantities that depend on $Q_n(v)$. Since $Q_n(v)$ depends on the degree of covariance between the parameters n and v, additional assumptions about the dependence of the content of gating units on CMS size are required to evaluate the equations derived in the preceding section. Two limiting situations may arise:

(a) There is no covariance between n and v. At low surface densities of gating units there may be little correlation between CMS size and content of gating units. This implies that

$$Q_n(v) = Q(v) \tag{20}$$

and consequently from eqs. 12–15 and 18 it then follows that

$$\bar{v}_n = \bar{v}, \bar{s}_n = \bar{s}, \xi_n = P_n. \tag{21}$$

From eqs. 11 and 21 one obtains

$$\bar{n} = \sum_n P_n n. \tag{22}$$

The mean surface density can then be obtained by substitution eq. 22 into the expression

$$\bar{\rho} = \left(\frac{\bar{n}}{\bar{s}}\right) \tag{23}$$

which results from eq. 16 after substitution of eq. 21. Finally, eq. 19 can be expressed as

$$[\langle e^{-kt}\rangle^n]_v = \int_0^\infty \langle e^{-kt}\rangle^n Q(v)\,dv. \tag{24}$$

(b) The content of gating units is proportional to CMS surface area. At high surface densities of gating units there will be strong covariance between CMS size and content of gating units. Assuming that the surface density on all CMS is given by the constant value $\bar{\rho}$, eq. 23 will again hold. Furthermore, this implies that

$$\bar{s}_n = \left(\frac{\bar{n}}{\bar{\rho}}\right) \tag{25}$$

and thus from eq. 10 that

$$\bar{v}_n = \left(\frac{1}{6\pi^{1/2}}\right)\left(\frac{n}{\bar{\rho}}\right)^{3/2} \tag{26}$$

From eqs. 10, 18, 23 and 25 one obtains

$$\bar{n} = \left[\sum_n P_n n^{-1/2}\right]^{-2} \tag{27}$$

$$\xi_n = P_n\left(\frac{\bar{n}}{n}\right)^{3/2} \tag{28}$$

Substituting eq. 27 for $\bar{n}$ in eq. 23 yields $\bar{\rho}$. The distribution function $Q_n(v)$ is given by

$$Q_n(v) = \delta(v - \bar{v}_n) \tag{29}$$

where $\delta(v - \bar{v}_n)$ is the delta function. Eq. 19 then becomes

$$[\langle e^{-kt}\rangle^n]_v = \langle e^{-k_n t}\rangle^n \tag{30}$$

where the n-dependent rate constant $k_n = k'/\bar{v}_n$, with $\bar{v}_n$ given by eq. 26, must be substituted for k in eq. 3 for $\langle e^{kt}\rangle$.

3. Mean single channel flux amplitude contributions

3.1. Inactivation of gating units

Frequently, gating reactions occur in several phases, where an initial activation (i.e., net channel opening) phase is often followed by one or more inactivation (i.e., net channel closing) phases. Specific examples are the rapid activation, and the intermediate and slow inactivation phases of in vivo channel gating by AcChR [9]. If the successive phases occur on widely separated time scales, each phase will constitute a separate reaction mode. When a given gating reaction phase j reaches equilibrium or a steady state, $\langle e^{-kt}\rangle$ will approach the limiting value

$$\langle e^{-kt}\rangle_{eq}^{(j)} = e^{-k_{eq}^{(j)}t} \tag{31}$$

where $k_{eq}^{(j)}$ is the effective flux rate constant associ-

ated with the equilibrated or stationary gating phase. As shown previously [1], $k_{eq}^{(j)}$ is given by

$$k_{eq}^{(j)} = \alpha_{j,eq}^{(o)}\left[\left(1/k_c^{(j)}\right)+\left(1/k_{eq}^{(j-1)}\right)\right]^{-1} \quad (32)$$

where $\alpha_{j,eq}^{(o)}$ is the fraction of open channels after equilibration of the jth gating phase given by $\alpha_{j,eq}^{(o)} = k_o^{(j)}/(k_o^{(j)}+k_c^{(j)})$ where $k_o^{(j)}$ and $k_c^{(j)}$ are, respectively, the apparent rate constants for channel opening and channel closing during the jth phase, and $k_{eq}^{(j-1)}$ is the effective equilibrium flux rate constant for the preceding $(j-1)$th) phase. Adopting the value $k_{eq}^{(o)} = k$ for the fastest gating phase, iterative application of eq. 32 can be used to find the expression for $k_{eq}^{(j)}$ for any phase $j > 0$.

For an inactivation phase j (net channel closing), one has $k_c^{(j)} > k_o^{(j)}$. From eq 32 it then follows that $k_{eq}^{(j)} < k_{eq}^{(j-1)}$. If $k_c^{(j)} \gg k_o^{(j)}$, there will be an apparent cessation of flux, leading to the appearance of a less than maximum overall flux amplitude due to incomplete emptying of the tracer content of the CMS. In practice, unless $k_o^{(j)} = 0$, flux will not cease entirely, but the residual flux will be negligible on the time scale of measurement. When the phases of the gating reaction occur on widely separated time scales, the dominant contribution to the overall flux amplitude will come from the slowest inactivation phase $j = s$, leading to the apparent cessation of flux. The limiting value of $\langle e^{-kt}\rangle$ as phase s approaches equilibrium will be the apparent mean single channel flux amplitude contribution $\langle e^{-kt}\rangle_\infty$ given by

$$\langle e^{-kt}\rangle_\infty = \frac{k_c^{(s)}}{k_c^{(s)}+k_{eq}^{(s-1)}}. \quad (33)$$

Substitution of $\langle e^{-kt}\rangle_\infty$ for $\langle e^{-kt}\rangle$ in eqs. 5 and 6 then leads to expressions for the relative flux amplitudes R_∞ when flux is effectively terminated by inactivation of gating units. Since both $k_c^{(s)}$ and $k_{eq}^{(s-1)}$ are apparent rate constants (see part I of this series, section 2.3 [1]) that may depend on the concentration of a ligand that induces inactivation, the overall flux amplitudes may also implicitly be ligand concentration dependent.

3.2. *Irreversible inhibition of gating units*

For many gating systems special ligands exist, which bind to sites on the gating units in a practically irreversible fashion, thereby inhibiting activation. Flux can then occur only through the channles connected with uninhibited gating units. The term 'gating unit' is used to denote the smallest functionally independent molecular entity controlling flux through one or more channels. Occupancy of a single ligand site may, however, lead to the inhibition of more than one such unit. In order to characterize uniquely the mode of inhibition it is necessary to specify the number of sites l, for which single or multiple occupancy leads to an identical inhibition of flux through r associated channels. The significance of such l-site units is illustrated in fig. 1.

The degree of inhibition of l-site units can be expressed in terms of the fraction $\sigma_l(\alpha)$, of uninhibited l-site units, which depends on the fraction of the total sites, α, that are occupied by inhibitory ligand [3]. The α-dependent overall tracer content $X(\alpha, t)$ of a collection of CMS is given by eqs. 1 and 2 if the summation index n in these equation is taken to represent the number of l-site units per CMS rather than the number of gating units per CMS. $\langle e^{-kt}\rangle$ must then also be redefined as the mean flux contribution of the r channels associ-

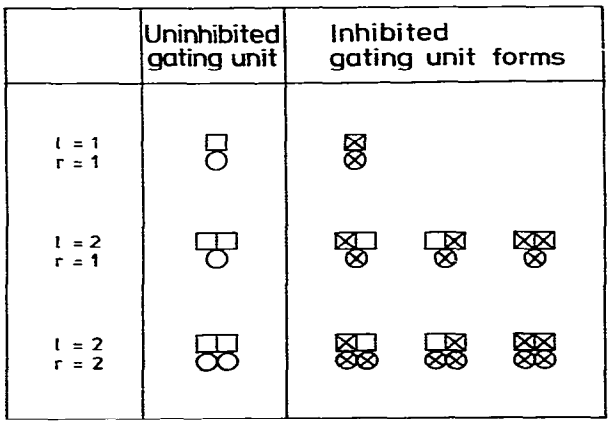

Fig. 1. Inhibition of channels resulting from occupation of gating unit inhibitory sites. An uninhibited gating unit contains l unoccupied inhibitory sites (□), and r uninhibited channels (○). Occupation of one or more inhibitory sites leads to inhibition of flux through all r channels, where □ denotes an occupied inhibitory site, and ⊗ an inhibited channel.

ated with an l-site. One therefore obtains

$$\langle e^{-kt}\rangle = [1-\sigma_l(\alpha)]\langle e^{-kt}\rangle_i^r + \sigma_l(\alpha)\langle e^{-kt}\rangle_u^r \quad (34)$$

where $\langle e^{-kt}\rangle_i$ and $\langle e^{-kt}\rangle_u$ are, respectively, the mean single channel flux contributions of inhibited and uninhibited channels. Statistical considerations for random ligand binding to equivalent and identical sites [3,4] lead to the expression

$$\sigma_l(\alpha) = (1-\alpha)^l. \quad (35)$$

Since the mean single channel flux contribution is defined as the mean factor by which the tracer content of a single CMS is changed due to flux through a single channel, one obtains $\langle e^{-kt}\rangle_i = 1$. Eq. 34 therefore becomes

$$\langle e^{-kt}\rangle = 1-(1-\alpha)^l(1-\langle e^{-kt}\rangle_u^r) \quad (36)$$

Finite α-dependent and time-independent flux amplitude contributions result when:

(a) Flux through channels associated with uninhibited l-site units is able to reach completion. This implies that $\langle e^{-kt}\rangle_u \to 0$. Eq. 36 then becomes

$$\langle e^{-kt}\rangle = 1-(1-\alpha)^l \quad (37)$$

(b) Flux through channels associated with uninhibited l-site units is terminated by inactivation of gating units. Eq. 36 then becomes

$$\langle e^{-kt}\rangle = 1-(1-\alpha)^l(1-\langle e^{-kt}\rangle_\infty^r) \quad (38)$$

where $\langle e^{-kt}\rangle_\infty$ is the mean single channel flux amplitude contribution given by eq. 33.

4. Flux amplitudes for AcChR-controlled flux

4.1. Mean single channel flux amplitude contribution

Previously, the ligand concentration-dependent flux amplitudes for tracer efflux from CMS derived from *Torpedo* electric organs were analysed on the basis of the assumption that a single inactivation process following AcChR activation leads to apparent cessation of flux [2]. The recent finding that inactivation occurs in (at least) two phases [9], of which only the slower leads to a cessation of flux [10], requires a more elaborate treatment. From eq. 33 the mean single channel flux amplitude contribution resulting for the slowest receptor inactivation phase is given by

$$\langle e^{-kt}\rangle_\infty = \frac{k_i}{k_i + k_{eff}} \quad (39)$$

where k_i is the effective forward rate constant for the slowest inactivation mode, and k_{eff} the effective flux rate constant after equilibration of the more rapid gating phases. Since the final inactivation phase occurs on a much slower time scale than the other phases, and since flux does not reach completion prior to final inactivation, it follows from eq. 32 that for the more rapid phases $k_c^{(j)} \gg k_{eq}^{(j-1)}$. This implies that, after successive iterative application of eq. 32, one obtains

$$k_{eff} = k \prod_j \alpha_{j,eq}^{(o)} \quad (40)$$

where the product is over all reaction modes preceding final inactivation. The effective rate constant for tracer flux through a single channel will therefore be less than the maximum value k, for flux through a permanently open channel, if the equilibrium fraction of open channels $\alpha_{j,eq}^{(o)}$, for any of the more rapid modes is less than unity. For ligand-induced activation and inactivation both k_i and k_{eff} may be ligand concentration dependent. Noting the implicit volume dependence of k expressed by eq. 4, eq. 39 can be rewritten as

$$\langle e^{-kt}\rangle_\infty = \frac{v}{v + (k'_{eff}/k_i)} \quad (41)$$

where, in analogy to eq. 40, a volume-independent effective flux rate constant may be defined as $k'_{eff} = k' \prod_j \alpha_{j,eq}^{(o)}$.

A simplified treatment is possible in the limiting case of high activator ligand concentrations. The reaction pathway corresponding to the reaction scheme of minimum complexity, consistent with present information about receptor processes,

$$2A + R \overset{K_1}{\rightleftharpoons} \ldots A_2R' \overset{K^*}{\rightleftharpoons} A_2R^* \underset{k_{-d}}{\overset{k_d}{\rightleftharpoons}} A_2R^{**} \quad (42)$$

is then expected to dominate, where A denotes the ligand, R the unbound receptor in the resting state, A_2R' the active receptor state, A_2R^* and A_2R^{**}, respectively, the intermediate and final inactive states [9], K_1 and K^* the (overall) dissoci-

ation equilibrium constants for the respective transitions, and k_d and k_{-d}, respectively, the forward and reverse rate constants for the slowest inactivation reaction step. Solution of the kinetic equations for this reaction scheme, assuming that final inactivation is much slower than all preceding steps, lead to the expression for the time constant τ_s for the final slow inactivation mode

$$\frac{1}{\tau_s} = \beta^{-1} k_d + k_{-d} \tag{43}$$

where

$$\beta = 1 + K^* \left(1 + \frac{K_1}{[A]^2}\right).$$

Since on the time scale of flux measurements a cessation of flux is observed at high ligand concentrations, it may be inferred that $\beta^{-1} k_d \gg k_{-d}$. The limiting effective forward rate constant for flux k_i, for the final inactivation mode, is then given by

$$k_i = \lim_{[A]\to\infty} \beta^{-1} k_d = \frac{k_d}{1 + K^*} \tag{44}$$

where $K^* = [A_2R']/[A_2R^*]$. Since the two activation steps in scheme 42 involve a bimolecular encounter, the corresponding equilibrium fractions of open channels $\alpha^{(o)}_{1,eq}$ and $\alpha^{(o)}_{2,eq}$ are expected to approach the value 1 in the limit of high activator ligand concentrations. The fraction of open channels $\alpha^{(o)}_{3,eq}$, following equilibration of the intermediate inactivation phase, is then given by $\lim_{[A]\to\infty} \alpha^{(o)}_{3,eq} = 1/(1 + K^*)$. From eq. 40 it therefore follows that

$$\lim_{[A]\to\infty} k_{eff} = \frac{k}{1 + K^*}. \tag{45}$$

Substituting eqs. 44 and 45 into eq. 39, and noting eq. 4, one obtains the expression valid in the limit of high activator ligand concentrations

$$\langle e^{-kt} \rangle_\infty = \frac{v}{v + (k'/k_d)} \tag{46}$$

where k' is the true volume-independent flux rate constant.

4.2. Overall relative flux amplitudes

A ligand concentration-dependent reduction in flux amplitudes is observed in flux experiments when inactivation or inhibition of AcChR occurs [2,5–7] and can be expressed in terms of the overall relative flux amplitude R_∞ defined by eq. 9. R_∞ is obtained from eqs. 5 and 6 after substituting the appropriate expressions $\langle e^{-kt} \rangle_\infty$ for $\langle e^{-kt} \rangle$. For simplicity only the equations for efflux will be presented. Entirely analogous expressions result for influx. Three separate cases need to be considered:

(a) Reduction of efflux upon irreversible inhibition of receptors is measured under conditions leading to complete emptying of all CMS containing uninhibited receptors. R_∞ will then depend on α, the fraction of total receptors inhibited. Substitution of eq. 37 into eq. 5 yields

$$R_\infty(\alpha) = \sum_n P_n \left[1 - (1 - \alpha)^l\right]^n \tag{47}$$

where n is the number of l-site units on a CMS. Occupation of a single site by inhibitor suffices to inhibit flux mediated by the entire l-site unit. No volume averaging is required, since $\langle e^{-kt} \rangle$ given by eq. 37 is volume independent.

(b) Reduction of efflux upon activator ligand-induced inactivation of receptor (i.e., desensitization). R_∞ will depend on activator concentration [A]. In the limit of low surface densities of receptors, eqs. 5, 24 and 41 yield

$$R_\infty([A]) = \sum_n P_n \int_0^\infty \left[\frac{v}{v + (k'_{eff}/k_i)}\right]^n Q(v)\, dv \tag{48}$$

where the rate constant ratio (k'_{eff}/k_i) implicitly depends on [A]. In the limit of high surface densities, substitution of eq. 41 into eq. 30 yields the expression

$$[\langle e^{-kt} \rangle^n]_v = \left[\frac{\bar{v}_n}{\bar{v}_n + (k'_{eff}/k_i)}\right]^n \tag{49}$$

where $\bar{v}_n$ is given by eq. 26. Upon some rearrangement, substitution of eq. 49 into eq. 5 yields

$$R_\infty([A]) = \sum_n P_n \left[\frac{n^{3/2}}{n^{3/2} + c}\right]^n \tag{50}$$

where

$$c = \left(\frac{3}{4\pi}\right)(4\pi p)^{-3/2} (k'_{eff}/k_i).$$

(c) Reduction of efflux upon irreversible inhibi-

tion of receptors is measured under conditions where inactivation of receptors occurs. R_∞ will then depend on both α and [A]. In the limit of low surface densities of receptors eqs. 5, 24, 38 and 41 yield

$$R_\infty(\alpha, [A]) = \sum_n P_n \int_0^\infty \langle e^{-kt} \rangle_\infty^n Q(v)\, dv \tag{51}$$

with

$$\langle e^{-kt} \rangle_\infty = 1 - (1-\alpha)^l \left(1 - \left[\frac{v}{v + (k'_{eff}/k_i)}\right]^r\right) \tag{52}$$

where inhibitory occupation of an l-site unit leads to inhibition of flux through r channels. In the limit of high surface densities of receptors, eqs. 5, 30, 38 and 41 yield the corresponding result

$$R_\infty(\alpha, [A]) = \sum_n P_n \left\{1 - (1-\alpha)^l \left(1 - \left[\frac{n^{3/2}}{n^{3/2} + c}\right]^r\right)\right\}^n \tag{53}$$

5. Discussion

The flux data obtained in tracer flux experiments can be expressed in terms of the time-dependent, overall relative tracer content $R(t)$ of a collection of CMS. Eqs. 5 and 6 show that $R(t)$ is a weighted sum of component flux contributions $[\langle e^{-kt} \rangle^n]_v$, from subfractions of CMS having n gating units per CMS where each unit controls one or more channels. The weight factors P_n given by eq. 18 are characteristic parameters of a collection of CMS. They depend on the distributions of CMS size, and content of gating units. Aside from the implicit dependence of the flux rate constant k on the nature of the tracer species, $R(t)$ does not depend on the amount or type of tracer used in the flux experiments.

When flux through gated channels is able to reach completion, $R(t)$ will vary between the limiting values $R(0) = 1$ (efflux) and $R(0) = 0$ (influx) at $t = 0$, and $R(\infty) = 0$ (efflux) and $R(\infty) = 1$ (influx) when $t \to \infty$. Finite time-independent flux amplitudes R_∞, where $0 < R_\infty < 1$, result when inhibition or inactivation of gating units leads to effectively irreversible net channel closing. Eqs. 5 and 6 express the dependence of R_∞ on the mean single channel flux amplitude contribution $\langle e^{-kt} \rangle_\infty$.

When random, near irreversible binding of an inhibitory ligand to gating unit sites on the CMS takes place, a mixed population of fractionally inhibited CMS is generated. CMS having all their gating units inhibited will not contribute to flux. Under conditions where flux through channels controlled by uninhibited gating units is able to reach completion, these totally inhibited CMS will retain their tracer content in efflux experiments, and will remain empty in influx experiments — thus giving rise to less than maximum flux amplitudes. In previous publications [3,4], the inhibition of gating units controlling a single channel, which contain a single binding site for inhibitory ligands, was considered. The dependence of the fraction $\mu_n(\alpha)$ of totally inhibited CMS on the fraction α of total sites inhibited was found to be given by the power law $\mu_n(\alpha) = \alpha^n$. The qualitative trend expected from this relationship — a steep decline in the fraction of totally inhibited CMS with increasing n — is illustrated in fig. 2. Eq. 47 expresses the dependence of the overall flux amplitudes $R_\infty(\alpha)$ on α, in the more general case where binding of a ligand leads to inhibition of an l-site unit controlling flux through r channels. $R_\infty(\alpha)$ is thus a weighted sum of polynomials in α. In practice, α is known, so that curve fitting of experimental data for $R_\infty(\alpha)$ using eq. 47 permits determination of the weight factors P_n and the unknown parameter l.

When flux is measured under conditions where, in addition to inhibition, activator ligand-induced inactivation of gating units occurs, the flux amplitudes $R_\infty(\alpha, [A])$ will depend on both α, and the concentration of activator ligand [A]. General expressions for $R_\infty(\alpha, [A])$ are then obtained by substituting eq. 38 into eqs. 5 and 6. In the special case where flux is controlled by AcChR, considerations based on present knowledge of receptor processes lead to the more detailed eqs. 51 and 53. Eq. 51 applies when there is no covariance in CMS size and receptor content, while eq. 53 holds in the alternative situation where receptor content is proportional to the CMS surface area. Assuming prior knowledge of $Q(v)$, $\bar{p}$, the parameters P_n and l, curve fitting of these expressions to the experimen-

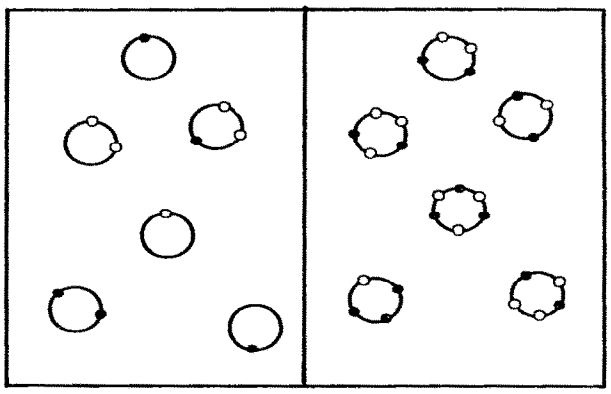

Fig. 2. Illustration of the mixture of fractionally inhibited CMS generated when random binding of an inhibitory ligand to gating units localized on CMS leads to the inhibition of the fraction $\alpha = 1/2$ of total gating units. Two separate collections of CMS, differing in the mean content of gating units per CMS, are considered. ● and ○, respectively, denote inhibited and uninhibited gating units. The probability that a given CMS has all its n gating units inhibited, and thus no longer contributes to flux, is seen to decrease with increasing n.

tal data for $R_\infty(\alpha, [A])$ can be used to determine the rate constant ratio (k'_{eff}/k_i) and the unknown parameter r. The quality of the overall fit will show whether eq. 51 or eq. 53 is applicable. This will indicate which of the two limiting case represented by these equations is more nearly realized. The quantities P_n and $Q(v)$ can then be used to determine the characteristics parameters of the CMS, $\bar{v}$, $\bar{s}$, $\bar{p}$, $\bar{n}$, $\bar{v}_n$, $\bar{s}_n$ and ξ_n from the equations presented in section 2.3. Knowledge of (k'_{eff}/k_i) suffices to determine uniquely the volume-functional $\langle e^{-kt} \rangle_\infty$ given by eq. 41.

Flux amplitude analysis, as outlined above, can be employed as a general technique for characterizing those factors of a CMS suspension that may vary from preparation to preparation. In order to determine $\langle e^{-kt} \rangle$ for an arbitrary gating process the following steps are then necessary:

(1) Characterization of the CMS suspension. The reduction in the overall flux amplitudes $R_\infty(\alpha)$ following inhibition of gating units, measured under conditions where flux regulated by uninhibited gating units reaches completion, can be analyzed using eq. 47. This permits determination of the parameters P_n. Separate measurements are necessary to determine $Q(v)$ (see part III of this series [8]).

(2) Performance of the actual flux experiment using aliquots of the same suspension.

(3) Analysis of the resulting flux data, expressed as an overall relative amplitude change $R(t)$, using eqs. 5 and 6 with the previously determined P_n. Knowledge of $Q(v)$ permits evaluation of the volume-averaged terms $[\langle e^{-kt} \rangle^n]_v$ once the expression $\langle e^{-kt} \rangle$ appropriate to the gating process under investigation has been adopted [1]. The two limiting cases for the dependence of CMS size and content of gating units must be considered.

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