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# Reviews

## Single channel recording and gating function of ionic channels

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*Summary.* This review discusses several aspects of the kinetic analysis of the gating function of membrane channels, as carried out on single channel data obtained by the patch clamp method. In particular, the following three aspects of channel behavior are reviewed in some detail: 1) estimate of the number of states or conformations the channel can enter; 2) the lifetime of each state as a function of transmembrane voltage (voltage-gated channels), or of ligand concentration (ligand-gated channels); 3) estimates of the rates at which the transitions between the various states occur, and assessment of reaction mechanisms. An introductory description of conformational transitions of channel proteins is also provided.
*Key words.* Ionic channel; patch clamp; gating; single channel analysis.

### *Introduction*

Ionic channels are integral membrane proteins regulating the passive transmembrane flow of ions [12]. A large number of different channels that mediate these fluxes have been identified. Although channels may differ greatly in structure and function, they all share the distinctive feature of gating, the mechanism whereby a channel switches from its non-conducting (closed) to conducting (open) conformation. The switching (gating process) may be governed by several different mechanisms. Two such examples include: 1) the changes in membrane voltage that induce transitions from one configuration to another (voltage-gated channels); 2) a specific ligand that binds to the receptor site of the channel and causes it to open or to close (ligand-gated channels). Although in principle these two mechanisms are not mutually exclusive, in practice most channels use either one or the other of them. Only rarely do channels use both gating modes: The Ca-activated K channel is an example of this double sensitivity [2]. In this case, an increase of $Ca^{2+}$ concentration at the cytoplasmic side of the membrane activates the channel, and membrane potential, additionally, modulates it. Understanding the gating mechanism of an ionic channel primarily means to define: 1) the number of states or conformations the channel can enter; 2) the time spent in each state (lifetime) as a function of voltage (voltage-gated channels) or ligand concentration (ligand-gated channels); 3) the rates at which transitions between the various states occur, and the underlying reaction mechanism.

These aspects of gating have classically been studied by electrophysiological methods. These consist of measuring: 1) the macroscopic currents through the entire cell membrane, i.e., through a large number of channels (total currents) [13]; 2) the fluctuation of the macroscopic current around its mean value, due to the random opening and closing of individual channels (fluctuation analysis) [6, 20]; 3) the gating currents, due to the displacement of charges within the channel protein that may accompany the closed → open and the open → closed transitions of the channels [1, 16, 24]. Although these methods are still commonly and profitably used, the method of patch clamping, which allows the direct measurement of ionic currents flowing through a single open channel, has recently been introduced in the study of channel behavior (fig. 1a) [11, 19].

This review discusses how the information content of single channel data can give clues to the gating behavior of ionic channels. This follows a review of the various patch clamp configurations used to obtain single channel data, which appeared in an earlier issue [9]. The present review describes the first-step analysis of single channel data. It focuses on procedures for estimating the number of states a channel protein can enter, and rate constants of the transitions between these states. Use of chi-squared and maximum likelihood statistical analyses on single channel data is discussed as a means to determine appropriate reaction mechanisms. The discussion is preceded by a short description of conformational transitions of channel proteins. Advantages and disadvantages of the single-channel recording method, as compared to other electrophysiological methods, are discussed elsewhere [9, 21, 25].

### *Conformational transitions of membrane channel proteins*

#### *General*

Ionic channels are integral membrane proteins. Like all proteins, a channel protein may assume a large number of conformational states among which it spontaneously fluctuates [15]. The frequency of the fluctuations (i.e., the number of transitions per unit time) pertaining to different domains of the protein depends on the group considered, and covers several orders of magnitude. As summarized by Hille [12], methyl groups, for instance, make a half rotation on average every 10 ps, while amino acid side chains change conformation with a frequency ten times slower. The peptide backbone shows a frequency of motion of the order of nanoseconds. Since these fluctuation rates are several orders of magnitude faster than the transit time of an ion through the channel pore (10–200 ns), these groups may rearrange hundreds or thousands of times during the passage of the ion. These very fast conformational changes, therefore, do not appear to be relevant factors to the gating process, which instead seems to be linked to the much slower tertiary and quaternary conformational changes of the channel protein.

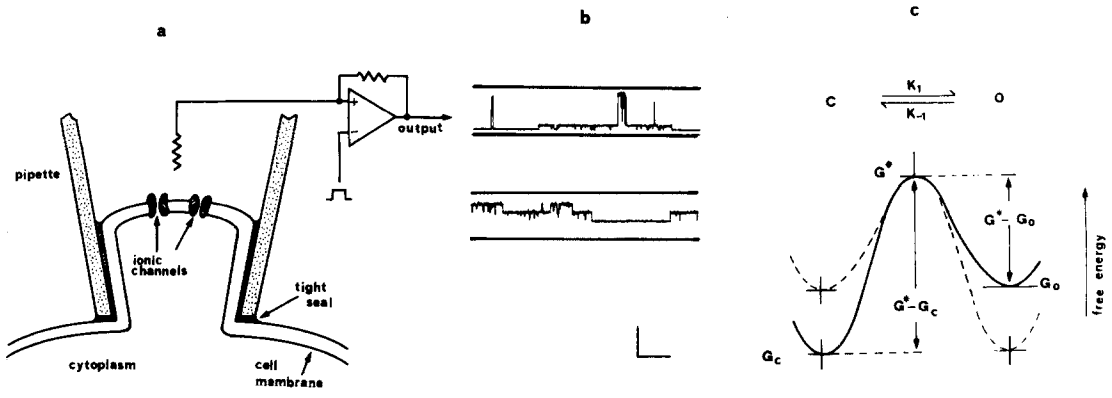


Figure 1. *a* Schematic drawing of the pipette-membrane arrangement, as obtained with the patch clamp technique, together with a simplified equivalent circuit. Two channels are shown in the patch membrane. Current flowing through the channels appears at the output, *b* in the typical square-wave signal of the single channel recording. The slow, 1 pA step is due to the $Cl^-$ ions going inward through anion-selective channels. The superimposed brief 8 pA step is instead the result of outward going $K^+$ ions moving through Ca-activated K channels. The record was taken in cell-attached configuration. Pipette solution was standard Ringer; holding potential was + 30 mV. Lower panel shows chloride currents through anion-selective channels. The clearly resolved three current levels indicate the presence of at least two channels in the patch. The membrane patch, in the outside-out configuration, separates symmetrical 140 mM CsCl solutions. Holding potential, + 60 mV. Calibration for both traces: horizontal, 25 ms; vertical, 8 pA. *c* Free energy profiles for ionic channel transitions in terms of energy barriers and wells. Solid line shows a hypothetical energy profile at hyperpolarized voltages, when the closed conformation has lower free energy, as compared to the open conformation, and the channel higher probability of being closed (see eqn 3). Broken line shows the energy profile at a depolarized voltage, when the situation is reversed.

As these major readjustments of the protein molecule occur in the frequency range of microseconds to milliseconds and seconds, the lifetimes of their conformational states are long enough to observe experimentally. On the basis of recording accessibility, they are referred to as macroscopic conformational states, as opposed to microscopic states which escape our experimental observation. Macroscopic states, the conformational states of the channel protein relevant to the gating and permeation processes, are therefore those referred to throughout.

*Thermodynamics of conformational transitions of channel proteins*

Let us consider a channel protein that fluctuates between the closed (C) and the open (O) state. In the common representation with energy profiles, the two states C and O, with free energies $G_c$ and $G_o$ respectively, correspond to energy wells separated by an energy barrier, the Gibbs free activation or transition energy, G* (fig. 1 c).

Suppose the channel is in the closed state. Because of the thermal energy, the channel molecule vibrates continuously with frequency $kT/h\ s^{-1}$ (at 20 °C, $6.11 \cdot 10^{12}\ s^{-1}$). (Here, k is Boltzmann's constant, h is Planck's constant, and T the absolute temperature.) This frequency can be thought of as the number of times the channel protein attempts, by stretching and bending its bonds, to exceed the Gibbs free energy barrier, and fall into the open state. The percent of successes in gaining the open state is only dependent (at T constant) on the height of the energy barrier, G*, separating the two wells. Transition rates between the two macroscopic states, as well as the relative probabilities of finding the channel protein in the closed or in the open state are quantitatively described by Eyring rate theory[8, 10, 18]. Briefly, the closed → open, and the open → closed transition rates, $k_1$ and $k_{-1}$ respectively, are exponential functions of the energy barrier to be overcome: $(G^* - G_c)$ for the closed → open transition, and $(G^* - G_o)$ for the open → closed transition.

$$k_1 = kT/h \exp[-(G^* - G_c)/kT] \quad (1)$$

$$k_{-1} = kT/h \exp[-(G^* - G_o)/kT] \quad (2)$$

The probability of finding, at equilibrium, the channel protein in the open state, $p_o$, is instead determined by the difference between the free energies of the two (closed and open) states, $(G_o - G_c)$. (Closed-state probability, $p_c$, is obviously the complement to that of $p_o$).

$$p_o = \langle 1 + \exp[(G_o - G_c)/kT] \rangle^{-1} \quad (3)$$

The lower the free energy associated with a given state, the higher the probability of finding the molecule in that state (that is, the longer the relative amount of time a channel spends in that state). Since the mean length of time a channel spends in a given state is the inverse of the rate constant leading away from that state, it follows that the two most relevant parameters characterizing the kinetics of an ionic channel (namely, occupancy of each state, and rate constant among states) depend on the free energies of its closed ($G_c$), open ($G_o$), and excited (G*) states.

In a thermodynamic system, the free energy associated with a given state is not a fixed value, but depends on the thermodynamic parameters such as pressure (P) and temperature (T), and on the electric field or voltage (E), provided the transition between the two states occurs with a variation of volume, a change in temperature, or a displacement of charge, respectively. Displacement of charge as a result of spatial reorientation of charged groups and dipoles within the channel protein, which may accompany the closed → open and the open → closed transitions has been shown to take place in the voltage-dependent Na channel, and is assumed to occur in all voltage-gated channels. Therefore, as long as pressure and temperature are kept constant, the electric field applied across the membrane is the only parameter governing the equilibrium distribution of open and closed states, as well as the transition rates between them. In general, at resting (negative) membrane potential, closed states reside at the lowest energy, and are thus the favorable states. Upon depolarization of the membrane, the open state(s) becomes the lowest in energy, thus favoring the transition of closed channels into their open conformation.

*Kinetic analysis of gating*

*General*

Since ionic channels are proteins that randomly undergo transitions between their various conformational states, their kinetic behavior can be described by the stochastic

analysis of random processes[4]. The simplest case is a channel that switches between one closed and one open state

$$\text{closed} \underset{k_{-1}}{\overset{k_1}{\Leftrightarrow}} \text{open} \qquad (4)$$

With reference to the thermodynamic treatment of channel transitions described above, the following assumptions may be made: 1) the channel exists either in the closed or in the open state, and the transitions between them are governed by a random process; 2) the probability that a given transition occurs is constant with time, i.e., it does not depend on the past history of the channel, thus the probability that an open channel closes within a given length of time is the same whether the channel has spent 1 or 1000 ms in the open state; 3) the rate constant of a given transition is only determined (T constant) by the probability of the transition occurring, which is constant with time, as shown above.

Two consequences arise from these assumptions. First, the lifetime of each state is a random variable. This means that on each occasion that a channel enters a given state, the lifetime is different for each excursion into that state, although the mean lifetime remains constant. Second, since the rate constant between two states represents the number of transitions per unit time, this parameter is linked to the mean lifetime of a given state. For the simplest reaction mechanism above, (4), the rate constant for leaving a state is the inverse of the mean lifetime of that state, i.e., the rate constant of the closed → open transition, $k_1$, is 1/(mean closed time), and the rate constant of the open → closed transition, $k_{-1}$, is 1/(mean open time).

An example of analysis which shows several features of the kinetic behavior of ionic channels follows.

*Mean open and mean closed time*

Since the switching of a channel from closed to open, or vice-versa, is always signalled by a discrete current jump (figs 1 b and 2 a), the measurement of the time the channel spends in the closed or in the open state is in principle unambiguous and relatively easy to do. One simply measures the duration between two successive current jumps (fig. 2 a).

In practice, however, the analysis is less simple and unambiguous. When a channel opens, a small current of the order of a picoampere flows through it. Because of the electrical background noise, the single channel current can be resolved only if the signal is subjected to low-pass filtering that excludes the high frequency component of the noise. Low-pass filtering, however, has the side effect of distorting the original square-wave signal typical of the single channel current. The distortion, due to the attenuation by the filter, makes the response follow the input current step due to single channel transition with some delay. While for long (as compared to the cut-off frequency of the filter setting) input current pulses, the output will only show exponential rising and decaying

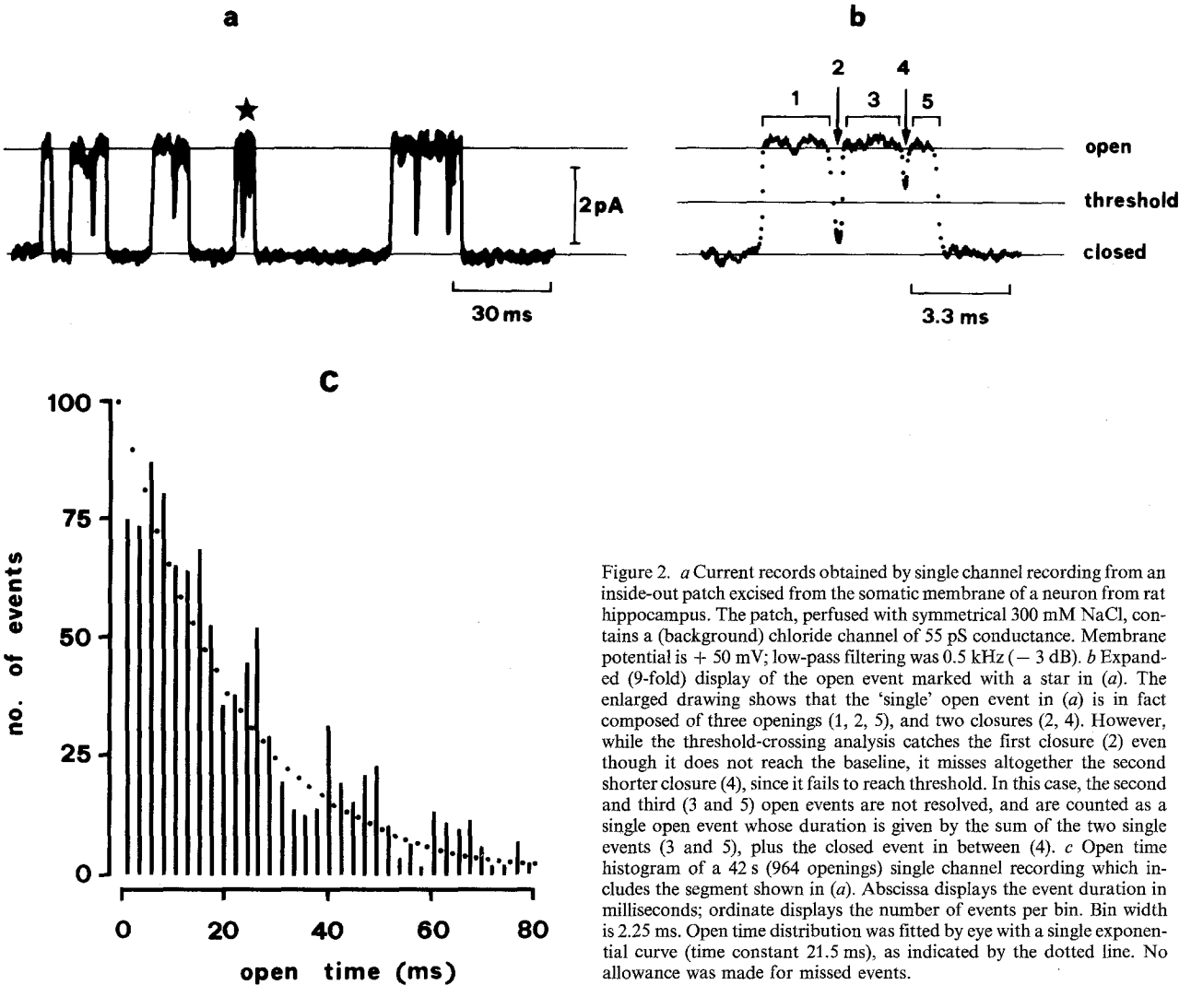


Figure 2. *a* Current records obtained by single channel recording from an inside-out patch excised from the somatic membrane of a neuron from rat hippocampus. The patch, perfused with symmetrical 300 mM NaCl, contains a (background) chloride channel of 55 pS conductance. Membrane potential is + 50 mV; low-pass filtering was 0.5 kHz (− 3 dB). *b* Expanded (9-fold) display of the open event marked with a star in (*a*). The enlarged drawing shows that the 'single' open event in (*a*) is in fact composed of three openings (1, 2, 5), and two closures (2, 4). However, while the threshold-crossing analysis catches the first closure (2) even though it does not reach the baseline, it misses altogether the second shorter closure (4), since it fails to reach threshold. In this case, the second and third (3 and 5) open events are not resolved, and are counted as a single open event whose duration is given by the sum of the two single events (3 and 5), plus the closed event in between (4). *c* Open time histogram of a 42 s (964 openings) single channel recording which includes the segment shown in (*a*). Abscissa displays the event duration in milliseconds; ordinate displays the number of events per bin. Bin width is 2.25 ms. Open time distribution was fitted by eye with a single exponential curve (time constant 21.5 ms), as indicated by the dotted line. No allowance was made for missed events.

time courses at the beginning and end of the pulse but no amplitude distortion, in the case of brief current pulses, the output may fail to reach full amplitude. The amplitude reached is a function both of pulse length and filter setting. Because of the large number of single channel events needed for statistical accuracy, essentially all methods developed to measure channel lifetimes make use of automatic computer processing of data. In most of these methods, an estimate of channel amplitude is used to set a threshold level, most commonly made equal to half amplitude of the single channel current (see Sachs et al. [23], for the convenience of using half-amplitude threshold). Every crossing of the threshold is interpreted as an opening or a closing of the channel. Times between two consecutive threshold crossings represent state lifetimes. This method clearly misses altogether brief events which, after low-pass filtering, do not reach threshold (fig. 2b).

The major consequence of missing events is the overestimate of state lifetimes (see legend to fig. 2b). For example, two (or more) consecutive closures separated by a brief (below threshold), missed opening are in fact counted as a single closure. Similar considerations apply to consecutive open events separated by missed closures. The overestimate of channel lifetimes due to missed events is usually serious, and appreciable errors in estimating underlying kinetic mechanisms are introduced. Recently, however, several possible methods have been proposed for correcting single channel data for missed events [3, 5, 22, 23].

Once the open times of an appropriate number of openings have been measured, the rate constant leaving the open state (i.e., the rate constant of the open → closed transition) can be derived from the mean open time as $k_{-1} = 1/(\text{mean open time})$. However, the mean open time is not calculated by ordinary statistics of summing all the open lifetime values and dividing by the number of events considered. In single channel analysis, event count versus lifetime histograms are used to calculate the mean lifetime, since this representation of data also provides information on the reaction mechanism, as will be seen later. In this type of plot, the vertical scale gives the number of events whose lifetimes fall in the corresponding time interval (bin) into which the abscissa has been divided (fig. 2c). In the case of a two-state mechanism, the open time distribution will be fitted by a single exponential curve (as expected for a stochastic process) whose time constant, $\tau_o$, gives the mean lifetime of the open state. Similar considerations apply to the closed state.

The finding that channel lifetimes are distributed exponentially, with short lifetimes occurring more frequently than long ones, may appear to contradict one of the basic assumptions of channel kinetics; transition probability is invariant with time. A thermodynamic-statistical approach is presented to show the physical basis for the exponential distribution of state lifetimes. In order to gain the open conformation, a closed channel must overcome an energy barrier (fig. 1c and related section). A channel protein makes an attempt to jump over the energy barrier about every picosecond (more precisely, every $1/(kT/h)$ s, i.e., every $0.16 \cdot 10^{-12}$ s, on average). Considering that lifetimes of channel states (those that can be measured) are of the order of milliseconds to seconds, it follows that a channel in the closed state makes on average up to $10^{10}-10^{13}$ unsuccessful attempts before it opens. If each attempt to exceed the energy barrier is considered as a binomial trial, binomial statistics for discrete variables can be applied. Since the probability of opening, $p_o$, is very low, and the number of trials, n, correspondingly high, the more convenient Poisson distribution can be used. (This is justified because the Poisson distribution can be derived as the limiting case of the binomial distribution when n approaches infinity and $p_o$ approaches zero.) According to the Poisson distribution, the probability of failing (not opening) to exceed the energy barrier in a given time is $P_{(\text{failure})} = \exp(k_1 t)$, where t is time, and $k_1$ is the ordinary rate constant for the closed → open transition, in this instance to be viewed as the mean number of successes in unit time. The probability of having no success (no transitions) throughout the time from 0 to t is then the probability that the lifetime of that state is greater than t, thus giving an exponential distribution for channel lifetimes.

*Rate constants and reaction mechanisms*

So far the reaction mechanism of the most simple ionic channel has been considered: a channel with a single closed and a single open state. However, virtually all ionic channels so far described have more than two states. In general, channels show several closed states, and often more than one open state. Therefore, to examine a more realistic (although still rather simple) situation, let us consider a channel with two closed states and one open state. One possible reaction mechanism is

$$C_1 \Leftrightarrow O \Leftrightarrow C_2 \qquad (5)$$

In this case, the open state separates the two closed states, $C_1$ and $C_2$. Although the two closed states are experimentally indistinguishable (i.e., it is not possible by simply looking at the current trace to say which of the two closed states a given closed episode belongs to), this particular reaction mechanism is relatively easy to study. Since the two closed states communicate only by passing through the open state, each closed episode must represent a single sojourn either into the closed state $C_1$, or into the closed state $C_2$. Moreover, as the two states are thermodynamically distinct, the lifetimes of the two closed states, $C_1$ and $C_2$, will be distributed exponentially. Thus the total distribution of closed lifetimes will be a mixture of the two exponential distributions, in proportion determined by the frequency of sojourns in the closed states $C_1$ and $C_2$. If the individual components of the double-exponential distribution have sufficiently well-separated time constants (usually, a factor of four or more), the two individual distributions will be unambiguously resolved.

A channel with one open and two closed states may, however, have a different reaction mechanism. For instance, in the following mechanism

$$C_1 \Leftrightarrow C_2 \Leftrightarrow O \qquad (6)$$

the two closed states communicate. In this case, the interpretation of experimental data is somewhat more complex. As in the previous case, the closed states $C_1$ and $C_2$ are experimentally indistinguishable. Moreover, with such a reaction mechanism, a closed episode can be a single transition into $C_2$ (an open channel closes into $C_2$, then reopens; $O-C_2-O$), but could also be a compound sojourn in $C_2$ (twice), plus a sojourn in $C_1$ ($O-C_2-C_1-C_2-O$), or a compound sojourn in any combination of the two closed states between which the channel could make any even number of transitions before reopening. In this case, the overall distribution of closed times would be a mixture of all possible combinations of compound states. (A compound state refers in this case to multiple transitions between the closed states which are undetectable electrophysiologically.)

Although in principle the theoretically infinite number of possible compound states could generate a complex distribution of closed times, in practice such a reaction mechanism often produces only two exponential distributions of closed times. The distribution with shorter mean closed time would arise from sojourns in the closed state $C_2$ ($O-C_2-O$), and the distribution with longer mean closed time would arise mainly from sojourns in the compound state $C_2 \cdot C_1 \cdot C_2$, as a result of the transition $O-C_2-C_1-C_2-O$. Sojourns in more complex compound states are less frequent, and

seldom contribute to the overall distribution with distinct exponential components. (Compound states are discussed thoroughly by Colquhoun and Hawkes[4].)

So far, a reaction mechanism has been considered, and the expected open and closed time distributions derived from it. In ordinary experimental practice, however, the situation is reversed. From given distributions of closed and open times one has to estimate the number of closed and open states, as well as to envisage a reaction mechanism that could account for the channel gating behavior. As a rule, the number of single exponentials one can fit onto closed and open time distributions gives the minimum number of closed and open states the channel can enter. Proposing a reaction mechanism is less straightforward, since it must also account for the voltage or ligand concentration dependence.

An example of this type of analysis follows. Magleby and Pallotta[17] studied the kinetic behavior of the Ca-activated K channel. They found that the closed time distribution could be fitted by the sum of three exponentials, and the open time distribution by the sum of two exponentials, thus suggesting three closed and two open states. On the basis of the effect produced by changes of Ca concentration at the intracellular side of the membrane, $[Ca^{2+}]_i$, the following reaction mechanism was suggested

$$\begin{array}{ccc} R \Leftrightarrow CaR & \Leftrightarrow & Ca_2R \\ \Updownarrow & & \Updownarrow \\ CaR^* & \Leftrightarrow & Ca_2R^* \end{array} \qquad (7)$$

Here R represents closed states, and R* open states of the Ca-activated K channel. By measuring the lifetimes of the various states the channel can enter, the authors found that the mean lifetimes were differently sensitive to $[Ca^{2+}]_i$. A long mean closed time was shown to be a logarithmic function of $[Ca^{2+}]_i$, whereas a long mean open time was a linear function of $[Ca^{2+}]_i$. The other lifetimes were, instead, insensitive to $[Ca^{2+}]_i$. Another feature of the Ca-activated K channel is that although it shows intrinsic voltage sensitivity, it can only be gated into the open state in the presence of Ca ions ($[Ca^{2+}]_i > 10^{-8}$ M). These observations suggest that the short open distribution results from opening to CaR* following the binding of one Ca ion, and the long open distribution results from openings to $Ca_2R^*$ following the binding of two Ca ions (cf. reaction mechanism (7)).

To assess whether a proposed complex reaction mechanism and the attached rate constants are quantitatively consistent with the kinetic behavior of a given channel, mathematical expressions have been developed, that relate a set of rate constants attached to a given reaction mechanism and the expected distribution of open and closed times. The basic idea is to define a measure of goodness of fit of the theoretical distribution to the observed data. Different sets of rate constants associated with various reaction mechanisms will give different degrees of goodness of fit to the experimentally observed distribution. The principle is therefore to allow the parameters (rate constants and reaction mechanisms) to take any possible value, and see which set maximizes the goodness of fit. This set of parameters and the associated reaction mechanism will be taken as the most likely.

Two methods for optimizing this type of analysis make use of the statistical chi-squared function, $X^2$, which provides a measure of badness of fit, and of the likelihood function which gives a measure of the goodness of fit. Thus, the values of the parameters are adjusted by optimizing computer programs so as to minimize $X^2$, or to maximize the likelihood function. Both methods are commonly used and give similar results when applied to the appropriate amount of data. A sound discussion of this problem, and criteria for choosing between these methods, can be found in Dahiya[7], Colquhoun and Sigworth[5], and Horn and Lange[14].

*Conclusions*

A few aspects of the kinetic behavior of ionic channels as elucidated by analysis of single channel data have been discussed. After an introductory description of the biophysical meaning and interpretation of channel states and conformational transitions of channel proteins, the discussion has focused on procedures for estimating the number of states a channel can enter, and the rate constants of the transitions from the lifetimes of open and closed states. The use of statistical analyses on single channel data, namely the chi-squared function and the maximum likelihood function, have been mentioned to show how conclusions can be drawn about reaction mechanisms of ionic channels, and what these conclusions are.

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# Casein, a prohormone with an immunomodulating role for the newborn?

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*Summary.* Maternal colostrum and milk, the earliest food of the newborn, should not only be considered as supplying nutrients, but also as agents providing protection against aggressions from the new environment. Indeed by enzymatic digestion of the main milk proteins, the caseins, biologically active peptides are released; they may be implicated in the stimulation of the newborn's immune system. From this point of view a 'strategic active zone' has been characterized in β-casein. A possible role of casein as a 'prohormone' for the newborn is suggested.
*Key words.* Casein; immunomodulation; casomorphin; immunomodulators.

After birth, the newborn enters into a temporary period during which its immature immune system still depends on maternal help, particularly when host defenses are considered. Its polymorphonuclear leukocytes (PMNL) have a decreased chemotaxis function and deformability of their membranes, and show both depressed oxidative metabolic responsiveness and lower bactericidal activity, compared to adult PMNL[37,38]. The percentage of T lymphocytes is significantly lower in the first days after birth in healthy neonates compared to adult controls[51]. Even though the number of suppressor T cells has been reported to be significantly lower in cord blood compared to adult blood, with a corresponding increase in the proportion of helper T cells[19,57], global helper function manifested by newborn T cells is low compared to their suppressive function[18]; newborn B cells differentiate into plasmocytes in response to pokeweed mitogen stimulation less well than adult B cells and synthesize exclusively IgM[18]. Nevertheless, the newborn must protect himself from the aggressions of its new environment, and breast-feeding facilitates transmission of a passive immunity.

Multifunctional factors contained in the maternal colostrum and milk have a direct effect on the newborn's resistance to bacterial and viral infections and on the harmonious development of the bacterial flora of the gut. High levels of immunoglobulins are present, mainly secretory IgA (sIgA) but also IgM and IgG[14]. Enzymes such as lysozyme (EC 3.2.1.17)[1,24] and peroxidase (EC 1.11.1.7)[15] and iron-binding proteins such as lactoferrin and transferrin[23] play important bactericidal and bacteriostatic roles. Other factors are of cellular nature, such as macrophages, granulocytes, T and B lymphocytes[21,42]; they promote humoral and cellular immunity against enteric bacteria (like *E. coli*) and favor indirectly growth of *Bifidobacterium bifidum* type IV which is able to transform lactose into glucose and lactic acid.

Meanwhile the newborn immune system must try to establish its autonomy. We suggest that its evolution may also be influenced by milk components or their degradation products. The most abundant milk protein, casein, has been found to generate, during enzymatic digestion, short peptides endowed with biological activities. We wish to discuss their possible involvement with the immune system.

Casein consists of several proteins, $\alpha s_1$-, $\alpha s_2$-, β- and κ-caseins which are associated into micelles in milk; its coagulation is triggered by the action of chymosin on κ-casein. Bovine casein contains 45% α-casein, 30% β-casein and 15% κ-casein; human casein which contains 20% κ-casein differs by its high level of β-casein (50%) in contrast with its low content of α-casein (10%)[10].

## *Immunostimulating casein peptides*

Assuming that the first food of the newborn might contribute to its natural immunostimulation thanks to peptides from milk proteins, we decided to submit tryptic or chymotryptic fragments of human caseins to some tests of biological activity. We chose two in vitro screening tests: phagocytosis of opsonized sheep red blood cells (SRBC) by resident peritoneal mouse macrophages and secretion of hemolytic antibodies by spleen cells from mice which have been immunized in vivo by SRBC, and also an in vivo test: protection of mice against *Klebsiella pneumoniae* infection. Several fractions were found to be active in these tests[25] and we purified two peptides, an hexapeptide Val-Glu-Pro-Ile-Pro-Tyr (residues 54–59 of β-casein)[44] and a tripeptide Gly-Leu-Phe, not yet located in the known sequences of human caseins[5]. These peptides stimulated phagocytosis of mouse macrophages at a concentration as low as 0.1 μM (table 1) and exerted in mice a protective effect against *Kl. pneumoniae* infection when injected intravenously at 0.3 and 1 mg/kg, 24 h before lethal infectious challenge (table 2). An analogue of the tripeptide, Gly-Phe-Leu (residues 60–63 of human β-casein, just following the hexapeptide) exhibited weaker but significant activities. We also demonstrated that these peptides stimulated human macrophages to phagocytize senescent human red blood cells[12].

## *Other biological active casein peptides implicated in immunomodulation*

### *Inhibitors of angiotensin I – converting enzyme*

Angiotensin I-converting enzyme (ACE, EC 3.4.15.1) catalyzes the production of the vasoconstrictor angiotensin II as well as the inactivation of the vasodilatator bradykinin and of the enkephalins in the guinea pig ileum[3]. Inhibitors of this enzyme might increase bradykinin and enkephalin activities. Bradykinin, known as a mediator of the acute inflammatory process, is able to stimulate macrophages, to enhance lymphocyte migration and to induce the secretion of lymphokines from lymphocyte cultures[43]. Furthermore, the