USE OF WEAK ACIDS TO DETERMINE THE BULK DIFFUSION LIMITATION OF H^+ ION CONDUCTANCE THROUGH THE GRAMICIDIN CHANNEL

E. R. DECKER AND D. G. LEVITT
Department of Physiology, University of Minnesota, Minneapolis, Minnesota 55455

ABSTRACT The addition of 2 M formic acid at pH 3.75 increased the single channel H^+ ion conductance of gramicidin channels 12-fold at 200 mV. Other weak acids (acetic, lactic, oxalic) produce a similar, but smaller increase. Formic acid (and other weak acids) also blocks the K^+ conductance at pH 3.75, but not at pH 6.0 when the anion form predominates. This increased H^+ conductance and K^+ block can be explained by formic acid (HF) binding to the mouth of the gramicidin channel (K_m = 1 M) and providing a source of H^+ ions. A kinetic model is derived, based on the equilibrium binding of formic acid to the channel mouth, that quantitatively predicts the conductance for different mixtures of H^+, K^+, and formic acid. The binding of the neutral formic acid to the mouth of the gramicidin channel is directly supported by the observation that a neutral molecule with a similar structure, formamide (and malonamide and acrylamide), blocks the K^+ conductance at pH 6.0. The H^+ conductance in the presence of formic acid provides a lower bound for the intrinsic conductance of the gramicidin channel when there is no diffusion limitation at the channel mouth. The 12-fold increase in conductance produced by formic acid suggests that >90% of the total resistance to H^+ results from diffusion limitation in the bulk solution.

INTRODUCTION

The conductance of an ion channel may be limited by the rate that ions diffuse through the bulk solution up to and away from the channel mouth. In the limit where the rate of transport through the ion channel becomes very large, bulk solution diffusion may become the rate-limiting step. In the most detailed study of this effect, Andersen (1983*a–c*) showed that the bulk solution probably limited the cation conductance of the gramicidin channel under some conditions. However, these results were indirect and subject to other interpretations (e.g., the rate-limiting step is dehydration and not diffusion [Hladky and Haydon, 1984]).

The origin of the experiments described here was the hope that they would provide a simple quantitative measure of the contribution of the bulk solution to the total H^+ conductance of the gramicidin channel. The idea was that the addition of high concentrations of a weak acid (e.g., formic acid) would provide a sink and source for H^+ ions at the mouth of the channel, significantly reducing the resistance of the bulk solution. The experiments confirmed this prediction—the addition of 2 M formic acid at pH 3.75 increased the H^+ conductance at 200 mV by a factor of 12. (At this pH, which is equal to the pK of formic acid, there are equal concentrations of donors [HF] and acceptors [For^-] of H^+). However, when this experimental result was examined quantitatively, we realized that the original idea of a source and sink for H^+ ions had to be modified because the rate constant for the disassociation of formic acid was too slow.

The purpose of this paper is to determine how formic acid (and other weak acids) increases the H^+ conductance of the gramicidin channel. The results suggest that the neutral form of the formic acid (HF) has an affinity for the channel mouth. This bound form can then dissociate, supplying a H^+ ion to the channel. Although the original idea was incorrect, the experiments still provide a direct quantitative measure of the contribution of the bulk solution to the resistance. The results imply that most, if not all, of the resistance of the gramicidin channel to H^+ is in the bulk solution. The implications of this conclusion are the subject of another paper (Levitt and Decker, 1987).

METHODS

The bilayer (2% glycerol monoolein in hexadecane) membranes were formed on the end of Teflon tube (inside diameter 0.3 mm) and the single-channel conductance was measured as described before (Decker and Levitt, 1983). The bilayers had diameters of ~0.1 mm. The current was filtered through a sixth order low pass Bessel filter with a 3-Hz cutoff frequency. Only channels lasting 1 s or longer were used. The channels were measured by hand. The offset voltage was measured before and after the experiment. Offsets or drifts of more than ±1.5 mV were discarded.

Dr. Decker's current address is Department of Physiology and Molecular Biophysics, Baylor College of Medicine, Houston, TX 77030.

Please address all correspondence to Dr. David Levitt, Department of Physiology, 6-255 Millard Hall, 435 Delaware St. S.E., University of Minnesota, Minneapolis, MN 55455.

For each voltage (V) the channels were measured at $\pm V$ and averaged. Differences of more than 3% between the + and − were discarded.

The pH was measured with a glass electrode designed to be used with Tris and the cation activity was measured with a glass electrode. There was a slow drift in the pH of the tetramethylammonium (TMA) solution and the initial value was used. The formic acid was titrated to the desired pH by addition of TMA OH. Those solutions that did not contain formic acid were buffered with 5 mM aspartic acid. All solutions contained at least 2 mM $MgCl_2$. This (5 mM aspartic plus 2 mM MgCl) is a high enough ionic strength to screen out any surface charge or double layer polarization (Hainsworth and Hladky, 1987) because adding an additional 20 mM $MgCl_2$ did not significantly ($P > 0.05$) change the conductance. The gramicidin (ICN) was presumably a mixture of 72% A, 9% B, and 19% C (Bamberg et al., 1976). The addition of up of 2 M formic acid or formamide did not significantly alter the K^+ activity.

At pH 6.0, no channel activity was detected in 1 M solutions of formate, TMACl, cholineCl, Tris, etc. or any other of the "inert" electrolytes that were used, with the exception of formamide and malonamide. Although no channels could be detected in fresh solutions of these two solutes, small channels became measurable within a few hours of making the solution and they increased over a period of days. Amide solutions have been shown to undergo hydrolysis with the release of NH_4^+ (Morrison and Boyd, 1973). Cation activity was detected in the amide solutions using a glass electrode and the activity and single-channel conductance was consistent with an NH_4^+ contaminant. All the experiments reported here were performed using fresh solutions that had a single-channel conductance less than that of a 1 mM NH_4^+ solution.

RESULTS

Accuracy and Frequency Distribution of Single Channel H^+ Conductance

Fig. 1 shows a typical current recording for a solution at a pH of 3.75 and an applied voltage of 50 mV. (The size of the channels for most of the other conditions examined in this paper is larger and, correspondingly, easier to measure.) Only those channels of duration >1 s (indicated by arrows) were used. Fig. 2 shows a frequency histogram of single-channel conductance for this case. The distribution is similar to that reported for other measurements using pure gramicidin A (Busath and Szabo, 1981; Hladky and Haydon, 1972; Andersen, 1983 *a*). In the averaging of channel conductance, channels deviating by more than 25% from the median were not used. The standard error of the single-channel conductance for a single experiment was ~2% of the mean and was never more than 5%. This is usually small compared with the day-to-day variation. The standard deviation of the day-to-day variation in the average conductance was always <10% of the mean and for the cases where more than 3 d experiments were

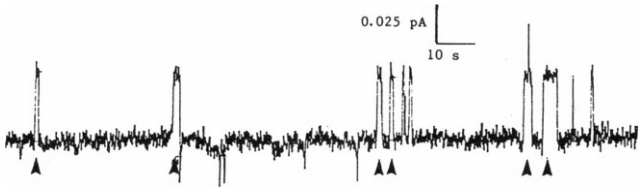

FIGURE 1 Recording of single-channel H^+ current at pH 3.75 and 50 mV. Only the channels with a lifetime >1 s (*arrows*) were used in the calculations.

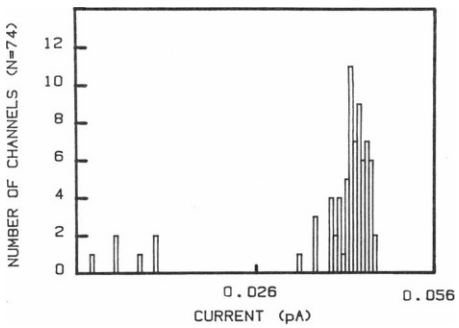

FIGURE 2 Histogram of frequency of channel events (pH 3.75; 50 mV). Bin size is 5×10^{-3} pA.

obtained, the standard error was <3%. The size of the symbols used in the figures provides an approximate measure of the standard error which was not more than 20% larger than the symbol.

Effect of Weak Acid on Single-Channel H^+ Conductance

Fig. 3 shows the voltage dependence of the single-channel current at pH 3.75 for the control solution (2 mM $MgCl_2$, 5 mM aspartic acid) and with the addition of varying amounts of formic acid. The voltage dependence of the H^+ current in the absence of formic acid is similar to that previously reported by Eisenman et al. (1980). It can be seen that formic acid produces a marked increase in the single-channel current, increasing the current by 12.5-fold at 200 mV (at 2 M). The addition of formic acid also changes the shape of the I-V curve from sub-ohmic to roughly linear. The possibility that the increased current in the presence of formic acid results from the conductance of either the formate ion (For^-) or an impurity in the formic acid is ruled out by the fact that no channels could be detected in 1 M formate solution at pH 6 (channels > 0.007 pA could have been detected). Also, this effect of formic acid was not the result of its osmotic or ionic

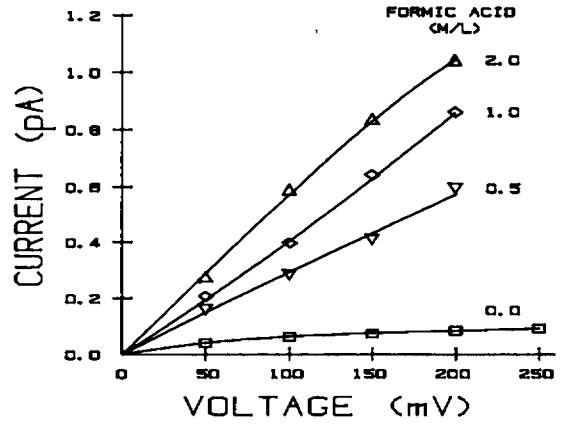

FIGURE 3 Single-channel current versus voltage at pH 3.75 for different concentrations of formic acid. The solid lines were fitted to the points by eye.

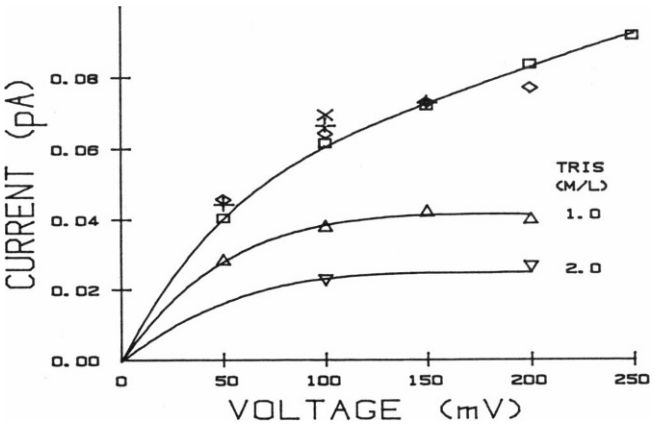

FIGURE 4 Influence of "inert" electrolytes on single-channel H^+ current at pH 3.75: (□) no inert electrolyte; (+) 1 M TMA; (◊) 1 M choline; (×) 2 M choline; (△) 1 M Tris; (▽) 2 M Tris. The top solid curve is drawn through the "no inert electrolyte" data.

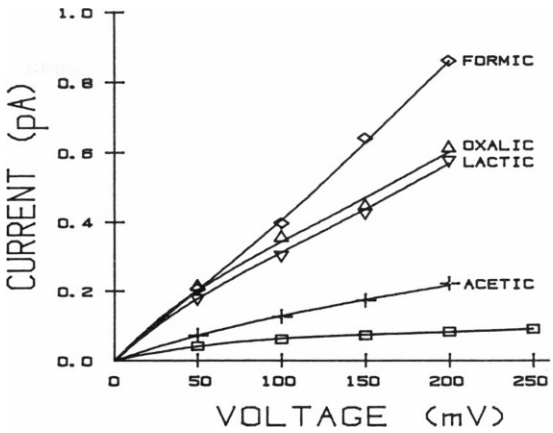

FIGURE 6 Effect of weak acids (1 M) on single-channel current at pH 3.75: (□) no inert electrolyte; (◊) formic acid; (△) oxalic acid; (▽) lactic acid; (+) acetic acid.

strength, because the addition of 2 M choline chloride or TMA (Fig. 4) changed the conductance by only a small amount. (Although the effect of choline is small, it is significant and is the subject of the second paper.) In contrast to choline and TMA, Tris (Fig. 4) produced a significant reduction in the H^+ conductance, suggesting that it is specifically binding to and blocking the channel and, therefore, cannot be regarded as an "inert" electrolyte. Fig. 5 shows the effect of formic acid at a pH of 2.75. The effect of 1 M acetic, lactic, and oxalic acid at pH 3.75 is shown in Fig. 6.

Asymmetric Weak Acid Solutions

In order to determine which side of the channel the weak acid acted on, the single-channel conductance was measured with 1 M oxalic acid in the front chamber and 1 M TMA in the back chamber, both at pH 3.75. Formic acid could not be used in this experiment because the neutral form has a high bilayer permeability (Walter and Gutnecht, 1984). Oxalic acid has a second carboxyl group (pK 1.2) that is charged at pH 3.75, so that it has a low bilayer permeability. The current is highly asymmetric (Fig. 7). Also shown in Fig. 7 is the current for symmetric TMA and symmetric oxalic acid solutions. At high applied voltages, the current in the direction from the oxalic acid to the TMA solution becomes equal to that in symmetric oxalic acid and the current from the TMA to the oxalic acid becomes equal to that for symmetric TMA. This indicates that the presence (or absence) of an oxalic acid bound to the opposite end of the channel does not alter either the simple H^+ or the oxalic acid induced H^+ flux.

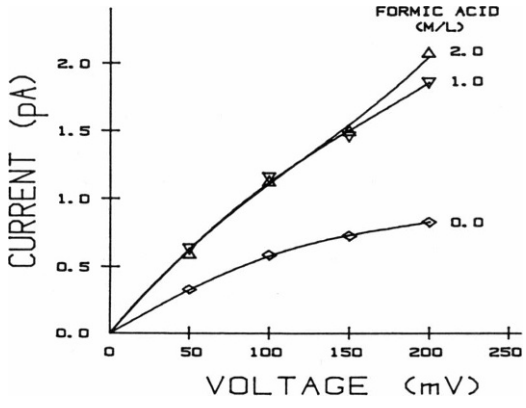

FIGURE 5 Single-channel current versus voltage at pH 2.75 for different concentrations of formic acid.

Interaction Between H^+, K^+, and Formic Acid

The single-channel current of a 100 mM KCl solution in the absence (pH 6.0) and presence of H^+ (pH 3.75 and 2.75) is shown in Fig. 8. The observed conductance at pH

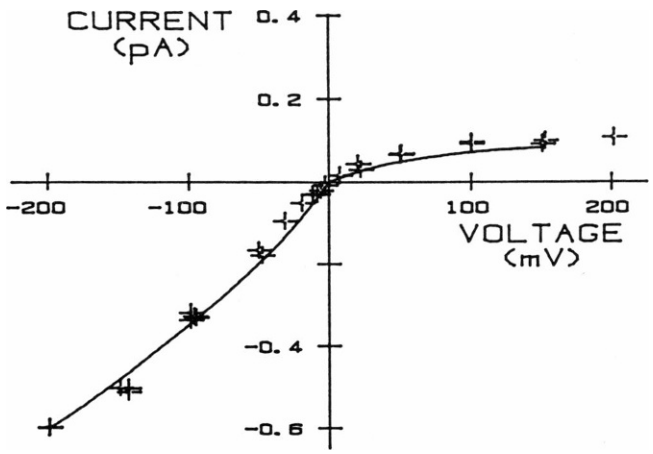

FIGURE 7 Single-channel conductance (+) at pH 3.75 in asymmetrical solutions: front chamber 1 M TMA; back chamber 1 M oxalic acid (a positive voltage means the front chamber is positive with respect to the back). The solid line for positive voltage is the current in symmetrical TMA (Fig. 4) and the solid line for negative voltage is the current in symmetrical oxalic acid (Fig. 6).

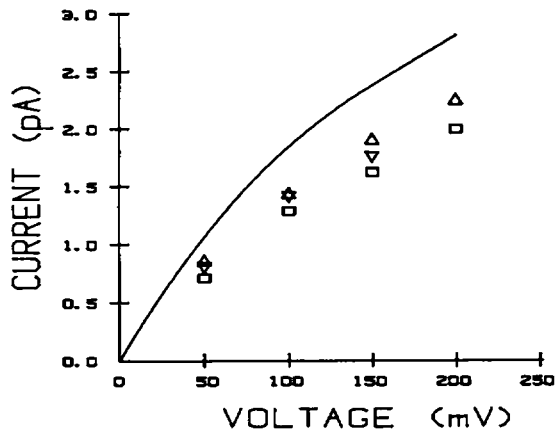

FIGURE 8 Single-channel conductance in 100 mM KCl at pH 6.0 (□); pH 3.75 (▽); and at pH 2.75 (△). The line is the theoretical sum of the conductance of 100 mM KCl at pH 6.0 (i.e., no H^+) plus the conductance at pH 2.75 and no K^+.

2.75 when both H^+ and K^+ are present is less than the theoretical sum of the conductances when only one of the ions is present (Fig. 8), indicating that there is some interaction between the two ions. Fig. 9, *A* and *B* shows the interaction between K^+ and formic acid at pH 3.75 and 2.75. If there were no interaction between formic acid and K^+, the addition of formic acid to a KCl solution should increase the H^+ and, therefore, the total conductance. In fact, the addition of formic acid decreases the total conductance, indicating strong interaction. This interaction is most obvious at pH 2.75 where the conductance decreases when 100 mM KCl is added to a 2 M formic acid solution! The simplest explanation for this interaction is that formic acid specifically binds to the gramicidin channel, blocking the K^+ conductance. The addition of 2 M formate at pH 6.0 (when 98% is in the formate ion form) did not significantly alter the conductance of a 100 mM KCl solution (not shown), demonstrating that only the neutral form of formic acid can bind to the channel. The other

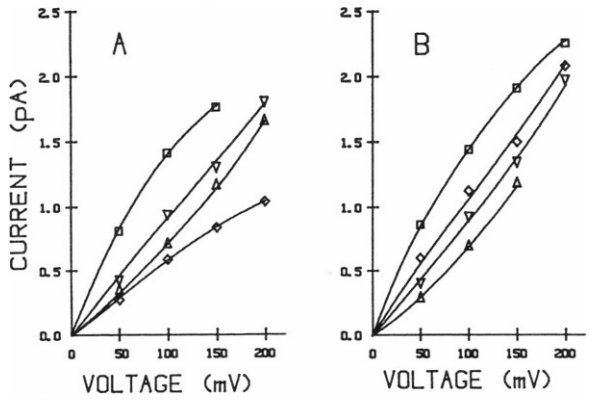

FIGURE 9 Interaction between K^+, H^+ and formic acid at pH 3.75 (*A*) and pH 2.75 (*B*). (□) 100 mM KCl; (◇) 2 M formic acid; (▽) 100 mM KCl with 1 M formic acid; (△) 100 mM KCl with 2 M formic acid.

weak acids had a similar but smaller block of the K^+ conductance (Fig. 10).

Interaction Between K^+ and Amides

The observation that the uncharged formic acid could bind to and block the gramicidin channel was the stimulus for looking at the effects of the (uncharged) amides on the K^+ conductance at a pH of 6.0. Formamide (2 M) also blocked the K^+ conductance, decreasing the conductance of a 100 mM KCl solution by 66% at 100 mV. Malonamide (1 M) produced a 26% block and acrylamide (1 M) produced a 9% block. This block of K^+ conductance does not seem to be competitive because 2 M formamide produced the same percentage block at 100 mM and 1 M KCl.

DISCUSSION

The original idea behind these experiments was that high concentrations of H^+ buffers in the solution would act as sources and sinks at the mouth of the channel, effectively short-circuiting the bulk solution contribution to the total channel resistance. The magnitude of this effect can be evaluated quantitatively by considering a simplified model in which it is assumed that transport within the channel is very fast with the rate-limiting step determined by the rate that ions can diffuse through the bulk solution up to the channel mouth. The position of the "mouth" is described by a hemispherical surface inscribed about the channel end with a "capture" radius *a*. For this model, as the voltage is increased, the flux rises and then levels off and the value of this saturating flux (J_H) is described by (see Appendix)

$$J_H = 2\pi a D_H[H](1 + a\lambda)$$

$$\lambda^2 = k_d[HA]/([H]D_H), \qquad (1)$$

where D_H is the bulk diffusion coefficient of H^+, *a* is the

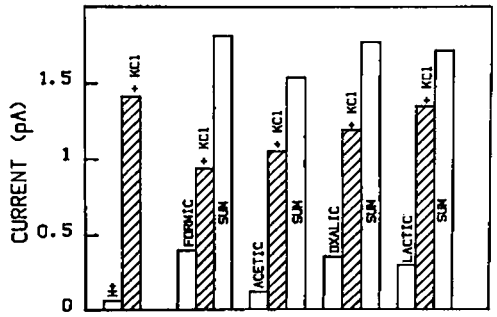

FIGURE 10 Interaction between K^+, H^+ and formic, acetic oxalic, and lactic acid. For each weak acid, the first bar is the single current for the weak acid alone, the second bar is the current for the weak acid with 100 mM KCl, and the third bar is the theoretical sum of the current when the weak acid and KCl are each present alone at pH 3.75. The third bar is the expected conductance if there were no interaction between formic acid, H^+ and K^+. The difference between the second and third bars is a measure of the block of the K^+ conductance by the weak acid.

capture radius, k_d is the dissociation rate constant of the weak acid, and [H] and [HA] are the bulk concentrations of H^+ and the neutral form of the buffer. This equation for the capture radius assumes that (*a*) the H^+ concentration at the capture radius is zero and (*b*) there are no voltage gradients in the bulk solution. The first assumption will be approximately satisfied if Eq. 1 is applied to the saturating flux (J_H) that occurs at high applied voltages. Although the second assumption is not rigorously correct, Eq. 1 should provide a qualitative estimate of the capture radius and effect of the formic acid buffer. A more accurate physical theory for the "capture" radius is presented in the second paper (Levitt and Decker, 1987).

The value of the capture radius can be estimated from the limiting conductance in the absence of weak acid ($\lambda = 0$). Using the current of 0.084 pA at 200 mV (Fig. 4) at pH 3.75 ([H] = 1.78×10^{-4} M) and $D_H = 9.3 \times 10^{-5}$ cm^2/s (Hille, 1984), yields an *a* of 0.87 Å. (This is similar to the values determined by Anderson [1983 *c*] for the monovalent cations.) Using this value of *a* and the dissociation rate constant of 8.6×10^6/s (Eigen et al., 1969), the value of λa for 2 M formic acid at pH 3.75 is 0.2. This implies that the addition of 2 M formic acid should increase the conductance at high voltages by only ~20%, much less than the 12-fold increase that is experimentally observed.

One possible explanation for the large increase in conductance is that formic acid increased the capture radius (*a*). An increase from the 0.87 Å value in the absence of formic acid to a value of ~6 Å would account for the 12-fold increase that is observed. Although this is a possible explanation, it does not seem likely because there is no physical reason to expect formic acid to increase the capture radius and it is not consistent with the observed block of the K^+ conductance by formic acid (see below). Another possibility is that the dissociation rate constant is increased by the voltage gradient at the channel mouth. This increase, known as the Wien effect, can be approximated by the equation (McIlroy and Mason, 1976):

$$k_d(E)/k_d(0) = 1 + 0.14\,E, \qquad (2)$$

where $k_d(E)$ is the dissociation rate in an electric field E (in mV/Å). The field at the channel mouth can be estimated from the electrostatic calculations of Jordan (1982) and at an applied voltage of 200 mV should have a value of ~5 mV/Å. This field would increase the dissociation rate (k_d) by ~70% (Eq. 2) which would increase the limiting flux (Eq. 1) by only 5%, a negligible effect.

The explanation that seems most consistent with the observations is that formic acid has a specific affinity for binding to the channel mouth. At high applied voltages, there will be a large voltage drop in the channel and each H^+ that dissociates will be transported through the channel. If the rate of binding and unbinding is fast, then at high concentrations, when the channel mouth was saturated with formic acid, the limiting conductance at high voltages will be equal to the dissociation rate (k_d) of the acid. The k_d of formic acid is 8.6×10^6/s, corresponding to a current of 1.38 pA. This is 17 times that of the conductance in the absence of formic acid and is greater than the maximum conductance (1 pA) observed in 2 M formic acid, indicating that this mechanism is, at least, consistent with the observations. The results with the other weak acids are also consistent. For example, the dissociation constant of acetic acid (7.8×10^5/s) is only 9% that of formic acid, and therefore the maximum current should only be 0.125 pA, slightly greater than the maximum observed current of 0.12 pA. This explanation also requires that the anion form (For^-) has a low affinity for the channel, rapidly dissociating and allowing another formic acid to bind. For this explanation, the electric field induced increase in the dissociation rate constant (Wien effect) could make a large contribution to the voltage dependence of the current that is observed. For example, the 70% increase in the rate predicted above for 200 mV should produce a 70% increase in current.

The observation that formic acid (but not For^-) blocks the K^+ conductance of the gramicidin channel provides direct support for this explanation. Qualitatively, this is seen in the decrease in conductance when formic acid is added to 100 mM KCl at pH 3.75 or 2.75 (Fig. 9). The observation that the conductance when both K^+ and formic acid are present together is less than the conductance when either is present alone indicates that both K^+ and formic acid must be able to bind simultaneously to the channel. Otherwise, the conductance would have to be some weighted average of the two conditions.

Quantitatively, the conductance is the sum of three terms: the conductance of H^+ alone, the conductance of H^+ in channels in which formic acid is bound, and the K^+ conductance. A simplified kinetic model is derived in the Appendix. The final expression for the conductance (G) as a function of the H^+ ([H]), formic acid ([HF]) and K^+ ([K]) concentration is

$$G = \frac{V_H h + V_F f}{(1+f)(1+2g)} + \frac{G_K}{(1+f)^2}$$

$$h = [H]/K_h \qquad f = [HF]/K_f \qquad g = [K]/K_k, \qquad (3)$$

where K_f and K_k are the equilibrium dissociation constants for binding of formic acid and K^+ to the channel end, $V_H h$ is the H^+ conductance in the absence of K^+ and formic acid, V_F is the maximum value of the formic acid stimulated H^+ flux, and G_K is the K^+ conductance in the absence of H^+ and formic acid. A comparison of this theoretical prediction and the experimental value of the conductance at 100 mV for 12 different combinations of H^+, K^+, and formic acid is summarized in Table I. Four parameters were used in fitting this data: (*a*) the experimental H^+ conductance (at 100 mV) at pH 3.75 in the absence of formic acid and K^+ ($V_H h = 0.62$ pS); (*b*) the experimental

TABLE I
COMPARISON OF THEORETICAL AND EXPERIMENTAL (IN PARENTHESES) CONDUCTANCE (pS) AT 100 mV FOR DIFFERENT SOLUTIONS OF H^+, K^+, AND FORMIC ACID

	H^+	Formic acid (no K^+)		100 mM KCl		
	(no K^+, HF)	1 M	2 M	No formic	1 M Formic	2 M Formic
pH 3.75	0.62 (0.62)	4.41 (4.0)	6.03 (5.88)	13.2 (14.1)	7.94 (9.37)	6.38 (7.1)
pH 2.75	6.2 (5.90)	8.9 (11.6)	10.0 (11.3)	16.0 (14.4)	8.02 (9.22)	6.59 (6.95)

K^+ conductance (at 100 mV) at 100 mM in the absence of H^+ or formic acid (G_K = 12.9 pS); (*c*) the empirically adjusted formic acid binding constant (K_f = 1 M); and (*d*) the empirically adjusted K^+ binding constant (K_k = 200 mM), which is within the range of values determined by fitting the single-channel conductance data (Hladky and Haydon, 1984).

The agreement between theory and experiment is quite good, lending support to the assumptions of the model that are summarized here: (*a*) At high formic acid (HF) concentration (when the channel mouth is saturated with HF) the rate-limiting step in the H^+ conductance is the dissociation rate of HF. This requires that the rate of binding of HF and unbinding of F^- must be fast compared with the dissociation rate ($k_d = 8.6 \times 10^6$/s) of formic acid. This condition would be satisfied if the binding of HF were diffusion limited since it would then have a binding rate of $\sim 4 \times 10^8$/s (assuming a capture radius of 1 Å, diffusion coefficient of 10^{-5} cm^2/s and HF concentration of 1 M). This assumption is also consistent with the experimental observations that support a superficial HF channel binding site (see *c* below). (*b*) The formic acid molecule has an affinity of ~1 M for the channel mouth. There is no interaction of formic acid between the channel ends, and the binding constant for the second formic is also 1 M. When the formic acid dissociates, releasing the H^+ ion into the channel, the formate ion immediately dissociates from the channel mouth which equilibrates with the formic acid in the bulk solution. The presence of a formic acid at the opposite end does not affect the rate of this formic acid-induced transport. (*c*) Formic acid can bind at either end of the K^+ occupied channel, regardless of the position of the K^+ ion. This implies that formic acid is binding superficially, external to the K^+ binding site. The most direct support for this assumption comes from experiments which indicate that the fractional block by formamide is independent of the [K^+] (100 mM to 1 M) concentration. This would not be expected if the presence of K^+ in the channel decreased the probability of formamide binding. (*d*) The rate that H^+ crosses the membrane is not affected by the presence of a formic acid bound at the opposite end of the channel. This is directly shown by the asymmetric oxalic acid experiment (Fig. 7). In contrast, the presence of a formic acid at either end, blocks the K^+ conductance. This assumption is necessary to explain the observation that the conductance in the presence of both K^+ and formic acid is less than when either one is present alone. A possible physical explanation for this difference between K^+ and H^+ is that movement of K^+ through the single file channel requires movement of all the channel water and could not occur when a formic acid is bound (Levitt, 1984). In contrast, H^+ moves by a hopping mechanism that does not involve net water movement, and might occur when HF is bound at the opposite end. (*e*) The fraction of channels occupied by H^+ is small at the highest concentration of H^+ used (pH = 2.75, [H] = 1.78 mM). This follows directly from the nearly 10-fold increase in conductance that results from the 10-fold increase in H^+ concentration (Figs. 3 and 4; conductance at pH 2.75/pH 3.75 = 9.4 at 100 mV, 10.0 at 150 mV, and 9.9 at 200 mV). If a significant fraction of the channels contained H^+, one would expect to see some nonlinearity resulting from the interaction between H^+ ions.

The specific binding of a neutral molecule (formic acid or formamide) to the gramicidin channel is a surprising result. The binding is of low affinity (~1 M) and we had hoped that it would be possible to find amides with a higher affinity. However, our preliminary investigation of a number of amides has been discouraging, with formamide (and formic acid) having the highest affinity (as determined by their block of the K^+ conductance). This suggests that the small size of the molecules somehow contributes to their relatively high affinity.

Although the simple hypothesis for the mechanism of action of formic acid was wrong, these experiments should still provide a measure of the contribution of bulk diffusion to the total H^+ resistance. The total resistance to H^+ in the absence of formic acid can be divided into five terms:

$$R_H = R_D^1 + R_e^1 + R_c + R_e^2 + R_D^2 = R_D + R_e + R_c, \quad (4)$$

where R_D is the bulk diffusion resistance, R_e is the resistance involved in entering and leaving the channel, and R_c is the intrinsic resistance of the channel. In the presence of a saturating concentration of formic acid, the resistance can be written as

$$R_F = R_k^1 + R_c + R_k^2, \quad (5)$$

where R_k is the resistance associated with the dissociation of the formic acid. Since the formic acid is binding at a very superficial site, the R_c in this expression should include the same channel region as in Eq. 4. It has been

shown that the H^+ transport through the gramicidin channel involves the same proton jump mechanism that occurs in bulk liquid (Myers and Haydon, 1972; Levitt et al., 1978). Since all that is required for the H^+ to enter the channel is the formation of a hydrogen bond with the water at the channel end, R_e should be very small. If one assumes that R_e is negligible compared with R_D, then

$$R_D/R_H > 1 - G_H/G_F \simeq 0.95,$$

where G_F, the conductance (R^{-1}) with a saturating concentration of formic acid, is equal to ~18 times G_H (the conductance without formic acid) at 200 mV (using the K_f of 1 M to extrapolate to infinite formic acid). Thus, >95% of the H^+ resistance in the absence of formic acid results from the diffusion limitation in the bulk solution.

APPENDIX

Effect of Formic Acid on the Diffusion Limitation of H^+

It is assumed that diffusion occurs along the radii drawn from the center of the channel mouth so that there is a uniform concentration on each hemispherical shell. In the bulk solution (at a long distance from the channel), the formic acid is in equilibrium:

$$HF \underset{k_a}{\overset{k_d}{\rightleftharpoons}} H^+ + F^- \qquad C_0 = k_d[HF]/k_a[F] \qquad (1A)$$

where C_0 is the bulk solution concentration of H^+. Since the formic acid is present at a concentration ~10,000 times that of H^+, the concentration of HF and F^- will not differ significantly from the bulk values. The diffusion limited rate of H^+ entering the channel is defined by the condition that the concentration of H^+ is zero on the hemispherical surface that has a "capture" radius a. This condition should occur in the limit of high applied voltage when there is large force that immediately transfers any H^+ reaching the channel mouth to the other side (assuming that there is no potential gradient in the solution external to the capture radius). The steady state H^+ concentration (C) is then described by the differential equation:

$$\frac{d^2C}{dr^2} + \frac{2}{r}\frac{dC}{dr} - \lambda^2 C = -\lambda^2 C_0 \qquad \lambda^2 = k_a[F]/D. \qquad (2A)$$

Boundary condition: $C = 0$ at $r = a$; $C = C_0$ at $r = \infty$ where D is the diffusion coefficient of H^+. This equation has a simple solution:

$$C/C_0 = 1 - (a/r)e^{-\lambda(r-a)}. \qquad (3A)$$

The final result for the diffusion limited H^+ flux at high applied voltage (J_H) is

$$J_H = 2\pi a^2 D \left.\frac{dC}{dr}\right|_{r=a} = 2\pi a D(1 + \lambda a)C_0. \qquad (4A)$$

Kinetic Model for the Dependence of Single-Channel Conductance on H^+, K^+, and Formic Acid Concentration

Only H^+ Present. It will be assumed throughout this appendix that the kinetics can be approximated by the one ion form of the gramicidin reaction-rate model (Hladky and Haydon, 1984). The low voltage H^+ conductance for the symmetric channel (with symmetric solutions) can then be written in the Michaelis-Menten form:

$$G_H = \frac{V_H h}{1 + h}; \qquad h = \frac{[H^+]}{K_H}; \qquad K_H = \frac{B}{2A};$$

$$V_H = (e^2/kT)\frac{BC}{2(B + 2C)}, \qquad (5A)$$

where A, B, and C are the rates of entering, leaving, and crossing the channel, respectively. The constant K_H is equal to ½ the equilibrium dissociation constant (B/A) for binding at one end. Although this expression is strictly valid only in the low voltage limit, it will be assumed that it is correct at any voltage with V_H and K_H functions of voltage. Since a 10-fold increase in H^+ ion concentration (pH 3.75–2.75) produced a 10-fold increase in conductance, the channel is apparently far from saturation ($[H^+] \ll K_H$) at the highest H^+ concentration used (pH 2.75):

$$G_H(0) \simeq V_H h, \qquad (6A)$$

where the 0 indicates the absence of formic acid.

H^+ With Formic Acid. The conductance is now the sum of two terms:

$$G = G_H(F) + G_F(F). \qquad (7A)$$

The first term is the H^+ conductance through the channels by the above mechanism (not due to formic acid) and the second term is the conductance associated with the formic acid. The first term is derived by assuming that H^+ can enter the left end of the channel in either the empty (P_0) state or the state that has a formic acid at the right end (P_{of}) (with similar conditions for the right end). It is assumed that if HF is bound at one end of the channel, then H^+ cannot enter the channel from that end, but the presence of an HF at the far end of the channel does not limit the H^+ flux towards that end. It is also assumed that formic acid binding to the channel ends is at equilibrium and there is no interaction between the ends (formic acid is uncharged) so that the binding of the first and second (at the opposite end) formic acid have the same equilibrium dissociation constant (K_f). With these assumptions, the first term is

$$G_H(F) = G_H(0)/(1 + f) \qquad f = [HF]/K_f, \qquad (8A)$$

where [HF] is the formic acid (HF) concentration. The basic assumption used in the derivation of the formic acid induced H^+ conductance is that the rate of H^+ crossing the channel from the left to the right end is described by $E \times (P_{fo} + P_{ff})$, where E is a rate constant (related to the dissociation rate constant of formic acid) and P_{fo} and P_{ff} are the probability of the states with a formic acid bound at the left end and both ends, respectively. (As above, it is assumed that the presence of formic acid at the opposite end does not influence the rate of H^+ crossing membrane.) Using the equilibrium binding of the formic acid, leads to a simple expression for this component of the H^+ flux:

$$G_F(F) = V_F f/(1 + f) \qquad V_F = (e^2/kT)E. \qquad (9A)$$

These two terms (Eqs. 4A and 5A) then describe the total conductance (Eq. 3A) in the presence of formic acid.

H^+ Plus Formic Acid Plus K^+. The conductance is now the sum of three terms:

$$G = G_H(F, K) + G_F(F, K) + G_K(F, K). \qquad (10A)$$

The first two terms are similar to those derived above, but now corrected for the fraction of channels that are occupied by K^+ (blocking the H^+ conductance), making the simplifying assumption that K^+ is at equilib-

rium at the channel ends:

$$G_H(F, K) = \frac{G_H(0)}{(1 + f)(1 + 2g)}; \qquad g = [K]/K_k,$$

$$G_F(F, K) = \frac{V_f f}{(1 + f)(1 + 2g)} \tag{11A}$$

where K_k is the equilibrium dissociation constant for the binding of K^+ at the channel end ($K_k/2$ is the apparent K_m for the one-ion K^+ channel). In the absence of formic acid, the K^+ conductance would be described by a one-ion equation of the form of Eq. 1A. (Since the H^+ ion concentration is not high enough to significantly saturate the channel, it does not influence the K^+ conductance.) This equation is modified by the presence of formic acid which is assumed to be able to bind to either end of the channel, whether or not K^+ is already bound, blocking the K^+ conductance. This means that each conducting state, e.g., P_{ko}, is in equilibrium with three other blocked states, e.g., P_{kf}, P_{fko}, and P_{fkf}. This leads to a reduction of each of the rate constants by the factor $(1 + f)^2$ and the following expression for the K^+ conductance:

$$G_K(K, F) = G_K/(1 + f)^2, \tag{12A}$$

where G_K is the K^+ conductance measured in the absence of formate. The total conductance (Eq. 3) is then the sum of Eqs. 11A and 12A (using Eq. 6A).

Received for publication 13 January 1987 and in final form 4 June 1987.

REFERENCES

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

Andersen, O. S. 1983*b*. Ion movement through gramicidin A channels. Interfacial polarization effects on single-channel current measurements. *Biophys. J.* 41:135–146.

Andersen, O. S. 1983*c*. Ion movement through gramicidin A channels. Studies on diffusion-controlled association step. *Biophys. J.* 41:147–165.

Bamberg, E., K. Noda, E. Gross, and P. Lauger. 1976. Single-channel parameters of gramicidin A, B, and C. *Biochim. Biophys. Acta.* 419:223–228.

Busath, D., and G. Szabo. 1981. Gramicidin forms multi-state rectifying channels. *Nature* (*Lond.*). 294:371–373.

Decker, E. R., and D. G. Levitt. 1983. Comparison of the gramicidin A potassium/sodium permeability and single channel conductance ratio. *Biochim. Biophys. Acta.* 730:178–180.

Eigen, M., W. Kruse, G. Maass, and L. De Maeyer. 1964. Rate constants of protolytic reactions in aqueous solution. *In* Progress in Reaction Kinetics. Vol. 2. G. Porter, editor. MacMillan Publishing Co., New York. 285–318.

Eisenman, G., B. Enos, J. Hagglund, and J. Sandblom. 1980. Gramicidin as an example of a single-filing ionic channel. *Ann. NY Acad. Sci.* 339:8–20.

Hainsworth, A. H., and S. B. Hladky. 1987. The effects of double-layer polarization on ion transport. *Biophys. J.* 52:109–113.

Hille, B. 1984. Ionic Channels of Excitable Membranes. Sinauer Associates, Inc. Sunderland, MA. 157.

Hladky, S. B., and D. A. Haydon. 1972. Ion transfer across lipid membranes in the presence of gramicidin A. *Biochim. Biophys. Acta.* 274:294–312.

Hladky, S. B., and D. A. Haydon. 1984. Ion movements in gramicidin channels. *Curr. Top. Membr. Transp.* 21:327–372.

Jordan, P. C. 1982. Electrostatic modeling of ion pores. Energy barriers and electric field profiles. *Biophys. J.* 39:157–164.

Levitt, D. G., Elias, S. R., and J. M. Hautman. 1978. Number of water molecules coupled to the transport of sodium, potassium and hydrogen ions via gramicidin, nonactin or valinomycin. *Biochim. Biophys. Acta.* 512:436–451.

Levitt, D. G. 1984. Kinetics of movement in narrow channels. *Curr. Top. Membr. Transp.* 21:181–197.

Levitt, D. G., and E. R. Decker. 1988. Profile of applied voltage in gramicidin channel determined from voltage dependence of H^+ ion conductance. *Biophys. J.* 53:000–000.

McIlroy, D. K., and D. P. Mason. 1976. Wien dissociation in very low intensity electric fields. *Faraday Soc. Trans.* II:590–596.

Morrison, R. T., and R. N. Boyd. 1973. *Organic Chemistry.* Allyh and Bacon, Inc., Boston, MA. 671–672.

Myers, V. A., and D. A. Haydon. 1972. Ion transfer across lipid membrane in the presence of gramicidin A. II. The ion selectivity. *Biochim. Biophys. Acta.* 274:313–322.

Walter, A., and J. Gutknecht. 1984. Monocarboxylic acid permeation through lipid bilayer membranes. *J. Membr. Biol.* 77:255–264.