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Brief closures of gramicidin A channels in lipid bilayer membranes

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Brief closures, so called flickers, gramicidin A channels were observed for glycerol monooleate/*n*-decane membranes for cesium chloride and hydrochloric acid solutions. The flickers, similar in nature to the flickers observed for physiological channels, were of the order of 1 ms and the interval between flickers was of the order of 50 ms. The flicker-duration and interval between flickers both decrease with voltage. The field dependence of the flickers is consistent with the hypothesis that the membrane forms a dimple when accomodating a dimer in the membrane and that the monomers, on breaking up, are associated over displacements of the order of 2 nm. For similar measurements for glycerol monoleate/hexadecane membranes only rare occurrences of flickers were observed. It is suggested that the flicker phenomenon is governed by the physical and chemical properties of the membrane and the influence of membrane thickness and interfacial free energy is emphasized.

Introduction

The ionic conductance of membrane channels formed by the hydrophobic polypeptide gramicidin A has been well characterized experimentally [1–4] and extensively studied on a theoretical [4–8] level. Less is known about the kinetics of the gating process, although there is a concensus that channel opening results from the head-to-head dimerization of gramicidin A monomers in the membrane [9]. The gramicidin channel is considered a model system for single-file channel conduction [4] and it may also serve well as a general model for channel gating [10].

More recently, efforts have been directed towards the investigation of the kinetics of physiological channels [11–16], and it has been suggested [14] that a common characteristic of these is the appearance of brief closures (so called flickers [15]), or bursts, of the open (conducting) state. This report concerns the observation of such flickers also for the 'model channel' gramicidin A, a feature particularly interesting in view of the alleged universality of the flicker behaviour of biological channels and pertinent to recent developments [17] in the understanding of gramicidin A dimerization-kinetics.

Methods

Single-channel events from glycerol monooleate *n*-decane lipid- (14 mg/ml) membranes were recorded for various salt concentrations and membrane-potentials. Measurements were performed at room-temperature (21°C). The lipid-membranes were formed on a hole (0.25 mm diameter) in a polypropylene-syringe as described previously [2]. Methanol, chloroform/methanol (2:1, v/v) and hexane were used for cleaning and the salts were proanalysi grade from Merck. The gramicidin A was kindly supplied by E. Neher from a batch prepared by H. Eibl.

To allow for the necessary time-resolution, the amplifier-head-stage current to voltage converter

(FMK 380) and the experimental chamber were completely enclosed in a Farady cage during recordings. Due to stray capacitance (5 pF) and the large feedback-resistor (1000 and 100 MΩ for the low- and high-concentration measurements, respectively) the signal was frequency-compensated, a procedure commonly used for work with the patch-clamp technique. The signal was then limited to a bandwith of 5 kHz and stored on magnetic tape (HP-3968A). The playback (at 1/4 the speed) signal was further filtered, sampled at 100 μs intervals, averaged over 1 ms and stored on floppy disc. This enabled digitization of continuous 32 s recordings at an effective resolution of 250 μs and an 'off line' analysis. The recording and analysis was accomplished using microcomputers (Cromemco sys 3 and Metric Card) and a signal-graphic-processing package developed by the author.

Results

Fig. 1 shows typical single-channel recordings for the glycerol monooleate/decane membranes at the salt concentrations used. The dominant feature of these registrations is the appearance of the same kind of brief closures of the open channel as is observed for biological receptor channels [11–16]. The duration of the channels are of the order of one second, and the duration of the closed state is of the order of milliseconds.

With most channels the distinction between a regular closing (i.e. end of event) and a flicker inherently presumes that, at the end of event, the

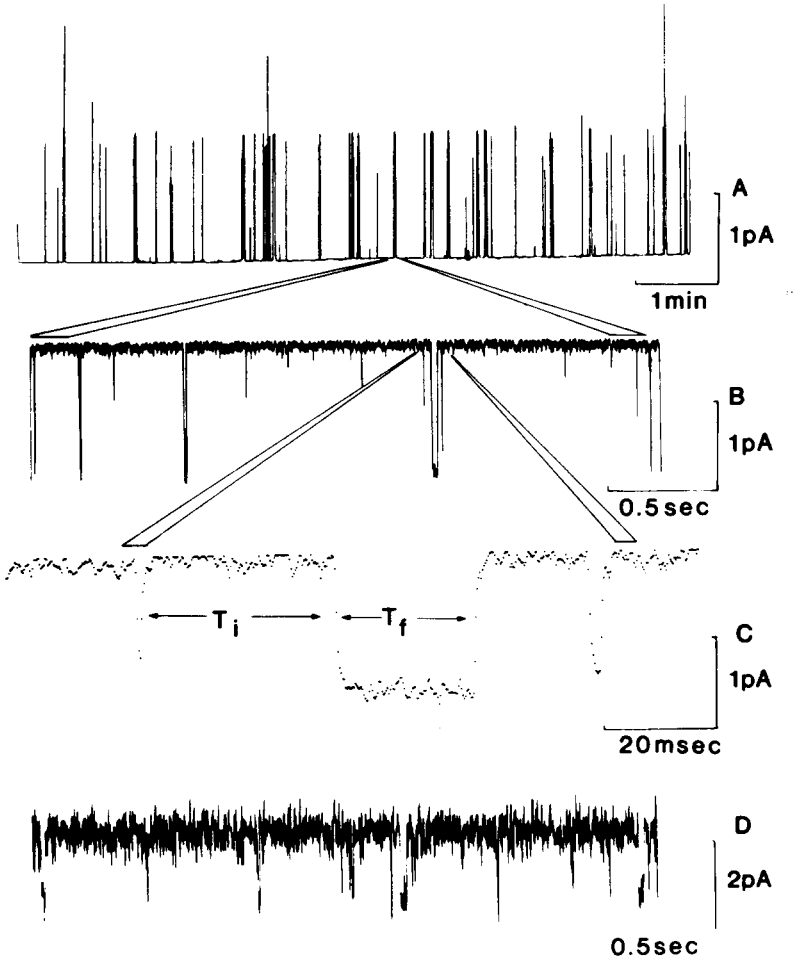

Fig. 1. Strip chart recording of single channels of gramicidin A (A) and digitized recordings (B–D) showing brief closures (flickers) of an open channel. (A) Each spike is a channel event, 100 mV, 0.2 M CsCl, 21°C. (B) The flickers observed during the channel event indicated. (C) The same event at increased time resolution showing an instant of three flickers and the effects of digitization and filtering. T_f indicates a flicker duration and T_i an interval between two successive flickers. (D) Flickers for the same experimental situation but with an applied potential of 20 mV. This is the worst recording (lowest potential and concentration) from the point of view of noise.

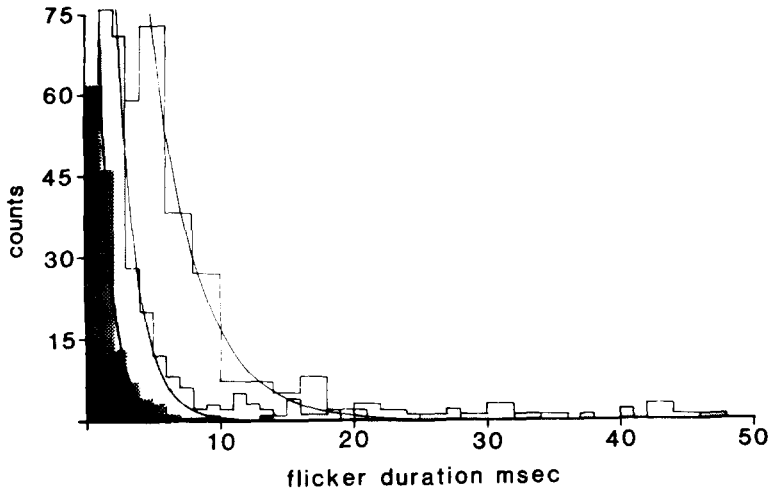

Fig. 2. Histograms of duration of flickers for the experimental situation of Fig. 1. White, grey and dark for applied voltages of 20, 50, and 100 mV, respectively. The curves indicate the best fit (least square, unweighted) exponentials. A 'tail' decaying more slowly than the exponential may be noted in the histograms.

channel makes a transition to a closed state, or class of states, and that the dwell time in this state is sufficiently longer than the average brief closing to be experimentally distinguishable from it. With a dimerization process, the average time between channel events is a quadratic function of the concentration of monomers and therefore not an intrinsic property of the channel. In the limit of very low gramicidin concentrations the measurement of flicker durations may therefore be well separated from the duration between channel events. In these recordings, the time between channel openings is three orders of magnitude (seconds) greater than what is here called flickers (milliseconds). The gramicidin concentration was also kept low enough to render unlikely the occurrence of superimposed channel events, which ensures a low probability for mistaking a new channel opening for a flicker.

The average flicker duration (T_f) for the closing-flickers is of the order of 0.1–5 ms and the interval between flickers (T_i) of the order of 50–100 ms. To obtain an objective quantitative measure of T_f and T_i, histograms of duration were compiled and the best-fit single exponential evaluated (Fig. 2). T_i was measured as the mean time-interval from the end of one flicker to the beginning of the next and also (with comparable results) from the exponential distribution. This value of T_i was then corrected for the estimated number of 'missing' flickers. The estimate for the number of missing flickers was obtained from the

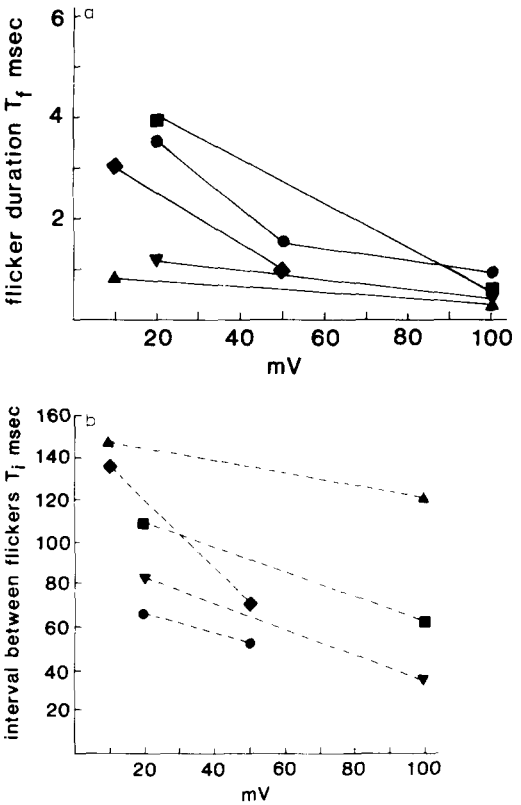

Fig. 3. Voltage dependence of flicker duration (a) and interval between flickers (b). ▼ ◆ and ▲, 0.5, 2 and 5 M HCl, respectively. ● and ■, 0.2 and 1 M CsCl, respectively. T_f and T_i are seen to decrease with voltage. Also, except for the extreme concentration of HCl (5 M) the flicker duration shows a tendency to increase with concentration. The number of events for each value of T_f was of the order of 200–300. The error in T_i is of the order of the correction due to missing flickers 50%.

best-fit exponentials of the T_f distribution. At low channel current levels, all flickers smaller than about 25% of the open-channel current-amplitude were discarded. This was imposed due to the limitations of bandwidth and noise (see Fig. 1).

Fig. 3 shows the dependence of T_f and T_i on voltage and both show a marked decreasing trend with voltage. Also, from Fig. 4, it is clear that since T_f/T_i decreases markedly, T_f has the greater voltage dependence.

For comparison, the average lifetime of channels (calculated from the autocorrelation function [18]) shows only a slight voltage dependence (Fig.5). In related experiments on glycerol monooleate/hexadecane membranes, for H^+ and high concentrations, a strong voltage dependence of channel-lifetime was observed [19]. However, since such a channel-lifetime voltage dependence is not found for the alkali cations at low concentrations whilst the voltage dependence of flickers is marked also for the lower concentrations of CsCl and HCl, it is not expected that this bears direct relevance to the flicker phenomenon.

Due to the limitations of bandwidth and noise, it was not possible to ascertain whether partial flickers (i.e. non-zero current) to subconducting states were present for the lower concentration registrations. However, except for the extreme concentrations of HCl (2 M, 5M) only solitary doubtful candidates for such transitions were noted. These, if present, were therefore rare enough not to significantly affect the evaluation of the average duration of complete flickers (i.e. to the closed state).

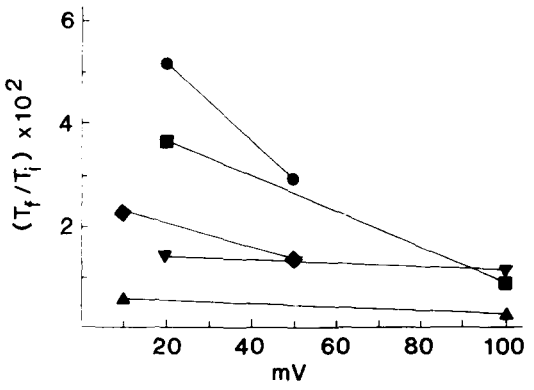

Fig. 4. T_f/T_i as a function of voltage. Symbols as for Fig. 3.

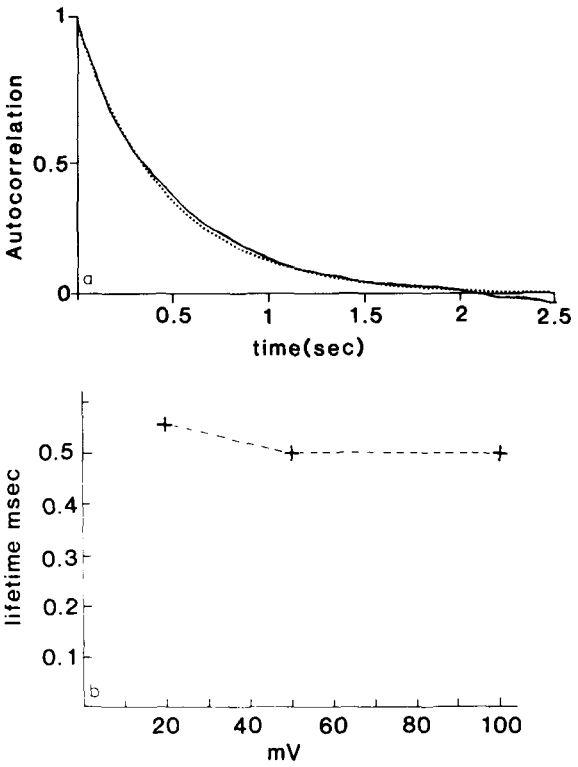

Fig. 5. The autocorrelation function (a) and the voltage dependence of open-channel lifetime (b) for the recording of Fig. 1 (and Fig. 2).

For 2 M and 5 M HCl, the channel events exhibit a wider range of characteristics, particularly in the conductance values, and in a few cases transitions between two well defined non-zero conductance levels were observed. These partial flickers could possibly involve transitions to the subconductance states recently described [20]. The channel events concerned were of a similar current amplitude and duration as the regular channel events, whilst the ordinary closing flickers were missing. The flickers were always directed from a high to a lower conductance level. This means that the channel spends most of the time in the most permeable state and suggests a correlation between stability and conductivity, a finding that fits well with the conclusions of recent work [18,19] dealing with the gramicidin-channel lifetime in general. In support of this is the observation that the distributions of gramicidin-channel conductance values generally show a sharp peak (i.e. high probability) near the maximum value for the con-

ductance and smaller peaks at lower conductances [20].

Discussion

The peculiarity of the flicker phenomenon for the gramicidin A channel is not likely to have passed unnoticed by others, but may be compared to that of the gramicidin subconductance states. As pointed out by Busath and Szabo [20], in the early work on gramicidin these miniature states were simply ignored. Also, experiments employing many-channel systems (for noise analysis) and excessive low pass filtering of single-channel recordings will clearly conceal the phenomenon. In some instances, despite low frequency cutoffs, gramicidin A flickers can be perceived in previously published sample recordings [20,21]. The recording of flickers from physiological channels on the other hand was provided for only through the advent of the high resolution patch-clamp technique [27].

With biological flickers, the channel kinetics is frequently modelled as a multiple-step process [22], and distributions fit with exponentials [12–15]. The existence of a slow component for the flickers presented here may be inferred from some of the individual distributions (e.g. see Fig. 2). The 'tail' of flicker durations longer than 20 ms are too numerous to be consistent with the assumption of a single-exponential process.

The lack of sufficient data did not permit a fit to a multi-exponential process for the individual histograms (i.e. corresponding to a certain concentration and voltage). An attempt at quantifying the slow decay was made by lumping the data and fitting with a double-exponential process. The data were therefore normalized using the value for the best fit lifetime obtained from the earlier single-exponential fits. In Fig. 6 a fit of two exponentials to the time scaled total of all the experiments clearly displays a second slower process.

The same data may be fit with a single process proportional to $1/T^x$ with $1.5 < x < 2$ (Fig. 7). Although unphysical for small T, since this would imply an infinite frequency of short duration flickers, this behaviour may be obtained over a limited frequency range (as is common for noise sources of electronic components) and it is similar to the

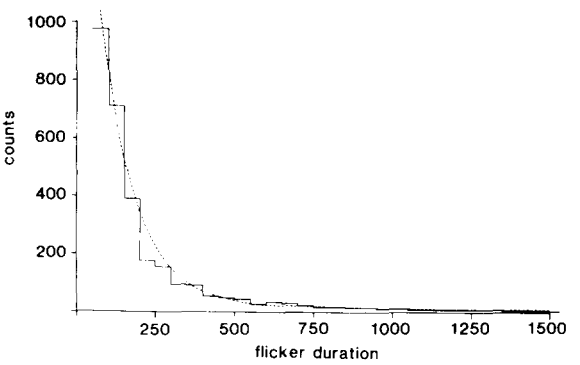

Fig. 6. Fit of two exponentials to the data. The flickers in each experimental situation of Fig. 3 have been normalized in time, using the evaluated time-constant of the exponential. The histogram has then been compiled from the total of all flickers, and a tail (visible also for some of the individual histograms, see Fig. 3) is clearly seen. The best fit here yields a time constant about 10-times larger than the fast component though this is an average for all the recordings.

$1/f$ type noise noted for the gramicidin open channel [23].

The question arises as to the origin of the gramicidin flickers and the possible relation to biological flickers. In fact, the framework for such a link has already been given by Haydon et al. [10,17,24] in a series of articles relating the action of general anaesthetics and the lifetime of the gramicidin channel to the thickness and tension of membranes. The 'dimple' [1] formed when coordinating a short channel in a thick membrane was suggested as the physical origin of the gramicidin-channel instability. The assumption is that (it is immaterial whether the 'loss of conduction' constitutes the breaking up of a dimer or some other pore destabilizing process) gating generally involves a conformational change which involves lipid rearrangement [10] and it is therefore modulated by changes in interfacial free energy [25].

The finding that gramicidin A also shows flickers and which also depend on lipid composition, reinforces this generalization. The dependence on lipid composition was not studied systematically, but a number of measurements were also made for glycerol monooleate/hexadecane membranes. Only scarce instances of flickers were found for glycerol monooleate/hexadecane systems and then only for extreme concentrations of HCl.

The detailed modelling of the flicker phenomenon in terms of molecular conformational changes is not possible on the basis of the data presented here alone and without reliable estimates of molecular interaction energies, since any general multi-step chemical process can be fit to this behaviour. As pointed out by Läuger et al. [26], channel formation could be a two-step process the first step being the formation of an inactive dimer. This conjecture was raised to explain the voltage dependence of the channel formation rate. The flickers would then, applying this model, result from a fast monomolecular transformation to a conductive dimer.

Also, a diffusion process towards a local energy minimum such as is expected to occur for the monomers in the glycerol monooleate/decane membrane can give rise to a flicker behaviour and this type of Markov process was easily simulated using a computer. Diffusion may be viewed as a multi-step process and, in the limit of lumping the steps, as a two-step process. The energy minimum could be due to the larger polarizability of gramicidin monomers as compared to lipid-molecules [28]. A monomer occupied with an ion would then lower the electrostatic energy on approaching a second gramicidin monomer. This effect has been proposed to account for gramicidin clustering at high ionic concentration levels [29] and is compatible with the observed increase of channel lifetime with increased ionic strength [1,18,29].

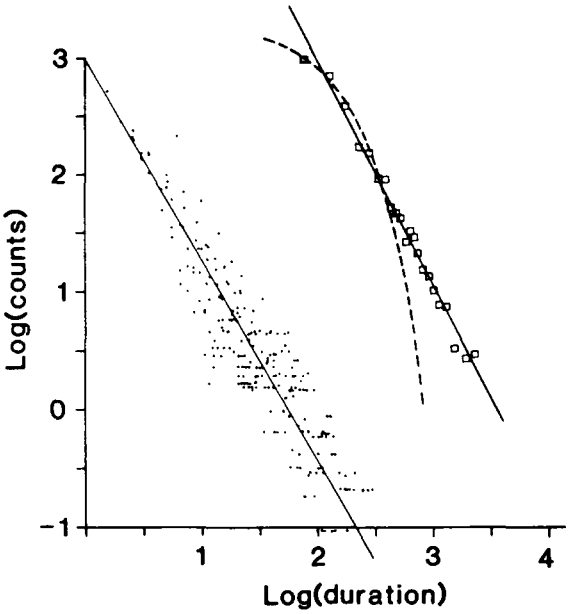

Fig. 7. The same data (squares) as those of Fig. 6. have been used to fit A/T^x giving $x = 1.7$. The logarithmic plot shows that the fit (continuous line) is excellent compared to the fit of a single exponential (dashed line). Also shown (dots), is the multiple regression of A_i/T^x, with the A_i individually varied for each individual histogram (experimental situation) and with x variable but common to all the registrations giving $x = 1.9$. Letting the A_i vary is equivalent to obtaining a best-fit time normalization of the data. For the graphical presentation, the data were then normalized by multiplying the counts of each bin with $1000/A_i$ (the A_i of the corresponding histogram). Each point therefore corresponds to the logarithm of the counts of one of the individual bins.

The conjecture of a multi-step process is of particular interest for the gating process for gramicidin in view of a recent study on gramicidin-channel lifetime [17], which concludes that the dimerized monomers, on separating, are associated over displacements (along the pore axis) of up to 1.8 nm as a consequence of the dimple. Since water is always present in the channel it may be expected for water molecules to bridge the gap between the monomers stabilizing this conformation. This model may be used to make specific predictions of the thickness- and voltage-dependence of the flicker process which may be experimentally tested.

For example, although the field effect on flickers may involve membrane- as well as channel-parameters [25,28,29], the change of the dissociation and redimerization rate constants may be estimated from Eyring rate theory. Considering a dimer with an ion in the gramicidin channel in either of the two internal sites, the flicker dissociation rate constant may be written as

$$K_D(U) = K_D(0)(\exp[(-d/h)(U/kT)] + \exp[(d/h)(U/kT)])/2 \quad (1)$$

where U is the membrane potential, d is the distance the monomers move upon separating, h is the thickness of the membrane, k is Boltzmans constant and T is the absolute temperature.

Using the estimate of 1.8 nm for d, a change from 20 to 100 mV across a 4.8 nm membrane implies an increase in rate constant of a factor of about 3.5 which is in general agreement with the results of Fig. 4.

Also since, in this model, the breaking up and redimerization will occur most frequently with an ion situated in the energetically most favourable position for each process (i.e. with the ion in the monomer on the 'far field' and 'near field' side, respectively), this also explains why both T_i and T_f decrease with increasing field, as may also be inferred from the symmetry of Eqn. 1.

The model also offers a tentative explanation of why flickers are predominant in glycerol monooleate/decane and not in glycerol monooleate/hexadecane membranes. For the alkane series it has been established that the membrane thickness decreases with increasing chain length. The thickness of a hexadecane membrane is close to that of a pure glycerol monooleate membrane whilst the glycerol monooleate/decane membranes, on the other hand, forms thick (4.8 nm) membranes, decane being the shortest alkane to form lipid bilayers with glycerol monooleate. Thus, the glycerol monooleate/hexadecane membrane is only slightly thicker than the length of the gramicidin A dimer (2.5–3 nm) and only a small dimpler is expected to form when coordinating the channel in this membrane. This means that the conditions for attaining a pseudo-stable closed-state (a flicker) for the glycerol monooleate/hexadecane membrane are not fulfilled for the model presented here.

The finding that the well characterized ionophore gramicidin A also shows flickers similar to the flickers found for complex biological channels may prove valuable to the interpretation and understanding of the kinetics of biological channels, irrespective of the fact that for gramicidin A the 'gating' involves a dimerization process. The interaction of the channel with the constituents of the lipid, the membrane potential and the electrolytic solutions involves common principles for proteins in membranes. Thus, Young and Poo [30] have shown that the acetylcholine-receptor-(AChR-) channel kinetics is influenced by the constituents of the environment. Also, the lifetime of the gramicidin A dimers have been shown [19] to be influenced by the ionic occupancy of the channel, similar to mechanisms that have been proposed for the AChR- [32,33], K-[34,35], Na-[36] and γ-aminobutyric acid-activated Cl-[37] channels.

The characterization of the flicker phenomenon for gramicidin A and the conditions for which it occurs should prove valuable in understanding the dimerization kinetics. Specific models may then be tested for the predictions of field-, surface-tension- and thickness-dependence of the flicker and lifetime parameters.

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