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Estimation of Rapid Kinetics of Formamide Block in Gramicidin A Channels

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ABSTRACT Blocking events in currents through biological ion channels occur over a wide range of characteristic times. The interruptions in single-channel currents from blocking events may by characterized by the direct measurement of gap durations or by analyzing open-channel current histograms, provided that the events are not much shorter than the time resolution of single-channel recordings (~10 μs). Here we present a method for the characterization of channel block on a much faster time scale by combining open-channel noise measurements with subsequent model fits according to a theoretical approach (Frehland, E. 1978. *Biophysical Chemistry.* 8:255–265). Although the bandwidth limitations in open-channel noise experiments are the same as in conventional single-channel experiments, from the dependence of the mean current and the spectral density of the noise on the concentration of the blocking agent, kinetics of very brief blocking events can be estimated. As an example we have analyzed the open-channel noise of K^+ currents through the gramicidin A channel in the presence of various concentrations of formamide, a weak blocker, at neutral pH. We estimate the blocking and unblocking rates to be $\sim 10^7 s^{-1}$ at 1 *M* formamide and discuss possible mechanisms for the blocking process.

INTRODUCTION

The binding kinetics of ligands that block ion channels have been studied by a variety of means. For ligands that block on the millisecond time scale, the blocking events may be measured directly by analyzing the gaps in recordings of single-channel currents (see, for example, Lansman et al., 1986) or the fluctuations in the current through populations of channels (for example, Li and Lindemann, 1983). Kinetics of faster blocking processes have been determined from the form of the amplitude distribution of single-channel currents; in this way Yellen (1984), using the theory of FitzHugh (1983), determined the time constants for the block of the Ca^{2+}-activated K^+ channel by Na^+ ions to be on the order of 100 μs. This method could also be applied for events which are an order of magnitude faster, but it would not give reliable estimates for time constants in the sub-microsecond range. We present here the analysis of a blocking process that occurs on a time scale of 100 ns, based on the measurement of excess fluctuations in the open-channel current. The blocker we have investigated is formamide, an uncharged molecule that blocks the gramicidin A (GA) channel with low affinity (Decker and Levitt, 1988).

Our analysis of fluctuations in single-channel currents is closely analogous to the earlier use of fluctuation analysis on recordings from populations of channels, but differs in two main aspects. First, since the fluctuations are very rapid, we cannot directly obtain information about the time scale of channel block from the corner frequency of the relevant Lorentzian component(s), expected to be on the order of 1 MHz. Instead, we make do with measurements of the spectral density within the 20 kHz bandwidth of our recording system. Second, the time scale of the blocking events is similar to that of the ion transport process itself; thus a proper analysis of the fluctuations should explicitly include the statistics of ion transport.

We start with a theory that describes the shot noise current fluctuations on the basis of a four-state kinetic scheme for ion transport in the GA channel, and then extend the theory to include kinetic transitions to blocked states. Estimates of the time scale of channel block are obtained through a comparison of the predictions of the theory with mean-current and spectral density data obtained at a variety of formamide concentrations. In principle there are many possible ways by which formamide can block the GA channel, and we have tested various models. Here we first consider a simplified theory involving

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one blocking site before going on to consider two models in detail. One of these assumes superficial binding sites for formamide outside the channel, as suggested by Decker and Levitt (1988); in the other model it is assumed that formamide can compete with K^+ for its binding sites within the channel.

METHODS

High-Resolution Recording from Micro-Bilayers

We used the experimental techniques for high-resolution recordings from micro-bilayers as previously described (Sigworth et al., 1987), and which we summarize briefly here. Lipids were spread onto the surface of the bath saline which filled a petridish. By passing the tip of a 1-mm teflon tube through this interface, spherical membrane bilayers could be formed. Lipid micro-bilayers were extracted from the larger bubble with borosilicate glass pipettes (Kimax), as commonly used for patch-clamp experiments. The pipettes (2–4 μm tip diameter) were coated with Sylgard$^{\mathrm{R}}$, and a treatment with dimethyl-dichlorosilane vapor immediately before the experiments assured the hydrophobicity of the pipette tips. Since the pipettes were free to move after the transfer of the lipids, they could be positioned such that only the tip was immersed into the bath solution, as is essential for low-noise experiments. Background noise levels of ~150 fA root mean square in the frequency band 0.3-3 kHz could be obtained.

The lipids were glycerol-monoolein (GMO) (Nu Chek Prep, Elysian, MN), 50 mg/ml in squalene or hexadecane. For noise experiments we preferred squalene as the solvent because it forms thinner bilayers than hexadecane or decane; the frequency of occurrence of brief (~10 μs) gaps, which must be masked out to avoid contamination of the power spectra, is much lower in the thinner membranes (Sigworth and Shenkel, 1988) while the open-times of the gramicidin channel are longer (Elliott et al., 1983). The Gramicidin A was a kind gift of Prof. D. Urry and was kept as a stock solution in ethanol.

In all of our experiments the bathing solutions consisted of either 640 or 500 m*M* KCl with a specified amount of formamide (Aldrich Chemical Co., Inc., Milwaukee, WI). We chose these KCl concentrations because they yield large single-channel currents (~8 pA at 200 mV), and because we observe a particularly low spectral density of ion transport noise with K^+ in this concentration range (Heinemann and Sigworth, 1988). The bath and pipette electrodes were Ag/AgCl wires. Currents were measured with an EPC-7 patch-clamp amplifier (List Medical, Darmstadt, FRG).

Data Storage and Analysis

Data recording and analysis were carried out as previously described (Sigworth et al., 1987). Briefly, data were digitized with a modified digital audio processor (PCM-501, Sony Corp. of America, Long Island City, NY) and stored digitally on a video tape at 44.1 kHz sampling rate. Although the analog signal was filtered with the patch clamp's 10 kHz, 3-pole Bessel filter, the actual limitation in bandwidth was the sampling rate of the storage device rather than the patch-clamp apparatus, because the spectra were corrected for the frequency response of the recording system.

For analysis on a PDP 11/73 (Digital Equipment Corp., Marlboro, MA) computer, the data were read continuously into a disk file at the full sampling rate, essentially as described by Bezanilla (1985). Sections of 3 min of raw data, filling up 16 MByte of memory, were processed at a time. After masking out disturbing flicker events with durations longer than ~15 μs (Sigworth, 1985), power spectra of the entire record were calculated in blocks of 4,096 points using an FFT algorithm. Portions of the digitized record corresponding to different channel states were then selected manually. Spectra of the open-channel noise were calculated by forming the difference between the open-channel spectrum and the baseline spectrum.

Noise Calculations from Kinetic Models

Let us first consider briefly the classical results for fluctuations from ion transport and blocking processes. The current through a channel is expected to have fluctuations due to the fact that a discrete charge movement occurs as each ion is transported. The simplest theory for these fluctuations is obtained by assuming a unidirectional flux in which the transport of each ion is accompanied by a single delta-function of current. Further, it is assumed that the ion transport events are independent, having no temporal correlations. In this case we expect the single-channel current i to be accompanied by shot noise fluctuations having the spectral density (Schottky, 1918)

$$S_{\mathrm{shot}} = 2\, e_0\, i, \qquad [1]$$

where e_0 is the unitary charge (1.609×10^{-19} c) in the case of a univalent charge carrier.

In the case of channel block, we make the usual assumption that the blocking is a first-order process, with rate of occurrence λ and lifetime τ. A rough estimate of the zero-frequency limit of the additional spectral density due to the current interruptions (e.g., Sigworth et al., 1987) is given by

$$S_{\mathrm{block}}\,(f = 0) \approx 4\,\lambda\,\tau^2\, i^2. \qquad [2]$$

This approximation holds if the blocking times are exponentially-distributed, represent complete closures of the channel, and cause only a small reduction in the mean single-channel current.

The assumptions behind Eqs. [1] and [2] do not hold in our experiments. It is known that substantial ion–ion interactions occur in GA channels (Andersen, 1984), so that ion transport events will show temporal correlations. Also, the transport of ions through the channel involves several different steps that can give rise to charge movements. Further, we are interested in a blocking process that could occur at both ends of the channel (i.e., two sites), that produces a substantial reduction in mean current, and which might show significant effects from correlations between blocking and ion transport events, which are on comparable time scales. For these reasons we use the theory of Frehland (1978) which predicts the noise spectrum for any discrete kinetic scheme of ion transport; this theory is readily extended to handle channel-blocking events by including additional kinetic states in the scheme to represent blocked channel configurations. Here we summarize the theory and describe our numerical implementation of the calculations.

Application of Frehland's Theory. Let us model the GA channel as a transport system of n discrete states. The transitions from state j to i, for example the movement of an ion from one site to another, occur with the rate m_{ij}. Around the steady-state solution of this dynamical system we can linearize the set of master equations giving the time development of the state occupancies n_i ($\langle n_i \rangle$ denotes the expectation value of n_i):

$$\frac{d\langle n_i\rangle}{dt} = \sum_{j=1}^{n} m_{ij}\langle n_j\rangle, \qquad [3]$$

where the steady-state solution $\bar{n}_j$ is obtained by solution of the linear equations:

$$\sum_{j=1}^{n} m_{ij}\bar{n}_j = 0. \qquad [4]$$

Now we consider the fluctuations α_i about $\bar{n}_i$:

$$\alpha_i = n_i - \bar{n}_i \qquad [5]$$

yielding the differential equations:

$$\frac{d\langle\alpha_i\rangle}{dt} = \sum_{j=1}^{n} m_{ij}\langle\alpha_j\rangle. \qquad [6]$$

The solutions of these equations under general initial conditions are expressed by the so-called fundamental matrix $\Omega(t)$, whose elements are defined by:

$$\omega_{ik}(t) = \langle\alpha_i(t)\rangle \qquad [7]$$

with the initial condition: $n_k(0) = 1$, $n_i(0) = 0$ for $i \neq k$.

(Here capital letters refer to matrices, and lower case to their elements.) The general solution for the expectation values of the α_i is therefore in matrix notation:

$$\langle A(t)\rangle = \Omega(t)\,N(0). \qquad [8]$$

With the flux ϕ_{ik} from state k to state i,

$$\begin{aligned}\langle\phi_{ik}(t)\rangle &= m_{ik}\langle n_k(t)\rangle \quad \text{for } i \neq k\\ \langle\phi_{ii}(t)\rangle &= 0\end{aligned} \qquad [9]$$

we can derive the expected fluctuation in flux under the initial condition that the channel is in state k

$$\begin{aligned}\langle\varphi_{ij}^{k}(t)\rangle &= m_{ij}\omega_{jk}(t)\\ &= m_{ij}\mathrm{Prob}\{j \text{ at } t \,|\, k \text{ at } t = 0\}.\end{aligned} \qquad [10]$$

The correlation of fluxes is expressed by a correlation matrix, which expresses the flux from state l to k, given that there was a transition from j to i at time zero:

$$c_{ij,kl}(t) = \langle\phi_{ij}(0)\,\phi_{kl}(t)\rangle - \langle\phi_{ij}\rangle\langle\phi_{kl}\rangle. \qquad [11]$$

Working out the ensemble average of the individual fluxes yields:

$$c_{ij,kl}(t) = m_{ij}\,\bar{n}_j[\delta_{ij,kl}\,\delta(t) + m_{kl}\,\omega_{li}(t)], \qquad [12]$$

where $\delta_{ij,kl}$ is the general Kronecker symbol and $\delta(t)$ Dirac's function. The first term in the brackets accounts for the correlations of transitions at $t = 0$ with themselves; the second one stems from the correlation of distinct transitions.

To obtain the corresponding electric currents, the fluxes are multiplied by the associated charge transfer of the corresponding transition. Introducing the antisymmetric charge matrix Γ, the observed current is

$$J = \sum_{i,j,=1}^{n}\gamma_{ij}\,\phi_{ij}. \qquad [13]$$

Together with the Fourier transform of [12] we obtain the noise spectrum of the observed electric current:

$$S_J(f) = \sum_{i,j,k,l=1}^{n}\gamma_{ij}\gamma_{kl}m_{ij}\bar{n}_j \times \left[2\delta_{ij,kl} + 4m_{kl}\int_0^{\infty}\omega_{li}(t)\cos(2\pi f t)\,dt\right]. \qquad [14]$$

Since we are only interested in the zero-frequency limit of the power spectra, we can simplify [14] by use of the following law (Rice, 1954):

$$\lim_{f\to 0}\left[\int_0^{\infty}\omega_{li}(\tau)\cos(2\pi f\tau)\,d\tau\right] = L\{\omega_{li}(\tau)\}_{\sigma=0}, \qquad [15]$$

where $L\{\ \}$ denotes the Laplace transform and σ the Laplace variable. Thus we can avoid calculating the integral in [14] explicitly if we can derive a simple expression for $L\{\Omega\}$. Imposing arbitrary initial conditions, [6] becomes:

$$\frac{d\langle A\rangle}{dt} = M\langle A\rangle + (N(0) - \bar{N})\,\delta(t). \qquad [16]$$

Laplace transformation of [16] yields:

$$\sigma L\{\langle A\rangle\} = M\,L\{\langle A\rangle\} + (N(0) - \bar{N}) \qquad [17]$$

or

$$L\{\langle A\rangle\} = (\sigma I - M)^{-1}(N(0) - \bar{N}),$$

where I is the unitary matrix. From the definition of Ω it follows:

$$L\{\Omega\} = (\sigma I - M)^{-1}. \qquad [18]$$

Thus we can solve Eqs. [14] and [15] without performing the time integration. The algebra is reduced to the inversion of $(\sigma I - M)$.

Numerical Implementation. The calculation of the mean current and spectral density for a specifc kinetic model starts with the transition matrix M, whose elements are simply the rate constants, and

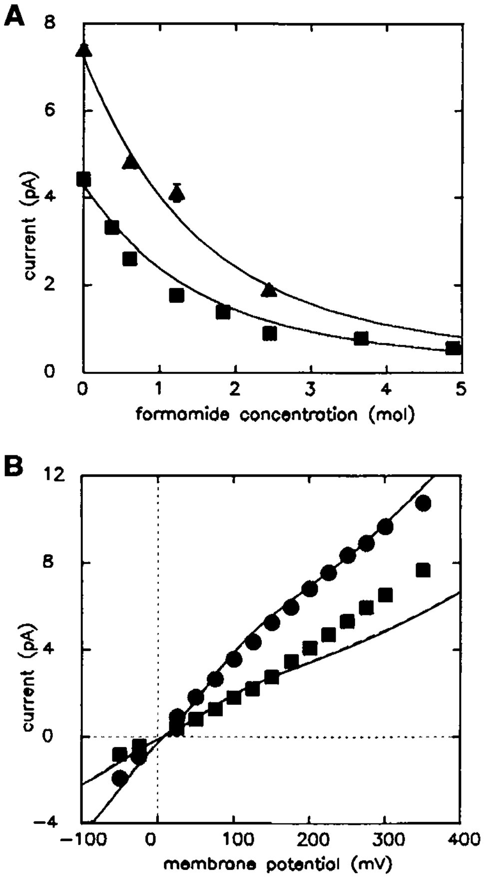

FIGURE 1 (*A*) Current through GA channels in GMO/hexadecane membranes in symmetrical 500 m*M* KCl solutions as a function of formamide concentration at 100 mV (*squares*) and 200 mV (*triangles*). (*B*) Current-voltage relationship of GA in GMO/hexadecane membranes with pure 500 m*M* KCl solutions (*circles*) and 500 m*M* KCl plus 1.2 *M* formamide (*squares*). The solid curves were obtained by a fit of the 16-state model (Fig. 4 *A*); the dashed curves represent a fit to the 9-state model (Fig. 4 *B*).

the charge matrix γ, which describes the charge movement corresponding to each possible state transition. The order of M is first reduced by using the normalization of probabilities,

$$\sum_{j=1}^{n} n_j = 1 \qquad [19]$$

to define a square matrix M' of the order n-1 and a vector C that satisfy

$$\sum_{j=1}^{n} m_{ij}\, n_j = \sum_{J=1}^{n-1} m'_{ij}\, n_j + c_i \qquad [20]$$

for $i = 1 \ldots n-1$. The inverse Q of M' is then used to find the steady-state occupancy vector given by [4], i.e., $\overline{N} = QC$

In most cases we are interested in the spectral density only at zero frequency, which means that we can set $\sigma = 0$ in Eq. [18]. The desired general solution matrix is then given directly by

$$L\{\Omega\} = -Q \qquad [21]$$

and the spectral density is evaluated from Eq. [14] as

$$S_J(0) = \sum_{i,j,k,l=1}^{n} \gamma_{ij}\gamma_{kl} m_{ij}\bar{n}_j \times [2\delta_{ij,kl} - 4m_{kl}q_{li}]. \qquad [22]$$

where:

$$q_{nj} = -\sum_{i=1}^{n-1} q_{ij}. \qquad [23]$$

This algorithm was implemented in FORTRAN on a VAXstation II (Digital Equipment Corp., Marlboro, MA). For a transition matrix of the order 16 the calculation of the single-channel current and its spectral density takes ~2 s using double-precision arithmetic. The data fits were done using a simplex minimization routine, imposing a least-square criterion.

RESULTS

Single gramicidin A channel events were recorded in symmetrical KCl solutions containing various concentrations of formamide. The formamide molecules, which are neutral at pH 7.0, block the K^+ currents (Fig. 1 *A*); the single-channel amplitude at 200 mV was reduced from ~8 to 3.3 pA by addition of 2.56 *M* formamide. For noise measurements this was the highest practical formamide concentration, because higher formamide concentrations yielded unstable recordings; however for the estimation of the single-channel conductance alone (at 100 mV) we could increase the formamide concentration to ~5 *M*, which reduced the current to 15% of control (lower curve in Fig. 1 *A*). Above 5 M formamide stable bilayers could not be formed. In Fig. 1 *B* the current-voltage relationships (*i*-V) of K^+ currents without formamide and in the presence of 1.2 *M* formamide are compared. With formamide the *i*-V curve tends to be more superlinear.

Formamide increased the open-channel noise, as can be seen in Fig. 2, which compares the raw current traces, raw power spectra of baseline and open-channel currents, and the difference spectra obtained in symmetrical solutions containing no formamide (*left panel*) and 640 m*M* formamide (*right panel*). GMO/squalene membranes were used for these and all other noise data shown here. It is seen

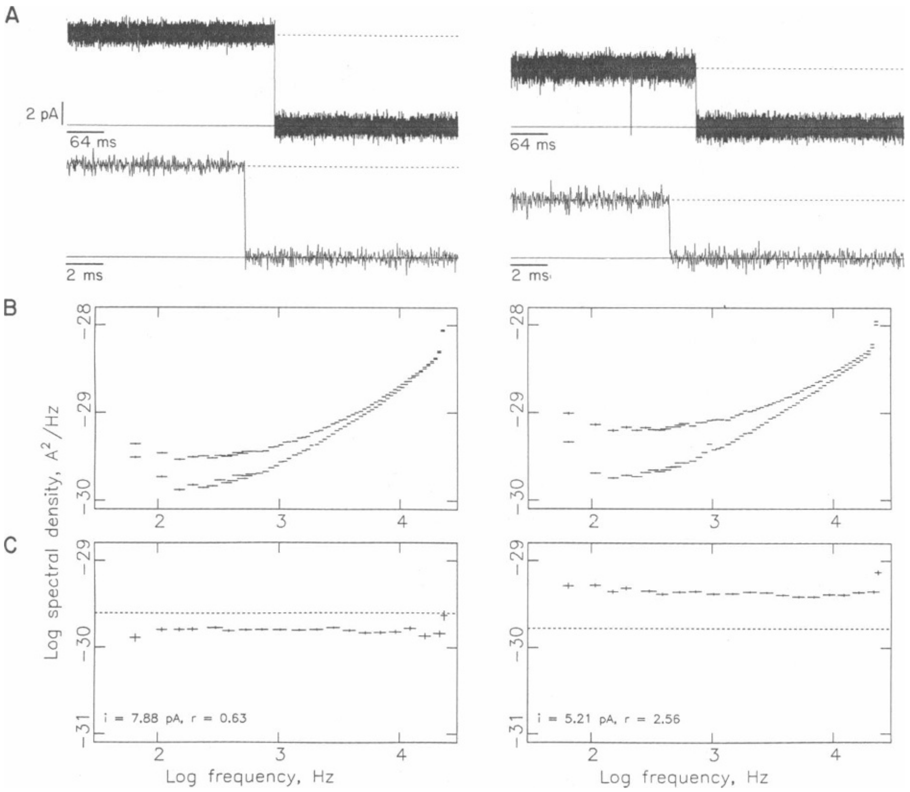

FIGURE 2 Comparison of open-channel noise measurements with symmetrical solutions of 640 m*M* KCl (*left panels*) and 640 m*M* KCl plus 640 m*M* formamide (*right panels*). (*A*) Step closings, shown at two different time scales, of single GA channels in GMO/squalene membranes at 200 mV membrane potential. Only one closing transition appears in these traces, because the mean open-time of GA in GMO/squalene membranes is several seconds. In these data, shown at the full recording bandwidth, little difference in noise level is visible between open and closed channel states. (*B*) Raw current spectra of baseline and open-channel segments. (*C*) Difference spectra. The spectra are white in the measured frequency range (0.04–20 kHz) for pure KCl solutions as well as for KCl plus formamide. According to the time constants for K^+ permeation and formamide block one would expect a Lorentzian component in the formamide-blocked currents with a corner frequency of ~1 MHz. The dashed lines indicate the shot noise level expected for independent ion transport (Eq. [1]). Values are shown for *i*, the single-channel current, and *r*, the ratio of the spectral density (averaged over 0.1 to 10 kHz) to the Schottky noise calculated from Eq. [1]. The value of *r* is clearly below unity for pure KCl solutions and increases when formamide is present. The absolute spectral densities of the open channel noise are 1.6×10^{-30} A^2/Hz and 4.3×10^{-30} A^2/Hz, respectively.

that the difference spectra are quite flat in the band 0.04-20 kHz, as was previously reported for Cs^+ currents through GA channels in diphytanoyl phosphatidylcholine/decane members (Sigworth et al., 1987). The dashed lines in Fig. 2 *C* indicate the classical shot noise spectral density, assuming the independent movement of ions (Eq. [1]). In the absence of formamide the ratio between the measured spectral density and S_{shot} is clearly below unity, indicating negatively-correlated ion transport events in K^+ solutions at this concentration (S.H. Heinemann and F.J. Sigworth, manuscript submitted for publication). The addition of formamide decreases the mean current but increases the noise spectral density, resulting in a spectral density ratio considerably above one.

Fig. 3 shows data at 200 mV membrane potential from a total of 18 recordings from 10 membranes; the mean current and the normalized spectral density are plotted as function of the formamide concentration. The dotted curve in the figure indicates a fit to a simple, one-site blocking model as mentioned earlier. Assuming that the transport of individual K^+ ions is not affected by the presence of formamide molecules one may describe the zero-frequency limit of the spectral density in this model by

$$S = 2\, i\, e_0 r_0 + 4 \lambda \tau^2 i^2, \qquad [24]$$

the sum of a shot noise term, in which r_0 is the ratio of the spectral density of the unblocked channel current to the Schottky-noise expression (Eq. [1]), and a blocking-noise term (Eq. [2]). The blocking rate λ is taken to be the association rate constant k_F times the formamide concentration. Further assuming that the association of a formamide molecule blocks the K^+ current completely, one obtains an expression for the single-channel current:

$$i = i_0/(1 + \lambda\tau), \qquad [25]$$

where i_0 is the current in the absence of formamide. The dotted curves in Fig. 3, *A* and *B* were drawn according to this equation with $\tau = 107$ ns and $k_F = 5.4 \times 10^6 s^{-1} mol^{-1}$. It is clearly seen that the fit does not describe the data well. A steeper concentration-dependence of the spectral density, for example due to the binding of more than one formamide molecule, is required.

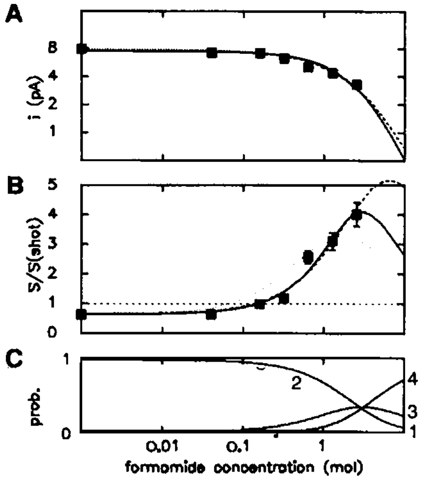

FIGURE 3 Block of GA currents at 200 mV membrane potential and 640 m*M* KCl. (*A*) Concentration dependence of K^+ current block by formamide. (*B*) Normalized spectral densities of open-channel currents. The dashed line indicates the Schottky-noise level. The solid curve is the result of a simultaneous fit of the 16-state model (Fig. 4 *A*) to both the current and noise data (for theory see Materials and Methods). As in comparison, the dotted curve represents a fit to a simple blocking model (Eq. [24], [25]) which clearly fails to describe the data well. The dashed curve is the result of a fit to the 9-state model shown in Fig. 4 *B*. (*C*) Channel occupancy as a function of formamide concentration. The curves indicate (1) probability of finding the channel empty and unblocked, (2) occupied by one or two K^+ ions and unblocked, and blocked by one (3) or two (4) formamide molecules. The occupancy curves were calculated for the 16-state model (Fig. 4 *A*).

Apart from describing the steepness of the observed concentration dependence of the blocking noise, a simple blocking theory is inadequate because the time scales of block and of permeation events are very similar. We therefore decided to use a more general approach in which the kinetics of the steps in ion permeation, along with the blocking steps, are combined in a single kinetic scheme. The stochastic behavior of this scheme is then used to

TABLE I
RATE CONSTANTS FOR THE STATE MODELS SHOWN IN FIG. 4

Forward Rate	Backward Rate
$[s^{-1}\, mol^{-1}]$	$[s^{-1}]$
A. Rate constants for K^+ transport (highlighted in Fig. 4)	
$fK1$ 6.9×10^7	$rK1$ 1.6×10^6
fKF 6.9×10^7	rKF 1.6×10^6
$fK2$ 1.8×10^8	$rK2$ 6.1×10^7
tK 1.2×10^7	
B. Rate constants for formamide block of the 16-state model (Fig. 4 *A*)	
$fF1$ 2.3×10^6	$rF1$ 1.4×10^7
$fF2$ 6.2×10^6	$rF2$ 1.0×10^7
C. Rate constants for formamide block of the 9-state model (Fig. 4 *B*)	
$fF1$ 1.3×10^7	$rF1$ 1.6×10^7
fFK 2.8×10^7	rFK 1.6×10^7
$fF2$ 4.0×10^7	$rF2$ 8.6×10^6
(tF 8.0×10^3)	

The rate constants in the part A of this table were determined by independent measurements of current and noise in symmetrical KCl solutions between 0.04 and 2.5 *M*. The rate constants involving movement of formamide were estimated by fitting the data shown in Fig. 3 to the 16-state model (part B of the table) and the 9-state model (part C). The lower case *f* denotes the forward rate, *r* the backward rate. *t* represents the transport rate form one binding site in the channel to the other one.

predict the mean current and spectral density as a function of blocker concentration.

The first step was to obtain a suitable model for K^+ permeation alone. We assumed a 4-state model (Finkelstein and Andersen, 1981) in which the GA channel is described as a symmetrical, single-file pore with two binding sites for cations. The relative electrical distance from the bulk solution to each binding site was assumed to be 0.06. Since our experiments were all performed at the same ionic strength this value was assumed to be constant, although in general it may be voltage and concentration dependent (Andersen, 1983). From fits to current and spectral density data in symmetrical KCl solutions of 0.04 to 2.5 *M* and at various membrane potentials, we obtained rate constants for association and dissociation of the first and second K^+ ions as well as for the translocation of ions through the channel as indicated in Table IA.

Then, in order to account for the block by formamide, we extended the 4-state model to a 16-state model illustrated in Fig. 4 *A*. The mechanism of formamide block is assumed to be binding to a superficial site outside the channel. In order to accommodate the symmetry of the channel we introduced two such sites. This yields 21 independent rate constants. Further assuming that the formamide binding does not depend on the occupancy of the channel by an ion reduces the number of free parameters to nine. The five parameters for the transport of K^+ had already been obtained independently and were used without further modifications. The remaining four rate constants were obtained by a least-squares fit to the data presented in Fig. 3. The resulting fits are shown as solid curves in Fig. 3, and the parameters are listed in Table IB.

The fit to Fig. 4 *A* yielded dissociation rate constants on the order of $1.0 \times 10^7\ s^{-1}$, and association rates that are indicative of cooperative binding: the association of the first formamide molecule was $fF1 = 2.3 \times 10^6\ s^{-1}mol^{-1}$, while the second association rate was $fF2 = 6.2 \times 10^6\ s^{-1}mol^{-1}$ (for notation see the legend of Fig. 4). Imposition a constraint which kept fF1 and fF2 equal during the fit, could not explain the experimentally-observed, steep increase of spectral density with formamide concentration.

In the 16-state model discussed so far it was assumed that no binding or movement of a K^+ ion could occur whenever a formamide molecule is bound. A slight variation of this model was also tested, in which the entry and exit of K^+ was blocked only on the side of the channel occupied by formamide, and gave very similar results.

Starting from the parameters for Fig. 4 *A*, similar fits were done for the data recorded from GMO/hexadecane membranes as shown in Fig. 1. The parameters involving formamide did not change significantly; however, to match the single-channel amplitude in the absence of formamide the relative electric distances of the binding sites were changed slightly, to 0.07. Since the other K^+ transport parameters were not modified further one cannot expect an ideal match between the data points even without formamide (see the upper *i*-V relationship in Fig. 1 *B*). The theory can provide a good fit to the concentration dependence of block at 100 mV (Fig. 1 *A*), but is not capable of duplicating the *i*-V relationship at high potentials in 1.2 *M* formamide.

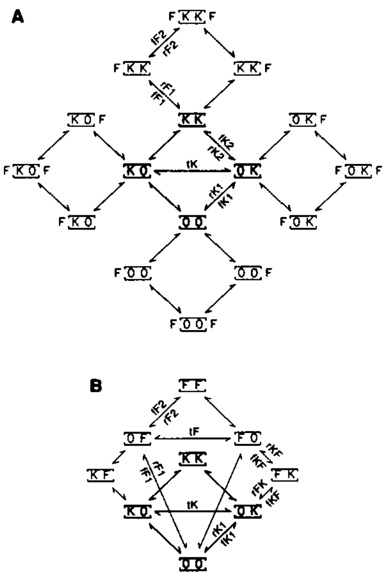

FIGURE 4 State diagrams for the blocking reactions. The small boxes indicate the GA channel with the two binding sites occupied by various combinations of particles. The 4-state scheme (Fineklstein and Andersen, 1981) that accounts for the conduction of potassium ions alone is shown highlighted. The models shown represent two possible mechanisms by which the channel could be blocked by formamide ("F"). In *A*, formamide can bind to two superficial sites outside the channel, while in *B* formamide competes with K^+ for its binding sites within the channel. The number of independent rate constants in each of the models is reduced by the fact that the GA channel is symmetrical. For the 16-state model we further assume that formamide binding does not depend on whether the channel is occupied by an ion. This yields a total of nine independent rate constants in *A*, of which five involve the transport of K^+ alone. Similar arguments yield a total of 14 independent rate constants in *B*, of which 7 involve K^+ transport only.

The dashed curves in Fig. 3, *A* and *B* were obtained by a fit to the 9-state model shown in Fig. 4 *B*. This model (Fig. 4 *B*) assumes competition of formamide for the two K^+ binding sites within the channel, and has 14 free parameters in the symmetrical case. However, assuming that the binding of K^+ to one site is the same whether the other site is empty or occupied by formamide, we already know seven parameters involving the K^+ transport alone. The remaining seven parameters that involve the movement of formamide were obtained by data fit (see Table IC). It is apparent that the rate constants for formamide dissociation and association do not depend strongly on whether or not there is a K^+ ion at the other end of the channel. In fact, introducing the constraints fF1 = fFK and rF1 = rFK also yielded a reasonable description of the data. However, as in Fig. 4 *A*, the binding of the second formamide molecule seems to be enhanced by the presence of the first one because the second association rate fF2 is consistently larger than fF1 and fFK, whereas the dissociation rate rF2 is found to be smaller than rF1 and rFK. The rate constant for the translocation of formamide through the channel, Ft, could not be determined from our data. Although the fit shown as the solid curve in Fig. 2, yielded $tF = 8.0 \times 10^3$ s^{-1}, far smaller and larger values for *tF* also gave reasonable fits.

DISCUSSION

Making use of a general theory for ion transport noise (Frehland, 1978), we have estimated the kinetics of block of K^+ currents in the gramicidin A channel by neutral formamide molecules. According to our analysis, the mean dwell time of formamide inside the GA channel is on the order of 50–100 ns, an interval which is comparable with the time between two ion transport events.

We have also demonstrated that the predictions of different mechanisms for channel block can be calculated readily by the application of Frehland's theory and have compared two of them with the measured data. Fig. 4 *A* assumes two superficial binding sites for formamide outside the channel, whereas in Fig. 4 *B*, formamide competes with K^+ for its binding sites within the channel. Our data do not allow us to discriminate between Fig. 4 *A* and *B*. Decker and Levitt (1988) have reported that there is little competition between formamide and K^+, which gives some support to Fig. 4 *A*, but further experiments at different K^+ concentrations will probably be necessary to clarify this question.

It is quite possible that neither of the models we have considered will hold true when the system is investigated more closely. The deviation between the calculated current-voltage relationships and the experimental data at high voltages (Fig. 1 *B*), and the apparent cooperativity of formamide binding predicted by both of the models lead us to expect that the mechanism may be more complex than modeled here. One explanation would be that formamide can enter and pass through the channel. In this case the occupation by more than two formamide molecules would be misinterpreted as cooperativity in models having only two binding sites. The deviation seen in Fig. 1 *B* is consistent with this idea; assuming a diffusion-limited, voltage-independent association of formamide, the dissociation could be increased at high potentials when the formamide molecules are dragged through the channel by the transport of ions and the associated water flow.

All of the theories considered here predict a reduction in the normalized spectral density at very high formamide concentrations; this occurs in the case where the channel spends most of the time in a blocked state, as illustrated by the plot of channel state occupancy (Fig. 3 *C*) for the fit to 4 *A*. The probabilities for finding the channel empty (*curve 1*), occupied with either one or two K^+ ions and not blocked (*curve 2*), blocked by one (*curve 3*) or two formamide molecules (*curve 4*) is plotted as function of the formamide concentration. It is seen that the spectral density (*solid curve in part B*) reaches its maximum at a concentration where curves 2 and 4 cross each other. Intuitively one can argue that the rare blocking events at low formamide concentrations create an excess noise by brief interruptions of the almost noiseless K^+ current, whereas at high concentrations the temporally-isolated K^+ ion transport events are expected to yield a spectral density that again approaches the Schottky value.

It should be emphasized that we have obtained kinetic information from the open-channel noise analysis, but not in the traditional manner in which the relaxation time constants are measured directly: instead, we have used only the low frequency asymptote of the noise power spectrum, measured at various ion concentrations. Although the spectral density depends on the same kinetic parameters that determine the single-channel current, they are weighted very differently, such that models giving very similar predictions for the mean current as a function of formamide concentration (e.g., in Fig. 3 *A*) can give very different predictions for the spectral density (Fig. 3 *B*)The analysis of open-channel noise and the subsequent test of models with the Frehland theory therefore promises to be a useful tool for determining the kinetics of processes of permeation and block in ion channels even if the relevant time scale is on the order of tens of nanoseconds.

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REFERENCES

Andersen, O. S. 1983. Ion movement through gramicidin A channels. Single-channel measurements at very high potentials. *Biophys. J.* 41:119–133.

Andersen, O. S. 1984. Gramicidin Channels. *Annu. Rev. Physiol.* 46:531–548.

Bezanilla, F. 1985. A high capacity data recording device based on a digital audio processor and a video cassette recorder. *Biophys. J.* 47:437–441.

Decker, E. R., and D. G. Levitt. 1988. Use of weak acids to determine the bulk diffusion limitation of the H^+ ion conductance through the gramicidin channel. *Biophys. J.* 53:25–32.

Elliott, J. R., D. Needham, J. P. Dilger, and D. A. Haydon. 1983. The effects of bilayer thickness and tension on gramicidin single-channel lifetime. *Biochim. Biophys. Acta.* 35:95–103.

Finkelstein, A., and O. S. Andersen. 1981. The gramicidin A channel: a review of its permeability characteristics with special reference to the single-file aspect of transport. *J. Membr. Biol.* 59:155–171.

FitzHugh, R. 1983. Statistical properties of the asymmetric random telegraph signal, with applications to single-channel analysis. *Math. Biosci.* 64:75–89.

Frehland, E. 1978. Current noise around steady states in discrete transport systems. *Biophys. Chem.* 8:255–265.

Heinemann, S. H., and F. J. Sigworth. 1988. Ion dependence of current noise in open Gramicidin A channels. *Biophys. J.* 53:330*a* (Abstr.)

Lansman, J. B., P. Hess, and R. W. Tsien. 1986. Blockade of current through single calcium channels by Cd^{2+}, Mg^{2+}, and Ca^{2+}. Voltage and concentration dependence of calcium entry into the pore. *J. Gen. Physiol.* 88:321–347.

Li, J. H.-Y., and B. Lindemann. 1983. Competitive blocking of epithelial sodium channels by organic cations: the relationship between macroscopic and microscopic inhibition constants. *J. Membr. Biol.* 76:235–251.

Rice, S. O. 1954. Mathematical analysis of random noise. *In* Selected Papers on Noise and Stochastic Processes. N. Wax, editor. Dover Publications Inc., New York.

Schottky, W. 1918. Über spontane Stromschwankungen in verschiedenen Elektrizitätsleitern. *Annalen der Physik* (*Leipzig*). 57:541–567.

Sigworth, F. J. 1985. Open channel noise. I. Noise in acetylcholine receptor currents suggest conformational fluctuations. *Biophys. J.* 47:709–720.

Sigworth, F. J., D. W. Urry, and K. U. Prasad. 1987. Open channel noise. III. High-resolution recordings show rapid current fluctuations in Gramicidin A and four chemical analogues. *Biophys. J.* 52:1055–1064.

Sigworth, F. J., and S. Shenkel. 1988. Rapid gating events and current fluctuations in gramicidin A channels. *Curr. Top. Membr. Transp.* 13:113–130.

Yellen, G. 1984. Ionic permeation and blockade in Ca^{2+}-activated K^+ channels of bovine chromaffin cells. *J. Gen. Physiol.* 84:157–186.