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Kinetics of pore-mediated release of marker molecules from liposomes or cells

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Abstract

We present a general mathematical treatment of marker efflux from liposomes or cells mediated by pore formation with the idea of using it in practice to obtain basic information about the underlying rates and mechanism. The approach encompasses permeation of molecules through any kind of pore-like defects in a cell membrane as they are induced by the action of some external agent. The approach broadens an earlier treatment to the more realistic general case in which a distribution of pore lifetimes must be taken into account. We derive a theoretical retention function describing the amount of marker remaining in the cells, formulated in terms of the pore activation and inactivation kinetics. The phenomenological efflux function evaluated directly from experimental data, is directly comparable with this retention function so long as the experimental signal is linearly related to the marker concentration. With the use of self-quenching dyes the relationship between signal and concentration is not, in general, linear, so that a more complicated treatment may be required. Even for these dyes, however, linearity holds under the frequently encountered condition of "all-or-none" release of dye from vesicles, a condition that can itself be verified experimentally.

Keywords: Channel gating; Lipid bilayer permeability; Membrane-active substances

1. Introduction

The appearance of pores or pore-like defects in the membranes of liposomes or cells can be probed by observing the concomitant efflux of an entrapped marker substance. This will be of particular interest for studies of pore formation induced by some membrane active agent or by the effect of certain external conditions, e.g. voltage or pH. Using various kinds of appropriate marker material (for instance self-quenching fluorescent dyes) the method has become quite popular in practice. So far, however, little has been done to investigate the measured rate of marker release with regard to a possible evaluation of the underlying activation and inactivation kinetics of pores. In the present article we shall discuss the necessary theoretical basis for a pertinent analysis of the experimental data in terms of mechanism, i.e. individual reaction steps and their rate laws.

Recently we have already dealt with the special case of an "all-or-none" depletion of liposomes (cells) [1]. Such a situation, implying a fast

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release through a sufficiently long-lived pore, appears to occur quite frequently among relevant systems, especially if small unilamellar lipid vesicles are used. Under these circumstances one obtains information about the opening of the first pore in any given liposome. To be more general, however, the previous approach needs an extension including slower release and premature pore closing. These aspects are naturally expected to gain in significance when the involved cells (liposomes) are fairly large.

2. Theoretical principles

2.1 Release through a single pore

In our context a cell is primarily considered as some domain of (possibly complex) solution being enclosed by a basically impermeable membrane. This comprises liposomes and particularly unilamellar vesicles as simple cell models. Pores are meant to be any kind of cell membrane-associated sites where the chosen marker can pass to a certain extent.

The flow of marker through an individual pore can be set proportional to the gradient between the effective marker concentrations inside and outside (denoted c_* and c, respectively). Accordingly

$$-\mathrm{d}n_*/\mathrm{d}t = \sigma_0(c_* - c) \tag{1a}$$

where n_* stands for the amount (i.e. number of moles) of marker inside and σ_0 measures the specific pore permeation coefficient. According to Fick's law of diffusion one obtains

$$\sigma_0 = A_\mathrm{p} D_0/d \tag{1b}$$

(A_p: pore cross-section, D_0: apparent marker diffusion coefficient upon passing the pore, d: pore length) under the condition that sufficiently fast equilibration of c_* and c occurs (which ordinarily holds true [1]). In the case of marker ions carrying z elementary charges, the permeation is related to the corresponding pore conductance g_0 at marker concentration c_0 by the relation

$$\sigma_0 = RT(g_0/c_0)(zF)^2 \tag{1c}$$

(R: gas constant, T: absolute temperature, F: Faraday constant).

We now introduce the internal cell volume $V_* = \frac{4}{3}\pi R_*^3$ where R_* defines an equivalent cell radius applicable to a possibly non-spherical shape. Having set $n_* = V_* c_*$ and observing that c remains practically constant (relative to c_*) eq. (1a) readily leads to

$$c_* - c = (c_{*0} - c)\exp(-t/\tau_0) \tag{2a}$$

involving a relaxation time

$$\tau_0 = \tfrac{4}{3}\pi R_*^3/\sigma_0 \tag{2b}$$

which characterizes single pore marker release. For a small unilamellar vesicle and a water-filled channel this falls in the time range of milliseconds and below [1,2]. Substantially longer τ_0 must be expected for larger cells (note the factor R_*^3) and/or pores of highly reduced permeability.

2.2 The retention function

Let us envisage a large number of like and equally sized cells. All pores are assumed to be equivalent and independent. According to eq. (2a) the amount of marker retained in a cell where one pore had been active (i.e. stayed open) for some period τ is reduced by an individual "retention factor" $\exp(-\tau/\tau_0)$. Those pores which had been active within a certain measuring time range t are generally subject to a statistical distribution of possible τ ($0 < \tau \le t$) which is to be described by the function $\omega(t, \tau)$. It implies that the probability of a pore having a period of activity with a duration between τ and $\tau + \mathrm{d}\tau$ is $\omega\mathrm{d}\tau$. The average retention factor of cells infected by only one pore then becomes

$$\rho(t) = \int_0^t \exp(-\tau/\tau_0)\,\omega(t, \tau)\,\mathrm{d}\tau \tag{3}$$

Since any two pores in the same cell membrane act independently their retention factors can simply be multiplied by each other. Hence the average retention factor of cells with n pores is generally equal to the nth power of $\rho(t)$.

Pores are Poisson distributed among the given cells. If an average of p pores per cell had been active, the probability of a cell with n pores can accordingly be written as

$$w_n = (p^n/n!)\ \mathrm{e}^{-p} \tag{4}$$

So we finally arrive at the retention function of the whole system, namely

$$R(t) = \sum_n w_n \rho^n = \mathrm{e}^{-p(1-\rho)} \tag{5}$$

describing the fraction of marker still retained in the cells at a measuring time t. Note that p as well as ρ are functions of t. They are determined by the rate laws of the pore kinetics.

2.3 Rate laws and pore characteristics

The concentration of membrane associated molecules and aggregates is suitably expressed by a variable r which stands for numbers of molecules (or aggregates) per lipid molecule (considering the lipid to be the solvent [3]). In particular, r_p (i.e. the molar ratio of pores per lipid) will be the appropriate pore concentration variable. The net production rate of pores is then fundamentally formulated as

$$\mathrm{d}r_\mathrm{p}/\mathrm{d}t = v_\mathrm{a} - v_\mathrm{i}$$

with v_a and v_i being the rates of formation (i.e. activation) and closing (i.e. inactivation), respectively.

The average number of pores per cell activated within the measuring time can therefore be calculated according to

$$p(t) = \nu \int_0^t v_\mathrm{a}(t')\ \mathrm{d}t' \tag{7}$$

(ν: number of lipid molecules per cell). If v_a does not change in the time range under consideration [1], then $\nu v_\mathrm{a} = \hat{k}_\mathrm{a}$ is a constant and we simply obtain $p(t) = \hat{k}_\mathrm{a} t$.

Turning towards a calculation of $\rho(t)$ one apparently must have a detailed knowledge of the function $\omega(t, \tau)$. This can be achieved in the following way. First the inactivation rate law ($\mathrm{d}r_\mathrm{p}/\mathrm{d}t = v_\mathrm{i}$) is integrated in order to determine the probability $\alpha(\tau)$ that an originally open pore is still active after a time τ has elapsed. Let us now consider $p_\alpha(t, \tau)$, i.e. the number of pores whose periods of activity within the measuring time t are τ or longer. Naturally they must be born at a time $\leq t - \tau$. This makes a number of $p(t - \tau)$ per cell. The fraction of those pores staying alive then at least a time τ is $\alpha(\tau)$ so that $p_\alpha(t, \tau) = \alpha(\tau) p(t - \tau)$. Thus the number of pores with a period of activity between τ and $\tau + \mathrm{d}\tau$ is $p_\alpha(t, \tau) - p_\alpha(t, \tau + \mathrm{d}\tau) = -p'_\alpha(t, \tau)\ \mathrm{d}\tau$ (p'_α: derivative of p_α with regard to τ). Taking into account that the total number of all pores having any possible value of τ (i.e. $0 < \tau \leq t$) must be equal to $p(t)$, the $\omega(t, \tau)$ appearing in eq. (3) generally becomes $-p'_\alpha(t, \tau)/p(t)$. We give the more detailed formulation

$$\omega(t, \tau) = -\mathrm{d}[\alpha(\tau) p(t - \tau)/p(t)]/\mathrm{d}\tau \tag{8a}$$

which directly refers to the functions $p(t)$ and $\alpha(\tau)$ as they can be derived from the rate laws.

In view of eq. (8a) it eventually follows from eq. (3) that

$$\rho(t) = -\int_0^t \alpha(\tau)[p(t - \tau)/p(t)] \times \exp(-\tau/\tau_0)\ \mathrm{d}(\tau/\tau_0) \tag{8b}$$

If the measuring time appreciably exceeds the single pore efflux time (i.e. $t \gg \tau_0$) we may practically set $p(t - \tau)/p(t) = 1$ and accordingly simplify eq. (8b). On the other hand, $\alpha(\tau) = 1$ is applicable in the event of comparable long-lived pores (relative to τ_0). When both conditions apply simultaneously we arrive at $\rho(t) \to 0$ ["all-or-none" case].

Ordinarily inactivation of pores should be subject to a first order kinetics, i.e. $v_\mathrm{i} = k_\mathrm{i} r_\mathrm{p}$. This results in $\alpha(\tau) = \exp(-\tau/\tau_\alpha)$ where the reciprocal rate constant $1/k_\mathrm{i} = \tau_\alpha$ is identical with the average pore life time. Then eq. (8b) takes on the simpler form

$$\rho(t) = 1 - \int_0^t [p(t - \tau)/p(t)] \times \exp(-\tau/\tau_{0\alpha})\ \mathrm{d}\tau/\tau_0 \tag{9a}$$

the composite relaxation time $\tau_{0\alpha}$ being defined according to

$$1/\tau_{0\alpha} = 1/\tau_0 + 1/\tau_\alpha \tag{9b}$$

In the basic case of a time-independent activation rate, i.e. $p(t) = \hat{k}_a t$ (as mentioned above), one obtains

$$\rho(t) = 1 - (\tau_{0\alpha}/\tau_0)\phi(t/\tau_{0\alpha}) \tag{10a}$$

involving the special function

$$\phi(x) = [\exp(-x) - (1-x)]/x \tag{10b}$$

Note that $\phi(x) = x/2$ at $x \ll 1$ and gradually approaches unity upon increasing x as described by $\phi(x) = 1 - (1/x)$ once $x \gtrsim 4$.

3. Experimental aspects

3.1 The efflux function

The release of marker may be monitored measuring the time course of an appropriate physical signal F (e.g. some optical property of the system). We define a directly observable efflux function

$$E(t) = (F_\infty - F)/(F_\infty - F_0) \tag{11}$$

with F_0 and F_∞ standing for the F-values at the start of the experiment ($t = 0$) and at complete release, respectively.

Generally a pertinent signal is composed of two parts, $F_1(c)$ and $F_2(c_*)$, depending on the marker concentrations outside and inside the cells, respectively. A possible distribution of c_* values among the cells is to be taken into account by introducing a relevant function $f(c_*)$ which describes the fraction of cells with an internal marker concentration between c_* and $c_* + \mathrm{d}c_*$ as $f(c_*)\,\mathrm{d}c_*$. Then we have

$$F = F_1(c) + \int_0^{c_{*0}} F_2(c_*) f(c_*)\,\mathrm{d}c_* \tag{12}$$

(c_{*0}: initial value of c_*). In many cases of practical interest, the system can be chosen such that F_1 as well as F_2 are linear functions of the respective concentrations (examples are pH and metal sensitive electrodes, physical separation of the two phases followed by conventional analyses, external indicator complexes, etc.). Under such circumstances mass conservation of the marker implies that the total signal F will depend linearly on c. Consequently the measured efflux function must be equal to the theoretical retention function $R(t)$ of eq. (5). Plotting $-\ln E(t)$ versus t accordingly would reflect the time course of

$$p(t)[1 - \rho(t)].$$

3.2 The use of self-quenching fluorescent dyes

Fluorescence emission of certain dyes, e.g. carboxyfluorescein and calcein, is substantially quenched at high concentrations ($\gtrsim 10$ mM). When being released from liposomes, dilution of such a dye will give rise to an increased fluorescence signal [4]. This kind of marker release experiment has become rather popular. Regarding an analysis of the measured efflux function we must, however, consider that in this case $F_2(c_*)$ is *not* a linear function of concentration, in contrast to $F_1(c)$. Upon dilution of c_* into the total volume, resulting in a marker concentration c'_*, one can experimentally determine the quenching factor $q(c_*) = F_2(c_*)/F_1(c'_*)$ [5]. If this turns out to be invariant at all times during a release experiment, one can conclude that an "all-or-none" case exists [5].

Under "all-or-none" conditions any signal can be linearly related to the amount of marker released. In addition, the retention factor, ρ, is zero. Therefore $-\ln E(t)$ is simply equal to $p(t)$ [1].

In a situation where ρ is not zero, the problem of converting $E(t)$ into useful quantitative information about the underlying pore kinetics appears to be more complicated. Nevertheless it is possible to develop at least approximate solutions taking advantage of special features that are present in certain situations. Let us envisage, as an example, pores having very short lifetimes such that $\tau_\alpha \ll \tau_0$. According to eq. (10) we then have $1 - \rho = (\tau_\alpha/\tau_0) \ll 1$. Thus a large number of pores is needed to deplete a cell. This implies that approximately the same number of pores had so far been activated in any cell at a given time. In other words, c_* decreases everywhere at about the rate of the overall retention function $R(t)$. So one eventually obtains the relation

$$E(t)=R(t)\left[1-q(c_{*0}R(t))\right]/(1-q_0) \qquad (13)$$

where $q_0=q(c_{*0})$. This allows $R(t)=(\tau_\alpha/\tau_0)\,p(t)$ to be calculated from a measured value of $E(t)$—provided the quenching factor $q(c_*)$ has actually been determined in the relevant range of c_*.

4. Discussion

We note that marker release experiments are preferentially carried out using a measurable signal which is linearly related to the amount of marker released. As was pointed out above, this allows the data to be readily converted into a theoretical retention function expressed in terms of the pore kinetics. Let us briefly discuss the basic case involving a time-independent activation rate v_a and a first-order inactivation as already introduced before (see derivation of eq. 10). The pertinent efflux function ($E(t)=R(t)$) may be calculated according to

$$-\ln E(t)=k_a\left[t-\tau_{0\alpha}\left(1-\exp(-t/\tau_{0\alpha})\right)\right] \qquad (14)$$

with $k_a=(\tau_{0\alpha}/\tau_0)\nu v_a$. In fig. 1(A) we present the corresponding release of marker (as originally seen in the experiments) for some values of $k_a\tau_{0\alpha} > 1$. The curves apparently exhibit a sigmoidal shape with an initial lag phase, turning to run single exponentially later (k_a being the time constant). This implies cases where appreciable release of marker occurs in the time range $t \lesssim \tau_{0\alpha}$. Alternatively, if $k_a\tau_{0\alpha} \ll 1$, one has to wait until $t \gg \tau_{0\alpha}$ in order to see pronounced efflux. The lag phase practically disappears under such circumstances and the curves become virtually single exponential. Plotting the data semilogarithmically ($-\ln E(t)$ vs. t) as demonstrated in Fig. 1(B) has the advantage that a single exponential time course will be reflected in a straight line. The parameter $\tau_{0\alpha}$ may be linearly extrapolated on the time axis whereas the slope is equal to k_a (a procedure expected to be most feasible if $k_a\tau_{0\alpha}$ assumes values of the order of unity).

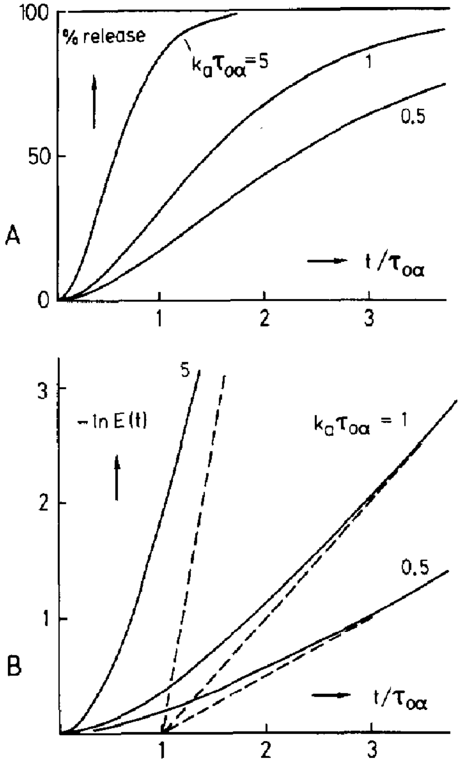

Fig. 1. Efflux functions according to eq. (14) describing the time course of marker release for a basic case of pore kinetics when a linear signal is employed. (A) Simulated signal, plotted as percentage of release for various values of $k_a\tau_{0\alpha} \gtrsim 1$. (B) Semi-logarithmic plot of the same curves. The dashed straight lines are extrapolated from the linear course approached at sufficiently advanced time.

Among the many reports on applications of the self-quenching fluorescent dye approach, straight-forward processing of data resulting in $p(t)=-\ln E(t)$ is most appropriate when the "all-or-none" conditions apply ($\tau_0 \ll \tau_\alpha$, t). This is likely with small unilamellar lipid vesicles (where τ_0 could well be below the millisecond range). Evidence for this has been given regarding the pore forming peptides alamethicin [1] and melittin (Popescu and Schwarz, unpublished results), as well as for some other agents [5–7]. A typical feature observed with all these systems is the substantial decrease of the slope of the $p(t)$

curve, which indicates a pronounced slowing down of the pore activation rate in the course of time. A detailed investigation of melittin behaviour is in progress (Zong and Schwarz, unpublished results). The present approach also appears promising for analysis of the action of bacterial toxins on lipid bilayers [8–11].

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