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Blocking of the Gramicidin Channel by Divalent Cations

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Summary. The conductance of the gramicidin channel in the presence of alkali ions is strongly reduced when divalent cations such as Ca^{++} or Ba^{++} are added to the aqueous solutions in concentrations between 0.1 and 1 M. Under the same conditions, carrier-mediated alkali ion transport is not affected by Ca^{++} and Ba^{++}. Different divalent cations differ considerably in their blocking action on the gramicidin channel; the effect of Mg^{++} or Zn^{++} is much smaller than that of Ca^{++} and Ba^{++}. Besides reducing the single-channel conductance, the blocking ions also change the current-voltage characteristic of the channel from a nearly linear to a strongly saturating behavior. These observations suggest that Ca^{++} or Ba^{++} (which are not permeable themselves) bind to a site at or near the channel mouth, thereby reducing the rate by which permeable ions enter and leave the channel. The blocking effect is analyzed in terms of the potential energy profile of the permeable ion in the channel. The saturating current-voltage characteristic may be explained by the assumption that in the presence of the blocking ion the passage over the entrance barrier is rate-limiting and, at the same time, only weakly voltage-dependent.

Gramicidin A has been used in recent years as a model compound for the study of ion transport through pore-like channels (Mueller & Rudin, 1967; Haydon & Hladky, 1972; Urry, 1973; Bamberg *et al.*, 1977; Eisenman, Sandblom & Neher, 1976). There is strong evidence that the channel is formed by association of two gramicidin monomers, but the precise structure of the dimer has not yet been established. One possibility consists in a head-to-head (formyl end to formyl end) association of two helical monomers (Urry, 1971; Glickson *et al.*, 1972). An alternative possibility, a double-stranded helix in which both peptide chains are coiled about a common axis, has been proposed by Veatch, Fossel and Blout (1974). A common feature of both models is the existence of a narrow pore along the axis of the dimer, which is lined with the oxygen atoms of the peptide carbonyls.

The gramicidin channel has been shown to be permeable to alkali ions as well as to a number of other small monovalent cations such as H_3O^+, NH_4^+, $HONH_3^+$, $H_2NNH_3^+$, $HC(NH_2)_2^+$ (Mueller & Rudin, 1967; Hladky & Haydon, 1972; Myers & Haydon, 1971; Eisenman, Krasne & Ciani, 1974; Bamberg, Noda, Gross & Läuger, 1976). The transport rate of these ions through the channel is rather high, in the order of $10^7\,sec^{-1}$ (in one molar solution at a voltage of 100 mV). It has also been observed (Hladky & Haydon, 1972) that the permeability of the channel for Ca^{++} is very low. From their studies of the single-channel conductance of gramicidin A in glycerolmonooleate membranes, Hladky and Haydon (1972) further reported that $CaCl_2$ "when added to KCl, ... produced a slight lowering of the conductance". In this communication we describe experiments which show that in phosphatidylcholine membranes (and also, to a lesser extent, in glycerolmonooleate membranes) divalent cations, such as Ca^{++} or Ba^{++}, have a strong blocking effect on the alkali ion permeability of the gramicidin channel. When high concentrations of Ca^{++} or Ba^{++} are added to a solution of the permeable ion, the single-channel conductance is strongly reduced. Furthermore, the current-voltage characteristic of the single channel, which is nearly linear in pure alkali ion solutions, becomes saturating in the presence of the blocking ions.

These observations are paralleled by recent findings that Tl^+ ions partially block the permeability of the gramicidin channel to alkali ions (Neher, 1975; Andersen, 1975; Sandblom, Eisenman & Neher, 1976). The principal difference between the blocking action of Tl^+ and of the divalent cations lies in the fact that Tl^+ itself is a permeable ion whereas Ca^{++} and Ba^{++} only block without being able to move through the channel.

Modification of ion permeabilities by calcium has been observed in several types of cells. For instance, a change in the extracellular calcium concentration shifts the conductance characteristic of the sodium and potassium system in the squid axon along the voltage axis (Frankenhaeuser & Hodgkin, 1957). Related observations have been made with certain epithelial tissues (Lindemann, 1968). These effects have been discussed in terms of a change in surface potential of the membrane induced by the divalent cations (McLaughlin, Szabo & Eisenman, 1971, and references cited in that paper). While this explanation seems appropriate for the nerve axon, it cannot be excluded that in other cases a direct and more specific interaction between divalent ions and the channel occurs (Frankenhaeuser & Hodgkin, 1957; Heckmann,

Lindemann & Schnakenberg, 1972). One of the well-documented cases of modification of a membrane channel by Ca^{++} is that of a cell-cell channel (Rose & Loewenstein, 1976). The gramicidin system studied here gives an example of how ion permeabilities may be regulated by a direct action of the modifying ion on the channel.

The phenomena described in this paper may also be compared with the observed deviation from the independence principle in the sodium and potassium channels of nerve (Bezanilla & Armstrong, 1972; Hille, 1972, 1975*a*; Woodhull, 1973; Cahalan & Begenisich 1976). These deviations include nonlinear conductance-concentration characteristics (saturation) as well as mutual blocking of different kinds of ions. They have been analyzed on the basis of the assumption that an ion binding to the channel prevents the entry of other ions. A similar model is used in this paper for the analysis of the blocking effects of divalent cations on the gramicidin channel.

Materials and Methods

Purified Gramicidin A was kindly provided by Dr. E. Gross (Bethesda). The peptide was added from a methanolic stock solution to the aqueous phases. 1,2-dioleoyl-*sn*-glycerol-3-phosphorylcholine (dioleoyllecithin) and 1,2-diphytanoyl-*sn*-glycerol-3-phosphorylcholine (diphytanoyllecithin) were synthetized by K. Janko (Benz, Stark, Janko & Läuger, 1973). Glycerolmonooleate was obtained from NuChek Preparation, Elysian, Minn. The sample consisted mainly of the α-isomer with small amounts of the β-isomer. The purity of the lipids was checked by thin layer chromatography. *n*-decane was from Merck, Darmstadt (standard for gas chromatography). All other reagents were analytical grade.

Black lipid membranes were formed in the usual way in a thermostated Teflon cell filled with aqueous electrolyte solution (Läuger, Lesslauer, Marti & Richter, 1967). The lipid was dissolved in *n*-decane (0.5–2%w/v). A series of Teflon cells were used with different diameters of the hole in the septum. The membrane area was determined with an eyepiece micrometer and was between 2×10^{-2}–8×10^{-2} cm^2 for the measurements of membrane potential and macroscopic conductance and about 3×10^{-3} cm^2 for the single-channel experiments. The temperature was 25 °C throughout.

For the measurement of the instantaneous current-voltage characteristic, voltage pulses of 0.5–1 msec duration and different amplitudes starting from zero voltage were applied to the membrane through silver/silver-chloride electrodes from a pulse generator with a rise-time of about 10 nsec. The time course of the membrane current was recorded with a storage oscilloscope (Tectronix Mod. 5103 N). The waiting times between the pulses were chosen to be at least ten times longer than the relaxation time of the channel-formation reaction, which is in the range of 10 msec to 1 sec (Bamberg & Läuger, 1973). With pulse amplitudes up to 500 mV there was no irreversible change in the membrane conductance. With membranes of low conductance a fast capacitive current-relaxation with a time constant of about 0.1 msec was observed at the higher voltages, which results

from the voltage dependence of membrane capacitance (Bamberg & Benz, 1976). In these cases the initial value of the conductive current was taken from the oscillogram after the decay of the fast relaxation process. For the measurement of membrane potentials calomel electrodes were used.

The single-channel experiments were carried out as described previously (Bamberg & Läuger, 1974). From the current fluctuations histograms were constructed by analyzing several hundred events for each experiment. The most probable fluctuation amplitude in the single-channel experiment was taken as representative for the conductance of the channel (Bamberg *et al.*, 1976).

Results

Single-Channel Conductance

When increasing amounts of $BaCl_2$ or $CaCl_2$ are added to a 1 M CsCl solution, the conductance of the gramicidin channel in a dioleoyl- or diphytanoyllecithin membrane becomes much reduced (Figs. 1 and 2). In the presence of 0.1 M $BaCl_2$ the conductance (at 100 mV) dropped to 53% and in 0.5 M $BaCl_2$ to 15% of the original value (Fig. 1). Similar, but somewhat smaller, effects are observed in the presence of $CaCl_2$ (Fig. 2).

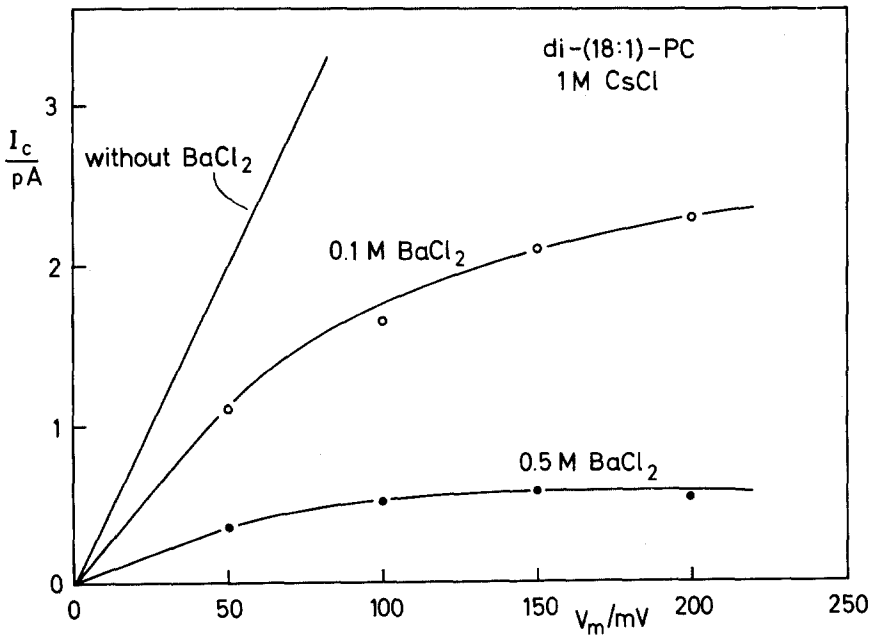

Fig. 1. Current J_c through the single channel as a function of the applied voltage V_m. Dioleoyllecithin (di-(18:1)-PC) membranes in *n*-decane, 1 M CsCl plus various concentrations of $BaCl_2$; $T=25$ °C. The data for 1 M CsCl alone have been taken from Bamberg *et al.* (1976*b*)

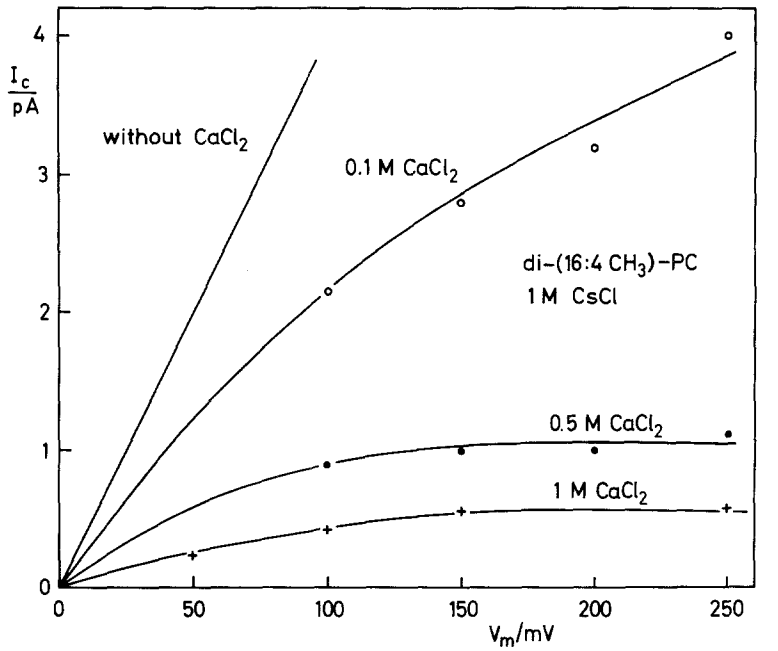

Fig. 2. Single-channel current J_c as a function of the applied voltage V_m. Diphytanoyllecithin (di-(16:4 CH_3)-PC) membranes in *n*-decane. 1 M CsCl plus various concentrations of $CaCl_2$; $T=25$ °C. The data for 1 M CsCl alone have been taken from experiments with dioleoyllecithin membranes (Bamberg *et al.*, 1976*b*)

Interestingly, it is found that the effect of divalent cations on the gramicidin channel conductance depends on the lipid composition of the membrane. This is shown in Fig. 3*A* and *B* where the results obtained from glycerolmonooleate membranes are represented. It is seen, for instance, that 0.5 M $CaCl_2$ reduces the conductance to 69 % in glycerolmonooleate membranes, whereas the corresponding value in a diphytanoyl-lecithin membrane is 21 %.

In all these cases the action of the divalent cation consists merely in shifting the probability distribution of the current fluctuations toward lower values without changing its general shape. This is shown in Fig. 4, where the histograms of the single-channel conductance Λ in 1 M CsCl in the presence and in the absence of blocking ions are compared. In 1 M CsCl the most probable conductance value occurs at 80 pS; upon addition of 1 M $BaCl_2$ the probability peak shifts to 29 pS and conductance events at the original value of 80 pS are no longer observed. Similar (but smaller) shifts of the probability peak are observed at $BaCl_2$ concentrations below 1 M. In other words, the conductance behaves as if

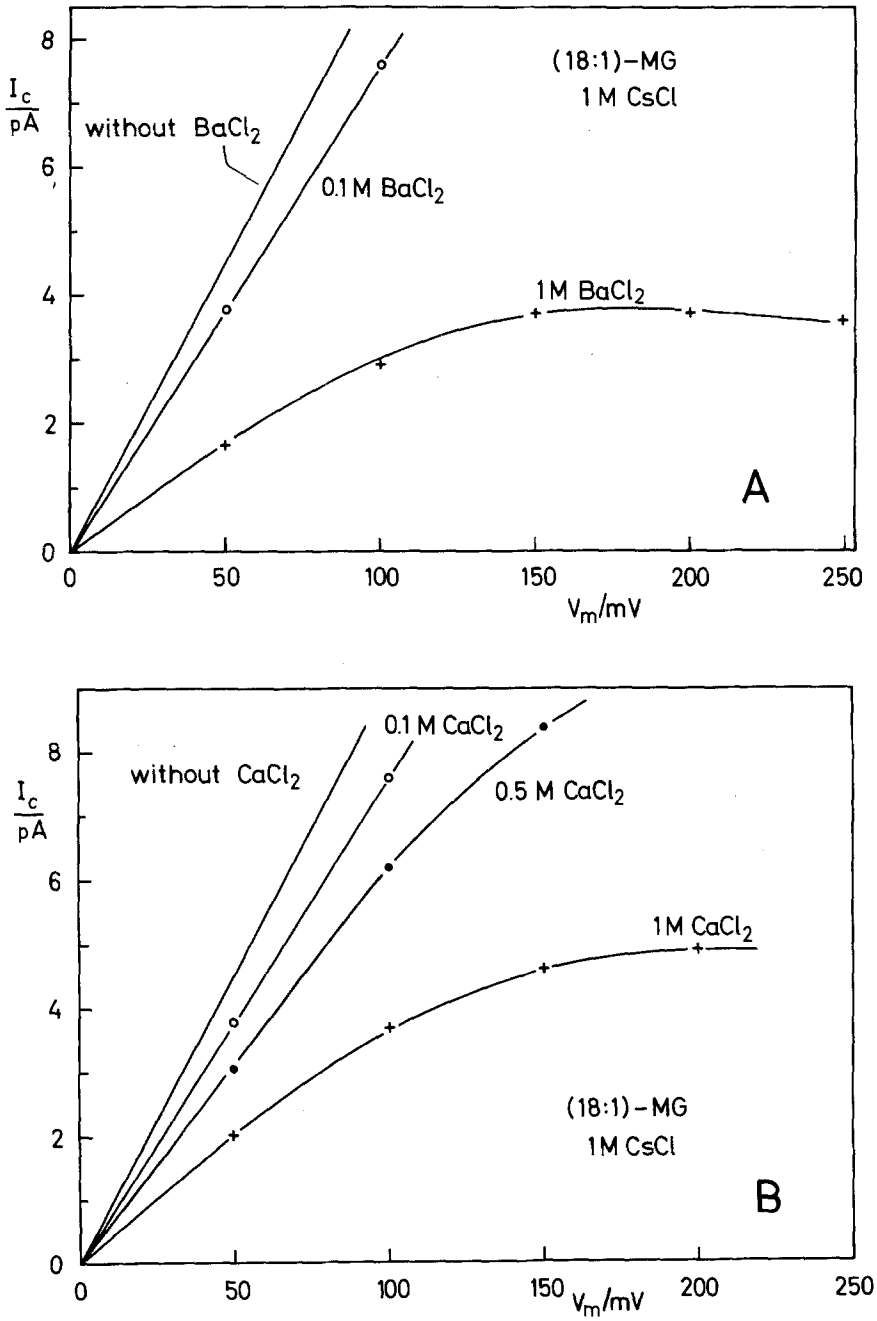

Fig. 3. Single-channel current J_c as a function of the applied voltage V_m. Glycerolmonooleate ((18:1)-MG) membranes in *n*-decane. 1 M CsCl plus various concentrations of divalent cations; $T = 25$ °C. The data for 1 M CsCl alone have been taken from Bamberg *et al.* (1976*b*). (*A*) Addition of $BaCl_2$. (*B*) Addition of $CaCl_2$.

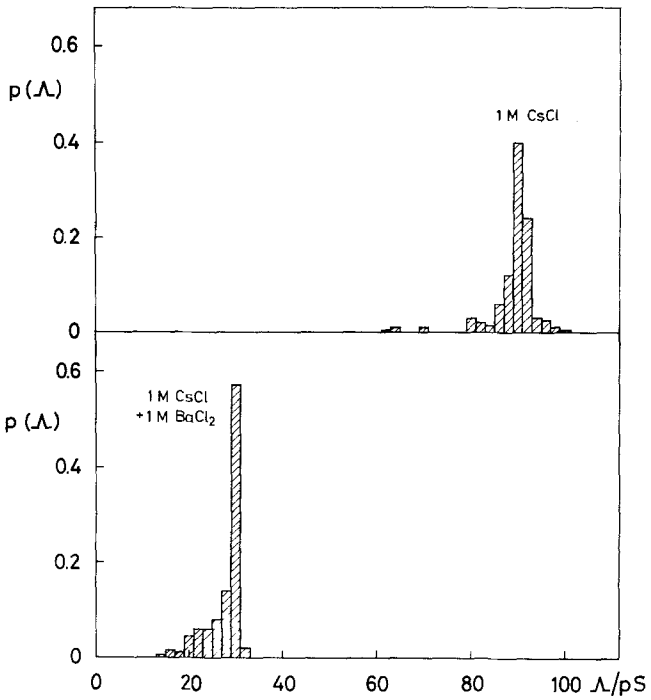

Fig. 4. Probability $p(\Lambda)$ of the occurrence of a conductance fluctuation of magnitude Λ. $p(\Lambda)$ is the number of events within an interval of width $\Delta\Lambda = \pm 2$ pS centered at Λ, divided by the total number n of events ($1\,\text{pS} = 10^{-12}\,\text{S} = 10^{-12}\,\Omega^{-1}$). Glycerolmonooleate/*n*-decane membrane, 25 °C. The values of n were $\geqq 800$

there was a graded (in contrast to an all-or-none) effect of divalent cations on the single channels; the histograms never show a mixture of blocked and normal channels.

Ca^{++} and Ba^{++} ions not only reduce the conductance, but also influence the current-voltage characteristic of the gramicidin channel. It is seen from Figs. 1–3 that the current-voltage curve which is almost linear in pure CsCl solution becomes strongly saturating at higher concentrations of the divalent cation.

Instantaneous Current-Voltage Characteristic

Direct measurements of the current-voltage characteristic of single channels up to several hundred millivolts are difficult to perform for reasons of membrane stability. An easier method consists in studying the

voltage dependence of the macroscopic conductance (in the presence of many channels), using brief voltage pulses. When a voltage is applied to a gramicidin-doped membrane, the transmembrane current starts at an initial value J_o and then approaches a stationary level J_∞, as new channels are formed under the action of the electric field (Bamberg & Läuger, 1973; Bamberg & Benz, 1976). The time constant of this relaxation process is of the order of 0.01–1 sec depending on the composition of the membrane. In order to obtain the current-voltage characteristic at constant number of channels, the initial current J_o is measured as a function of the applied voltage V_m; as long as interactions between channels may be neglected, the shape of $J_o(V_m)$ should be identical with the shape of the current-voltage characteristic $J_c(V_m)$ of the single channel.

In Figs. 5–7 the initial current J_o, obtained from the voltage-pulse method, is plotted in reduced form as the ratio of J_o divided by the (extrapolated) ohmic current at a reference voltage; the reference voltage was chosen to be $RT/F = 25.6$ mV (R is the gas constant, T the absolute

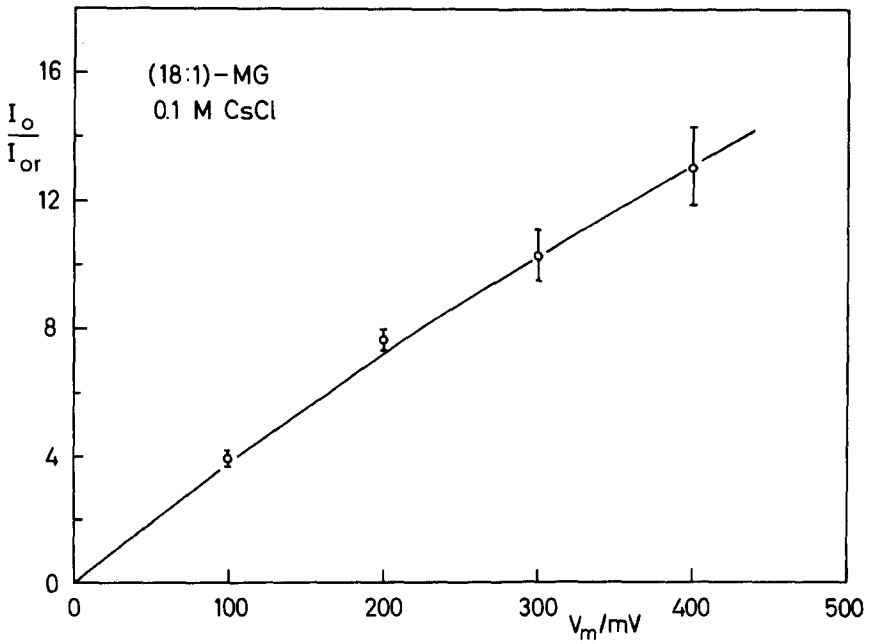

Fig. 5. Instantaneous current-voltage curve in 0.1 M CsCl. J_o is the initial current after a voltage jump of amplitude V_m; J_{or} is the current at a voltage of $RT/F = 25.6$ mV. Mean values, obtained from several membranes are given together with standard deviations. Glycerolmonooleate/*n*-decane membranes, 25 °C. The membrane conductances ranged between 10^{-2} and 10^{-5} S cm^{-2}. The theoretical curve has been calculated from Eq. (12) with n = 5, $\alpha = 0.2$ and $k_{pa}/k_i = 3$

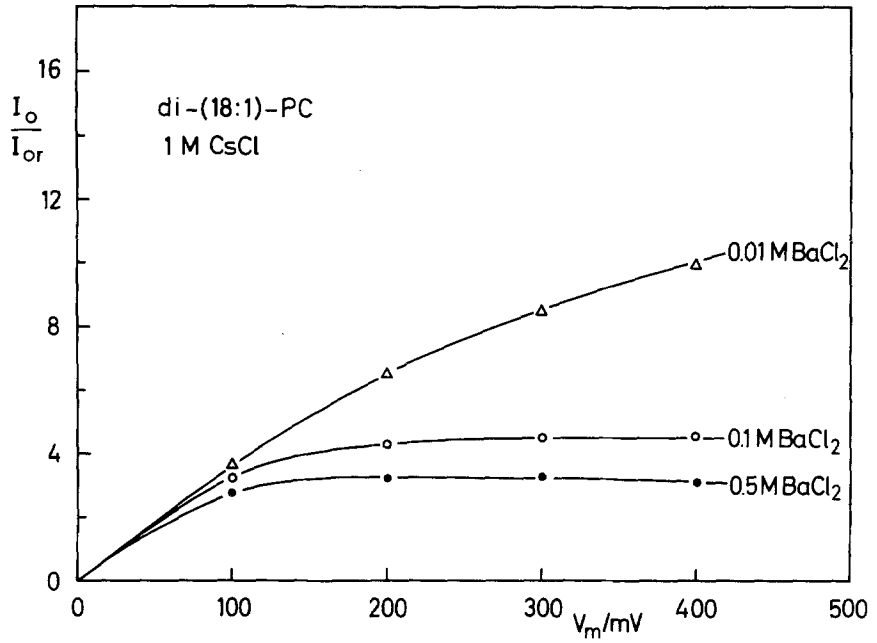

Fig. 6. Instantaneous current-voltage curves in 1 M CsCl plus various concentrations of $BaCl_2$. J_o is the initial current after a voltage jump of amplitude V_m; J_{or} is the current at a voltage of $RT/F = 25.6$ mV. Dioleoyllecithin/*n*-decane membranes, 25 °C. The membrane conductances ranged between 10^{-2} and 10^{-5} S cm^{-2}

temperature and F the Faraday constant). Mostly the current at $V_m = RT/F$ is still in the ohmic range and, if necessary, the value of J_{or} was slightly corrected for the nonlinearity. In this way the current-voltage curves measured at different channel densities may be represented in a single diagram. The current-voltage characteristic in pure CsCl solution (0.1 M) is shown in Fig. 5. The current increases almost linearly with voltage, showing a slight tendency towards saturation. This finding is consistent with the current-voltage behavior of single channels reported previously (Hladky & Haydon, 1972). When $CaCl_2$ or $BaCl_2$ is added, the current-voltage characteristic of the many-channel system becomes strongly saturating (Figs. 6 and 7) Again, the effect is larger for Ba^{++} than for Ca^{++} and larger with glycerolmonooleate membranes than with lecithin membranes.

The current-voltage characteristics of the single channel and of the many-channel system may be directly compared by calculating the ratio J_c/J_{cr} from the single-channel data (Figs. 1–3), where J_{cr} is the value of J_c at the reference voltage RT/F. It is found that J_c/J_{cr} and J_o/J_{or} agree within about 30 % in the experimental voltage range.

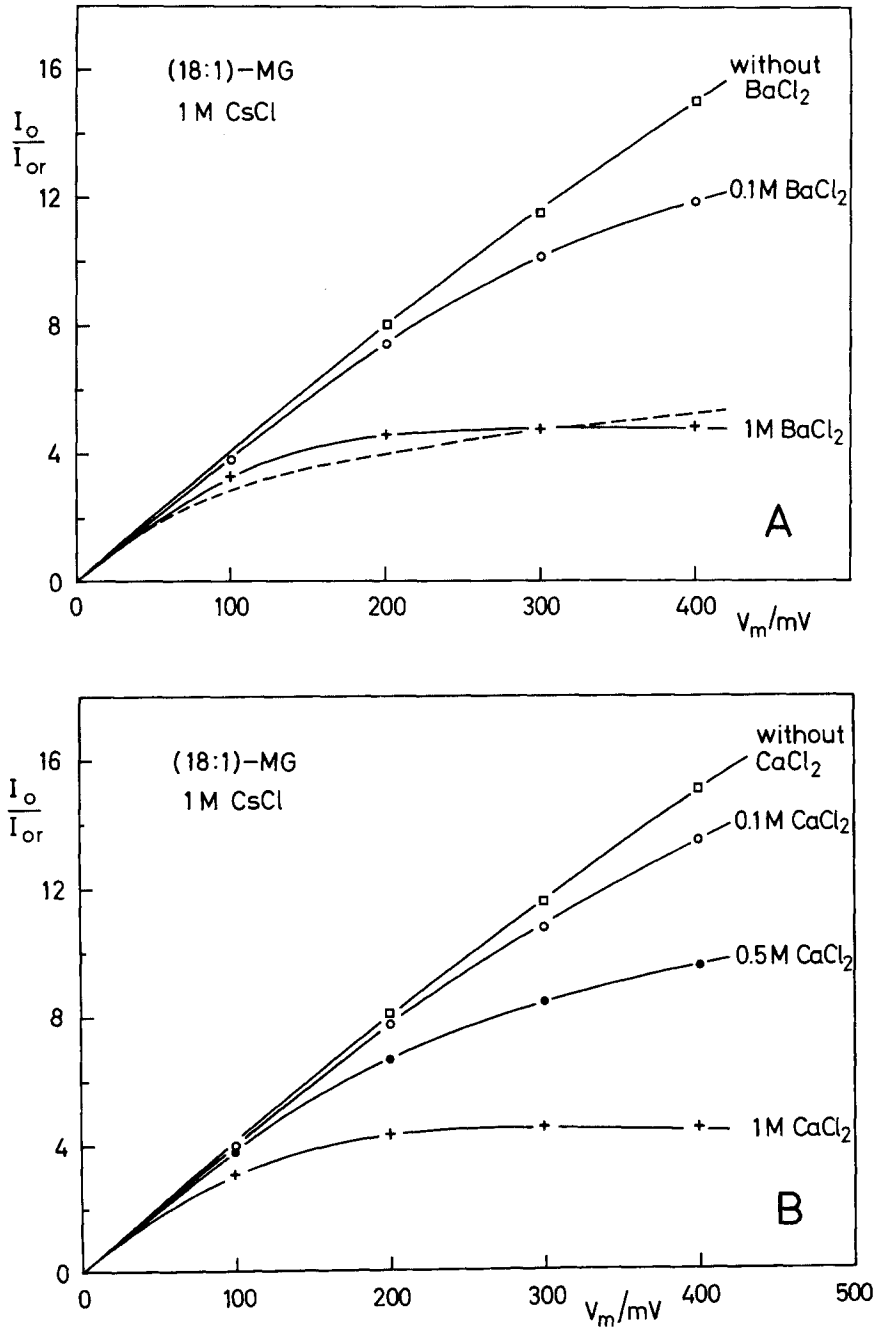

Fig. 7. Instantaneous current-voltage curves in 1 M CsCl plus various concentrations of $BaCl_2$ (*A*) or $CaCl_2$ (*B*). J_o is the initial current after a voltage jump of amplitude V_m; J_{or} is the current at a voltage of $RT/F = 25.6$ mV. Glycerolmonooleate/*n*-decane membranes, 25 °C. The membrane conductances ranged between 10^{-2}–10^{-5} S cm^{-2}. The dashed line in *A* has been calculated from Eq. (12) with $n = 5$, $\alpha = 0.2$ and $k^*_{pa}/k_i = 0.6$

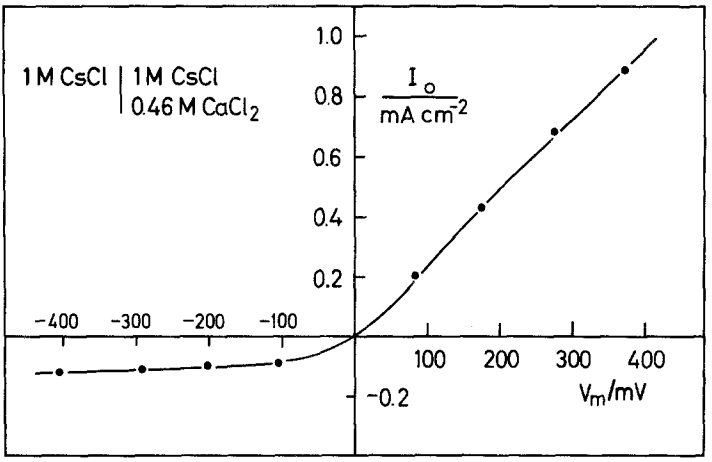

Fig. 8. Instantaneous current-voltage characteristic in asymmetric solutions. 1 M CsCl on one side, 1 M CsCl + 0.46 M $CaCl_2$ on the other. Diphytanoyllecithin/*n*-decane membrane, 25 °C. The sign of the J_o is positive for a current passing through the membrane from the calcium-free side to the calcium side. The curve has been corrected for a zero-current asymmetry potential of the Ag/AgCl electrodes of +14 mV which mainly results from the difference in the chloride activity between both solutions

Also a number of voltage-jump experiments was carried out with $CaCl_2$ present on only one side. In this case the current-voltage characteristic becomes asymmetric, the current being much larger when positive ions are driven from the calcium-free solution to the calcium solution than in the opposite direction (Fig. 8).

Further Experiments

To test whether the cation-to-anion selectivity of the channel is modified in the presence of divalent cations, the membrane potential V_m was measured at zero current in the system

1 M $BaCl_2$		1 M BaCl
	membrane	
10 mM CsCl		100 mM CsCl

using calomel electrodes. The membrane was formed with 1 M $BaCl_2$ + 10 mM CsCl on both sides, and then one solution was replaced by 1 M $BaCl_2$ + 100 mM CsCl. The membrane potential was found to be $V_m \simeq 56$ mV with the positive side in the more dilute CsCl solution. The

observed value of V_m is close to the theoretical potential of 59.2 mV for an ideally cation-selective membrane. The difference between the theoretical and the experimental value of V_m may result from different activity coefficients of Cs^+ in the aqueous phases or from incomplete exchange of the solution. These results allow the conclusion that the gramicidin channel retains its high cation selectivity in the presence of $BaCl_2$.

The reduction of the single-channel conductance by Ca^{++} and Ba^{++} could, in principle, result from a change of the activity coefficient of the permeable ion or from a binding of divalent cations to the lipid. In the latter case a positive surface potential would be created which would reduce the concentration of permeable cations in the vicinity of the membrane.

These possibilities have been studied by measuring the membrane conductance in the presence of a cation-carrier system such as nonactin/K^+ or valinomycin/K^+ (Ca^{++} and Ba^{++} are not transported to any appreciable extent by nonactin or valinomycin). In an aqueous solution of 10^{-7} M nonactin and 1 M KCl the membrane conductance (measured with a dioleoyllecithin membrane in the absence of gramicidin) was the same within the limits of reproducibility (50 %), whether or not 1 M $CaCl_2$ was symmetrically added to the solutions. This finding, of course, could simply mean that an increase of the surface potential is compensated by an increase of the partition coefficient of the carrier between water and membrane, but this possibility seems to be unlikely. Furthermore, when 0.5 M $CaCl_2$ is added to only one side in the presence of 10^{-7} M nonactin and 1 M KCl on both sides, the current-voltage characteristic remained symmetrical. Other experiments have been carried out with 10^{-4} M valinomycin, added to the membrane-forming solution (0.75 % dioleoyllecithin (w/v) in *n*-decane). Also in this case the membrane conductance was the same within about 30 % in 0.1 M KCl alone and in 0.1 M KCl + 1 M $BaCl_2$.

The effects of Ca^{++} or Ba^{++} which were studied in the presence of CsCl are not specific for the Cs^+ ion but are also observed with Na^+ or K^+ as permeable ions. A few experiments have also been performed in which Mg^{++} or Zn^{++} were present as divalent cations. Mg^{++} also blocks the conductance of monovalent cations, but the effect is much smaller than for Ba^{++} or Ca^{++}; in 1 M $MgCl_2$ + 1 M NaCl the instantaneous current-voltage curve of gramicidin in dioleolylecithin is only slightly saturating (the J_o/J_{or} *vs.* V_m plot approximately coincides with the curve for 0.01 M Ba^{++} in Fig. 6). 0.1 M Zn^{++} added to 1 M CsCl

left the current-voltage characteristic virtually unchanged as compared with 1 M CsCl alone.

Discussion

Two distinct effects on the gramicidin channel are observed when calcium or barium ions are added to the aqueous solutions: (i) a reduction of the single channel conductance, and (ii) a change of the current-voltage characteristic from an almost linear to a strongly saturating behavior. An explanation of the first effect in terms of a decrease of the activity coefficient of the permeable monovalent cations or an adsorption of Ca^{++} or Ba^{++} to the membrane and a concomitant change in the surface potential are unlikely, because such changes should also influence the rate of carrier-mediated cation transport; in the experiments with nonactin or valinomycin, however, no appreciable effect of divalent cations could be found.

A further argument against the above-mentioned interpretation is the observation that the current-voltage characteristic of the channel is drastically modified in the presence of divalent cations. That should not be the case if only the activity of the permeable ions in the vicinity of membrane were decreased. Furthermore, as the experiments with Mg^{++} and Zn^{++} have shown, different divalent cations differ considerably in their blocking action.

These findings strongly suggest that the interaction between the blocking ion and the channel is of a more specific nature. As the gramicidin channel is impermeable to both Ca^{++} and Ba^{++} it is unlikely that these ions are able to penetrate very far into the channel. Therefore it is assumed that the channel is blocked by the binding of a divalent ion at or near its entrance. The ligand groups interacting with the ion may be amide carbonyls of the peptide and/or may derive from the polar residues of lipid molecules surrounding the channel. The latter possibility cannot be excluded, as the observed blocking effect is somewhat different in lecithin and in monoglyceride membranes.

For an explanation of the saturating current-voltage characteristic which is observed in the presence of divalent cations, several possibilities have to be considered. If the ion is bound to a site in the channel, part of the applied voltage drops between this binding site and the external solution. This means that for an ion located in the half-channel facing the aqueous phase which is made positive, the binding strength and therefore also the fraction of time spent by the ion at the binding site increases

with increasing voltage. Such a mechanism has been proposed by Heckmann *et al.* (1972) in their theoretical model for the blocking of ion channels by calcium. For the gramicidin channel, however, this mechanism can be largely excluded, because it is not consistent with the experimental current-voltage characteristic. The model predicts that at higher voltages the current should be a decreasing function of voltage. Under certain conditions a slight decrease of the current is indeed observed (*compare* Figs. 1, 3*A* and 6), but the effect is much too small. If the experimental current-voltage curve is fitted to the model at low voltage V_m, the theoretical curve goes through a pronounced maximum at higher values of V_m, which is not observed experimentally. It may be, however, that this mechanism, in which the binding of the divalent cation is stabilized by the applied voltage, plays a marginal role in observed blocking effect and may possibly account for the slight tendency of the current to decrease at the higher voltages.

In the following we show that the observed blocking effects are consistent with the assumption that the blocking ion binds to a site which is near the mouth of the channel, but which is different from the channel path. This means that permeable ions may pass by the divalent ion which, however, modifies their flow rate.

A transport system exhibits a saturating current-voltage characteristic, if the rate-limiting transport step is voltage independent (or only weakly voltage-dependent). According to this principle, the current voltage behavior of the modified gramicidin channel may be explained by the assumption that the blocking ion creates at the pore mouth a high coulombic energy barrier, which limits the access of permeable ions to the interior of the pore. If this energy barrier is located in the membrane-water interface, it is feasible that only a small fraction of the total voltage drops across the barrier. Under these conditions the transport of permeable ions across the entrance barrier is rate limiting and, at the same time, only weakly voltage-dependent.

In order to discuss in more detail how the blocking ion may modify the transport of permeable ions, the kinetics of association and dissociation of the divalent ion at the binding site has to be considered. In the presence of divalent ions B^{++} the binding site S fluctuates between an empty and an occupied state according to

$$B^{++} + S \underset{k_b}{\overset{k_f}{\rightleftarrows}} BS^{++} \tag{1}$$

k_f and k_b are the rate constants for the forward (association) and backward (dissociation) reaction, respectively.

The average lifetimes of the empty (τ_e) and the occupied state (τ_o) are then given by

$$\tau_e = 1/k_f c_B \tag{2}$$

$$\tau_o = 1/k_b. \tag{3}$$

c_B is the concentration of the blocking ion. τ_e and τ_o have to be compared with the average transit time τ_t of the permeable ion, i.e., the average time which an ion that is located in the potential-energy minimum in front of the channel on one side needs to enter the channel and to leave it on the opposite side. The transit time τ_t determines the time which is required for the establishment of a stationary concentration profile in an ensemble of channels after a perturbation such as a sudden change in the height of the entrance barrier by the blocking ion (for a more detailed discussion of nonstationary states in ion channels, *see* Frehland & Läuger, 1974). τ_t is approximately equal to or longer than the reciprocal of the maximum transport rate ρ of the gramicidin channel which is of the order of 10^{-7} sec (Läuger, 1973). Depending on whether τ_t is small or large compared with τ_e and τ_o, two limiting cases may be distinguished:

(A) $\tau_t \ll \tau_o, \tau_e$. If the transit time is much shorter than the lifetimes of both the blocked and the unblocked states, then the channel fluctuates between two distinct and stationary conductance states.

(B) $\tau_t \gg \tau_o, \tau_e$. If the transit time is much longer than the lifetimes of the blocked and unblocked states, the channel stays all the time in an intermediate conductance state (between the fully blocked and the unblocked state); in other words, the permeating ion "sees" an entrance barrier of average height.

From sound absorption studies Eigen and Maass (1966) concluded that the bimolecular reaction rate constant between Ca^{++} or Ba^{++} and organic ligands approaches the limiting value of a diffusion-controlled reaction; this means that k_f in Eq. (1) is of the order of $10^9\,\text{M}^{-1}$. Thus, at $c_B = 1$ M the lifetime τ_e of the unblocked state is about 10^{-9} sec and at $c_B = 10^{-1}$ M about 10^{-8} sec. In a similar way the lifetime of the blocked state may be estimated: from the analysis of the blocking effects (see below) the binding constant $K_B = k_f/k_b$ of the divalent ion is estimated to be of the order of $10\,\text{M}^{-1}$ (similar values of K_B may be directly inferred from Figs. 1–3). This yields values of $\tau_o = 1/k_b = K/k_f$ of the order of 10^{-8} sec.

These estimated values of τ_e and τ_o are shorter than the average transit time of a permeable ion ($\tau_t \simeq 10^{-7}$ sec) which suggests that a time averaged barrier height (case *B*) may be used for an approximate description of the blocking effects. As the difference between τ_t on one hand and τ_e and τ_o on the other is not more than one order of magnitude, a more exact treatment should be based on a general theory including both limiting cases *A* and *B*. It was impossible, however, to fit the data with a model based on case *A*, the reason being that for the parameter values (binding constant of B^{++}, rate constants of the blocked channel) needed to account for the reduction of ohmic conductance by B^{++}, channels which are blocked on the exit side dominate the overall $J(V_m)$ behavior, leading to a superlinear $J(V_m)$ characteristic. As we shall describe below, a reasonable fit is possible using time-average entrance barriers (case *B*). The above considerations also explain, of course, why only one population of channels is seen in the presence of blocking ions.

Theoretical Treatment

(a) *Current-Voltage Characteristic of the Unmodified Channel.* For a quantitative analysis of the data we describe the movement of ions across the channel as a pasage over a series of activation-energy barriers. This barrier model of ion transport, which is based on the theory of absolute reaction rates developed by Eyring and coworkers (Zwolinski, Eyring & Reese, 1949), has been repeatedly applied in the theory of ion channels (Woodbury, 1971; Heckmann, 1972; Heckmann *et al.*, 1972; Läuger, 1973; Chizmadjev, Khodorov & Aityan, 1974; Hille, 1975*a*, *b*; Eisenman *et al.*, 1976). Rather little is known about the potential energy profile of an ion in the gramicidin channel; as long as detailed structural data are lacking, one has to rely on more or less plausible assumptions about the shape of the energy profile. Urry (1971) discussed two possible arrangements of the carbonyl oxygens around the cation in the channel, either a fourfold or a sixfold coordination. Similar configurations seem feasible for the channel structure proposed by Veatch *et al.* (1974). If a sixfold coordination is assumed, the number of potential-energy minima in the channel becomes $n=5$; for a fourfold coordination $n=7$ is obtained. The choice of n is not critical, however; we adopt a value of $n=5$ in the following (Läuger, 1973). The general shape of the potential profile is obtained by superposition of all energetic interactions of the ion with its surroundings; the most important contributions probably are the image-

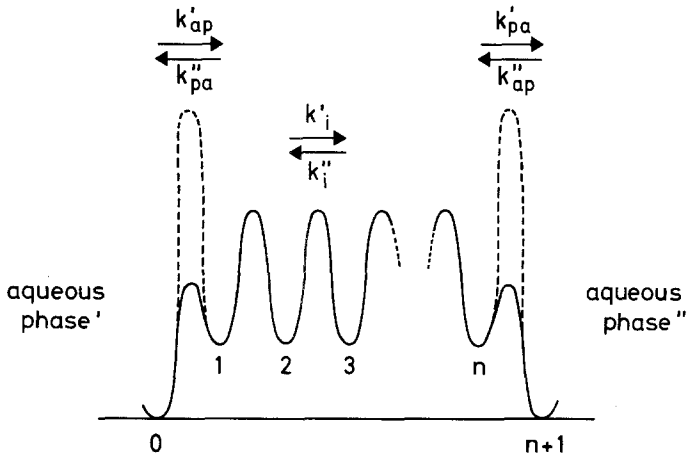

Fig. 9. Potential energy profile of the channel at zero voltage in the unmodified state (full line) and in the blocked state (dashed line). All internal barriers are assumed to have identical heights

force interaction with the membrane dielectric and the electrostatic interactions with the ligand system of the peptide helix (Läuger & Neumcke, 1973). The dielectric energy curve strongly depends on position near the membrane-solution interface, but has a rather flat top in the middle of the membrane (Neumcke & Läuger, 1969). Furthermore, the interaction with the ligand system creates a series of $n+1$ more or less regularly-spaced barriers, which may be assumed to have, except for the outermost barriers at either end of the channel, similar heights. A plausible potential energy profile which is obtained by superposition of the single contributions is schematically depicted in Fig. 9.

The number of possible shapes of the potential profile is restricted by the requirement that it has to be consistent with the current-voltage characteristic of the channel. In 0.1 M CsCl the current-voltage curve is nearly linear to slightly saturating in the voltage range up to 400 mV (Fig. 5; *see also* Hladky & Haydon, 1972). An energy profile with a high barrier in the center of the membrane, for instance, would yield a superlinear current-voltage characteristic. For the comparison with the observed current-voltage behavior we explicitly assume a symmetrical potential profile with equidistant internal barriers of identical height (Fig. 9); the entrance barrier on either side of the membrane is assumed to be, in general, different from the internal barriers. As the entrance barrier is located in a plane in (or near) the polar layer of the membrane where the dielectric constant is higher than in the hydrocarbon zone, the

voltage drop across the entrance barrier is likely to be smaller than across the internal barriers. The fraction of the applied voltage which drops across either entrance barrier is therefore written as $\alpha/(n+1)$ where α is a dimensionless quantity ($0 \leqq \alpha \leqq 1$); $\alpha = 1$ corresponds to the case where the voltage drop is uniformly distributed over all barriers. It is easily seen that the fraction of the voltage, which drops across each of the $(n-1)$ internal barriers is equal to $(n+1-2\alpha)/(n+1)(n-1)$. The rate constants for the jumps over the barriers (Fig. 9) are then given by (Läuger, 1973):

$$k'_{ap} = k_{ap} r; \quad k''_{ap} = k_{ap}/r \tag{4}$$

$$k'_{pa} = k_{pa} r; \quad k''_{pa} = k_{pa}/r \tag{5}$$

$$k'_i = k_i s; \quad k''_i = k_i/s \tag{6}$$

$$r = \exp\left[\frac{\alpha u}{2(n+1)}\right] \tag{7}$$

$$s = \exp\left[\frac{n+1-2\alpha}{2(n+1)(n-1)} u\right] \tag{8}$$

$$u = \frac{V_m}{RT/F} = \frac{\psi' - \psi''}{RT/F}. \tag{9}$$

k_{ap}, k_{pa} and k_i are the rate constants at zero external voltage ($V_m = 0$); ψ' and ψ'' are the electrical potentials in the left and right aqueous phase, respectively.

In the following we restrict the analysis to low concentrations c of the permeable ion where the channel conductance is a linear function of c. As shown in the Appendix, the current J_c through the single channel is then given by

$$J_c = \frac{v c e_o k_{ap}(1-e^{-u}) r}{1 + \frac{1}{r s^{2(n-1)}}\left[\frac{k_{pa}}{k_i} s \frac{s^{2(n-1)}-1}{s^2-1} + \frac{1}{r}\right]}. \tag{10}$$

v is a constant with the dimension of a volume per mole, $v c k_{ap}$ being the frequency of jumps at zero voltage from the solution into the channel.

In the ohmic limit ($u \approx 0$) Eq. (10) reduces to

$$J_{co} \approx \frac{v c e_o k_{ap} u}{2 + (n-1) k_{pa}/k_i}. \tag{11}$$

The current J_{cr} at the reference voltage $V_m = RT/F$ ($u=1$) is, by definition, equal to J_{co}, taken at $u=1$. Furthermore, as shown previously, the reduced single-channel current J_c/J_{cr} may be identified with the reduced instantaneous current J_o/J_{or} from pulse experiments. Thus,

$$\frac{J_o}{J_{or}} = \frac{J_c}{J_{cr}} = \frac{[2+(n-1)\,k_{pa}/k_i](1-e^{-u})\,r}{1+\dfrac{1}{rs^{2(n-1)}}\left[\dfrac{k_{pa}}{k_i}\,s\,\dfrac{s^{2(n-1)}-1}{s^2-1}+\dfrac{1}{r}\right]}. \tag{12}$$

Besides the number n of potential wells, which may be estimated from structural arguments, Eq. (12) contains two adjustable parameters, the quantity α, which is implicit in r and s, and the ratio k_{pa}/k_i. By fitting the theoretical expression (12) to the experimental current-voltage curve, α and k_{pa}/k_i may thus be determined. With a preselected value of $n=5$ (*see above*), a satisfactory fit to the observed current-voltage behavior of the unmodified channel in 0.1 M CsCl is obtained with $\alpha=0.2$ and $k_{pa}/k_i=3.0$ (Fig. 5).

These parameter values mean that the entrance barriers are lower than the internal barriers and are rather insensitive to the applied voltage[1]. The good agreement between observed and calculated current-voltage curve does not prove, of course, that the proposed potential profile is the correct one, because in the experimentally accessible voltage range and within the limits of experimental error the choice of the energy parameters is probably not unique; it does show, however, that the general shape of the energy profile of Fig. 9 is consistent with the observed current-voltage behavior of the channel.

(b) *Blocking Effects.* When a blocking ion binds to the channel the heights of all barriers may, in principle, be affected. As we already have mentioned, however, it is likely that the divalent cation binds near the end of the channel at the membrane-solution interface. In this case the electric field of the blocking ion is directed almost exclusively towards the water phase which has a much higher dielectric constant than the membrane. Accordingly, it is assumed that, to a first approximation, only the outermost barrier at either side of the membrane is changed by the blocking ion (Fig. 9). This means that the action of the blocking ion consists mainly in decreasing the rate by which permeable ions enter and leave the channel. Furthermore, if the blocking ion is bound near the

1 In a previous paper (Läuger, 1973) somewhat different parameter values have been evaluated on the basis of the limited information on the current-voltage characteristic which was available at that time.

membrane-solution interface, both binding sites may be assumed to be independent and unaffected by the applied voltage. Under these assumptions the current through the blocked channel is described by expressions identical with Eqs. (10)–(12), except that now k_{ap} and k_{pa} have to be replaced by (time averaged) rate constants k^*_{ap} and k^*_{pa}, which are modified by the action of the divalent ion. We further assume that the parameter α is the same in the blocked and in the unblocked channel. Thus, the single-channel current is now given by:

$$J_c = \frac{vce_o\, k^*_{ap}(1-e^{-u})\, r}{1+\frac{1}{rs^{2(n-1)}}\left[\frac{k^*_{pa}}{k_i}\, s\, \frac{s^{2(n-1)}-1}{s^2-1}+\frac{1}{r}\right]}. \tag{13}$$

Expressions analogous to Eqs. (11) and (12) hold for J_{co} and J_c/J_{cr}. In the derivation of Eq. (13) we have assumed that saturation effects in the conductance-concentration behavior of the channel may be neglected. This is only approximately true, since at Cs^+ concentrations of 1 M where the experiments with blocking ions have been performed the relationship between single-channel conductance and Cs^+ concentration is no longer strictly linear (Hladky & Haydon, 1972). Furthermore, we disregard the possibility that monovalent cations compete with the blocking ions for the binding site and that the channel may be occupied by more than one permeable ion (Sandblom *et al.*, 1976).

It is found that Eq. (12) approximately describes the measured current-voltage characteristic in the presence of blocking ions, if suitable values of the ratio k^*_{pa}/k_i are used; an example is represented in Fig. 7*A*. The saturating behavior, however, is somewhat less pronounced in the theoretical curves than observed experimentally. A possible reason for this deviation could be the influence of voltage on the binding strength of the blocking ion. Indeed, if it is assumed that a small fraction of the voltage drops between binding site and aqueous solution, the fit of the theoretical curves can be considerably improved. An exact treatment of this effect, however, would require a detailed model for the dependence of the time-averaged rate constants k^*_{ap} and k^*_{pa} on the binding constant of the blocking ion, which is presently not available.

From the values of k^*_{pa}/k_i, which are determined from the reduced current-voltage curves (Figs. 6 and 7), together with $\alpha = 0.2$ and $k_{pa}/k_i = 3$ (obtained from J_o/J_{or} of the unblocked channel) the ratio k^*_{pa}/k_{pa} may be calculated (Table 1). In a similar way the ratio k^*_{ap}/k_{ap} of the rate constants for the entry into the channel may be obtained: If J^*_{co} and J_{co}

are the ohmic single-channel currents with and without blocking (measured at the same voltage and at the same concentration c of the permeable ion), then, according to Eq. (11), the ratio J^*_{co}/J_{co} is given by

$$\frac{J^*_{co}}{J_{co}} = \frac{k^*_{ap}}{k_{ap}} \cdot \frac{2+(n-1)\,k_{pa}/k_i}{2+(n-1)\,k^*_{pa}/k_i}. \tag{14}$$

Thus, k^*_{ap}/k_{ap} may be obtained from the experimental values of J^*_{co}/J_{co} and from k_{pa}/k_i and k^*_{pa}/k_i determined from the current-voltage characteristic. The values of k^*_{ap}/k_{ap} calculated in this way are given in Table 1.

According to our assumption that only the entrance barriers are modified by the blocking ion (Fig. 9), the ratios k^*_{pa}/k_{pa} and k^*_{ap}/k_{ap} should be approximately equal:

$$\frac{k^*_{pa}}{k_{pa}} \approx \frac{k^*_{ap}}{k_{ap}} \approx \exp(-\Delta f), \tag{15}$$

where Δf is the change in the height of the energy barrier (expressed in units of kT) by the binding of the blocking ion. A comparison of the calculated values of k^*_{pa}/k_{pa} and k^*_{ap}/k_{ap} therefore represents a check of the internal consistency of the proposed model. It is seen from Table 1 that k^*_{pa}/k_{pa} and k^*_{ap}/k_{ap} approximately agree, the differences being less than a factor of two. k^*_{pa}/k_{pa} tends to be somewhat larger than k^*_{ap}/k_{ap}; this indicates that also the level of the internal energy minimum adjacent to the interface ($v=1$ and $v=n$) is slightly shifted upwards upon binding of the blocking ion.

The binding constant K_B of the blocking ion determines the fraction of time p_e, during which the binding site is empty:

$$p_e = \frac{1}{1+c_B K_B}. \tag{16}$$

As already mentioned, an exact theoretical treatment of the relation between K_B and the time-averaged rate constants k^*_{ap}, k^*_{pa} is difficult, because it is not known in sufficient detail how the fast binding-unbinding reaction of the blocking ion interferes with the passage of the permeable ion through the channel. The simplest assumption on the averaging process leads to equations of the form

$$k^*_{ap} = p_e\,k_{ap} + (1-p_e)\,\tilde{k}_{ap} \tag{17}$$

$$k^*_{pa} = p_e\,k_{pa} + (1-p_e)\,\tilde{k}_{pa} \tag{18}$$

where $\tilde{k}_{ap}$ and $\tilde{k}_{pa}$ are the rate constants in the occupied (fully blocked) state. From the observation that at high concentration c_B of the blocking ion the single-channel conductance is reduced to about 15% of the normal value (dioleoyllecithin membranes, 1 M $Cs^+ + 0.5$ M Ba^{++}), a reasonable approximation consists in the assumption that $\tilde{k}_{ap} \ll k_{ap}$ and $\tilde{k}_{pa} \ll k_{pa}$. With this approximation Eqs. (17) and (18) simplify to

$$\frac{k^*_{ap}}{k_{ap}} \approx \frac{k^*_{pa}}{k_{pa}} \approx \frac{1}{1 + c_B K_B}. \tag{19}$$

The values of K_B which are calculated according to Eq. (19) from k^*_{ap}/k_{ap} are given in Table 1 (we have used k^*_{ap}/k_{ap} for the evaluation of K_B because, as outlined above, the values of k^*_{pa}/k_{pa} are probably affected by a change in the level of the first and n-th energy minimum). It is shown that the binding constant K_B is always less for Ca^{++} than for Ba^{++}. Furthermore, K_B is smaller for monoglyceride membranes than for lecithin membranes. The finding that the binding constant depends on the lipid environment of the channel is interesting; it could mean either that the lipid directly participates in the binding of the divalent cation or that the conformation of the channel is modified by the surrounding lipid.

Recently, Sandblom *et al.* (1976) have presented a theoretical model for the blocking effects of thallous ions on the gramicidin channel. They assumed that the channel contains four ion binding sites and that up to four univalent cations may be simultaneously present in the channel. An inherent property of such a model is that it contains a large number of parameters, i.e., binding constants and transition frequencies; but as Sandblom *et al.* have shown, the number of parameters may be considerably reduced by introducing a series of plausible assumptions. In particular, Sandblom *et al.* have shown that by a suitable choice of binding constants and transition frequencies the minimum in the conductance-concentration behavior of the channel in mixed K^+/Tl^+ solutions as well as the observed concentration-dependent permeability ratios may be reproduced by the model. For the analysis of blocking effects of divalent cations we have used a much simpler model, assuming that the blocking ion binds to a site which is separate from the pathway of permeating ions in the channel. In principle, the assumption of such a "regulatory" binding site could also explain the blocking effect of thallous ions on channel conductance, as well as the peculiar form of the Eadie-Hofstee plot of the conductance-concentration characteristics in

pure K^+ solutions observed by Sandblom *et al.* For a more critical test of the different blocking models more experimental information seems to be needed, in particular data on the current-voltage behavior of the channel in the presence of Tl^+.

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Appendix

Derivation of Eq. (10)

In a symmetrical system ($c' = c'' = c$) and at low ion concentration c the single-channel current J is given by (Läuger, 1973; Eqs. (21) and (26)):

$$J_c = \frac{e_o v c k'_o (1 - e^{-u})}{1 + \sum_{\nu=1}^{n} S_\nu}, \tag{A1}$$

$$S_\nu = \frac{k''_1 k''_2 \ldots k''_\nu}{k'_1 k'_2 \ldots k'_\nu}. \tag{A2}$$

k'_j and k''_j are the rate constants for jumps from the j-th energy minimum to the left and to the right, respectively; vck'_o is the frequency of jumps from the left aqueous phase into the channel. For the potential profile in Fig. 9 the rate constants are given by $k'_o = k'_{ap}$, $k'_1 = k'_2 = \ldots = k'_{n-1} = k'_i$, $k'_n = k'_{pa}$, $k''_1 = k''_{pa}$, $k''_2 = k''_3 = \ldots = k''_n = k''_i$, $k''_{n+1} = k''_{ap}$. Introducing relations (4)–(8) into Eqs. (A1) and (A2), Eq. (10) is obtained.

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