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Phallolysin
A mushroom toxin, forms proton and voltage gated membrane channels

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Abstract. Phallolysin, a water soluble protein of M_r 34,000 produced by the poisonous mushroom *Amanita phalloides*, causes lysis of various mammalian cell types. Lysis is thought to be initiated by the formation of ion permeable membrane channels. We therefore studied the interaction of phallolysin with solvent-free planar lipid bilayers. In the presence of low phallolysin concentrations (10–100 nM) single channel current fluctuations were observed. Unit channel conductances are 44 pS in 500 mM NaCl and 77 pS in 1 M NaCl. Although the channel does not significantly discriminate between alkali cations, its permeability to Cl^- is lower ($P_{K^+}/P_{Cl^-} = 4/1$). Gating kinetics display a pronounced bursting behavior and a dependence on membrane voltage, *cis* side pH-value, and on membrane lipid composition. An equivalence relation between membrane voltage and proton concentration was found, i.e. a pH change of one unit is equivalent to a corresponding voltage change of 130 mV. Dependence on the amount of negatively charged lipids is explained by changes of the actual pH due to surface charge effects.

Key words: Reconstitution, *Amanita phalloides*, mushroom toxin, ion channel, lipid dependence

Introduction

As early as 1891 Kobert described the hemolytic activity of aqueous extracts from the poisonous mushroom *Amanita phalloides*, the "deadly agaric". This activity was ascribed to phallolysin, a water soluble and heat labile protein (Faulstich and Weckauf-Bloching 1974; Odenthal et al. 1975; Seeger and Burkhardt 1976) which is structurally quite different from the well-known peptidic toxin families of phalloidin and amanitin (Wieland 1968). More detailed studies showed that cytolysis caused by this toxin is not restricted to erythrocytes; in fact, it induces cytolysis in a great number of other mammalian cell types at concentrations as low as 10 nM (Seeger and Lehmann 1973; Faulstich et al. 1974). A remarkable observation is the considerable difference in toxicity to related cells of different species, e.g. human or cattle erythrocytes (Seeger and Burkhardt 1976; Wieland and Faulstich 1978). Recently, phallolysin was purified to homogeneity and its physical and biochemical properties were studied in detail (Faulstich et al. 1983). At least two, sometimes three, different protein components (phallolysin A, B and C) were identified after separation by isoelectric focusing. Proteins A and B were assayed for their amino acid composition and found to be very similar with respect to their large content of polar amino acids and their molecular mass ($M_r \approx 34{,}000$).

Although much is known about its biochemical properties, the molecular mechanism of cytolysis caused by this toxin remains poorly understood. Neither proteolytic nor phospholipolytic activity could be detected. Saponin-like interactions with cholesterol or detergent-like effects on lipid membranes were excluded (Seeger and Burkhardt 1976). Kinetic studies showed that rupture of red cells after treatment with phallolysin followed alterations of cell membrane properties. Prior to lysis an increase in membrane permeability for cations such as K^+ and Na^+ was observed (Faulstich et al. 1983; Seitz et al. 1981). Unilamellar as well as multilamellar liposomes were found to release ^{14}C-glucose after exposure to phallolysin, provided that negatively charged lipids were part of its lipid composition (Bühring et al. 1983). These findings suggested that phallolysin interacts with lipid bilayers by forming transmembrane channels.

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Abbreviations: 1,3-SMPC 1-stearoyl-3-myristoyl-glycero-2-phosphocholine; 1,2-DOPS 1,2-dioleoyl-glycero-3-phosphoserine

In order to check this hypothesis we studied the effect of phallolysin on virtually solvent-free planar lipid bilayer membranes using electrochemical methods. Our experimental data (Wilmsen 1983) support the concept that the cytolytic activity of phallolysin results from the formation of channels within the lipid bilayer domains of cell membranes. Here we describe for the first time the functional properties of a mushroom toxin on the molecular level of single channel fluctuations. Gating behavior of the protein channel is similar to that described for gated ion channels in vertebrate cells (Sakmann and Neher 1983).

Experimental

Single channel experiments were carried out with partially purified phallolysin samples which were, however, free from peptidic toxins. After the ammonium sulfate precipitation step approximately 20% of the protein content is phallolysin (Faulstich et al. 1983). The apparent specific activity of the samples used was approximately 4,800 hemolytic units per mg protein. Parallel measurements with more highly purified preparations (13,000–16,000 hemolytic units/mg protein, 60–70% purity) revealed no qualitative change in channel activity pattern. Phallolysin was stored as lyophilized powder at 4 °C. For experimental use a stock solution of 1 mg phallolysin per ml of specific electrolyte buffer was prepared and kept at 4 °C. This solution was stable over a period of 4–5 days without loss of membrane-modifying activity.

1-Stearoyl-3-myristoyl-glycero-2-phosphocholine (1,3-SMPC) and 1,2-dioleoyl-phosphoserine (1,2-DOPS) were synthesized as described elsewhere (Eibl 1980; Eibl et al. 1983; Eibl 1984). Cholesterol (puriss.) was purchased from Fluka. Virtually solvent-free planar lipid bilayer membranes were formed according to the Montal-Mueller method (Montal and Mueller 1972) from a solution of 2 mg lipid per ml solvent (hexane/ethanol 9 : 1) on hexane/hexadecane pretreated sandwich septa (Schindler and Feher 1976). Aperture diameter was usually approximately 150 µm. Lipid composition for negatively charged membranes: 1,3-SMPC/1,2-DOPS/cholesterol 7 : 2 : 1 (molar ratio); for neutral membranes: 1,3-SMPC/cholesterol 9 : 1. Temperature: 37 °C, i.e. above the main phase transition temperature of 1,3-SMPC, $t_c = 30$ °C (Stümpel et al. 1983).

Substitution of 1,3-SMPC by the synthetic lipid 1-palmitoyl-2-oleoyl-3-phosphocholine (at 22 °C) led to comparable results. In addition, preliminary experiments were carried out by forming completely solvent-free planar bilayers on the tip of fire-polished glass pipettes (Boheim et al. 1983; Hanke et al. 1984). With this method and the two component system phallolysin/1,3-SMPC, a channel activity pattern similar to that described here was observed.

If not otherwise stated, we used 1 *M* alkali salt solutions supplemented with 10 m*M* buffer: pH 5.5, 10 m*M* Potassium hydrogen phthalate, adjusted with KOH; pH 6.0–7.5, 10 m*M* Hepes, adjusted with KOH; pH 8.1, 10 m*M Tris*, adjusted with HCl.

We refer to the two sides of the bilayer membrane as cis and trans. Phallolysin was always added to the cis side, the trans side was set to virtual ground. Thus the voltage sign refers to the cis side. Current is defined as positive if cations are translocated from cis to trans. Details of the electronic recording system are given elsewhere (Boheim and Kolb 1978; Hanke et al. 1984).

Data was recorded on a tape recorder (Racal Store 4DS). Signals were replayed into a computer (MNC DECLAB 11/23) at a sampling rate of 1,000 points/s. Statistical analysis of single channel current fluctuations followed the procedure described previously (Methfessel and Boheim 1982).

Results

Incorporation of phallolysin into planar bilayers

Phallolysin was added to the cis side compartment at a final concentration of 80 n*M*. Insertion of the protein into the lipid membrane was found to be significantly voltage-dependent. At positive as well as low negative voltages, channel incorporation could hardly be detected. However, after application of large negative voltages ($V \leqq -100$ mV) or of a series of fast jumps to large negative voltages, single channel current fluctuations could be observed within 5 to 15 min. As soon as channel activity was recognized, the negative voltage was reduced and current fluctuations were recorded. With this procedure, only one channel became incorporated in most cases. Once induced, current fluctuations of the particular channel could be observed for several hours, i.e. for the duration of the experiment. No long time inactivation occurred, which indicates the virtual irreversibility of protein incorporation. Presumably as a consequence of this procedure of voltage-induced protein insertion, the channels were found to be oriented nearly exclusively in the same direction within the membrane. This is judged from the asymmetry of voltage and pH dependence of its gating properties. Only 2 out of nearly 100 channels exhibited opposite polarity. Because of the strong dependence of phallolysin insertion on voltage pre-

treatment, it turned out to be difficult to establish a dose/response characteristic under fixed conditions. In some experiments at least four simultaneously active channels were observed.

Phallolysin channel states

The typical current fluctuation pattern at constant applied voltage of a single phallolysin channel is shown in Fig. 1. The upper two traces reveal that open channel events appear in bursts. Between bursts the channel adopts a resting state of zero conductance. Channel states are labelled R_r-"interburst resting state" and R_b – "bursting state", t_r and t_b are the lifetimes of the corresponding states. Their distribution is characterized by mean lifetimes τ_r and τ_b in the second time range. In contrast, transitions between the two conductance levels within bursts reflect much faster gating kinetics, as is demonstrated by the two lower traces in Fig. 1. It shows part of the upper trace at an expanded time scale. The bursting state divides into two discernible substates named R_0 – "open state" and R_c – "closed state". Corresponding lifetimes and mean lifetimes are t_0, t_c and τ_0, τ_c, respectively.

Whereas open state events can be unequivocally identified, this is impossible for closed and resting state events. Both states are characterized by the same zero conductance. Lifetime distributions of R_r and R_c state events might be different, but as a consequence of the statistical nature of the lifetime of single events this characteristic is not transferable to an individual event. Therefore, single channel analysis is based on the following statistical argument. If the transition rates out of the resting or closed state are sufficiently different, then nearly all zero conductance events within bursts consisting of more than 100 events constitute closed events. With respect to the slow interburst kinetics this means that state distributions of R_0 and R_c within bursts are described by the actual equilibrium distribution.

In contrast to the strong dependence of open-closed transition rates on membrane voltage and the pH value of the electrolyte, we did not observe a significant change in interburst kinetics due to alterations of these variables. Here we will, therefore, restrict ourselves to the quantitative analysis of the intraburst kinetics, i.e. the fast gating process. A quantitative evaluation of the slow gating process will be presented in a later publication.

Unit channel conductance

Open state current amplitudes were evaluated as a function of membrane voltage. In each case a linear relationship was obtained. The corresponding constant unit channel conductances are independent of the pH value of the compartment solutions and of membrane lipid composition.

Using the given symmetrical salt solutions the following conductances were measured: 80 pS (1.0 *M* CsCl), 83 pS (1.0 *M* KCl), 77 pS (1.0 *M* NaCl), 36 pS (1.0 *M* LiCl) and 44 pS (0.5 *M* NaCl).

In order to check for the current-carrying ion, the ratio of the channel permeabilities for K^+ and Cl^- (P_{K^+}/P_{Cl^-}) was determined from the reversal potential of the single channel current in a KCl concentration gradient by application of the Goldman equation (Goldman 1943). A reversal potential of 22 mV was extrapolated from the single channel current-

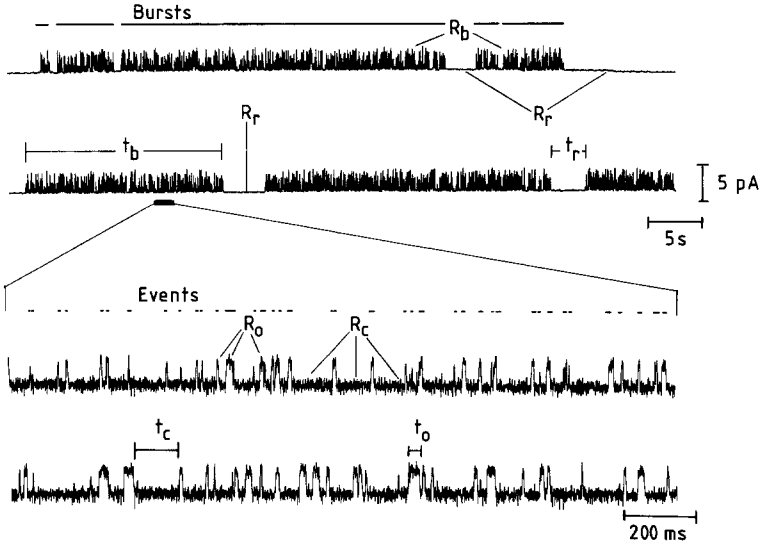

Fig. 1. Typical current fluctuation pattern of a single phallolysin channel in a planar lipid bilayer membrane. *Two upper traces:* Channel activity appears in bursts. Channel states are labelled R_r – "interburst resting state" and R_b – "bursting state", t_r and t_b are lifetimes of the corresponding states. *Two lower traces:* On an extended time scale two substates of the bursting state are resolved. These are labelled R_0 – "open state" and R_c – "closed state", respectively; t_0 and t_c are corresponding state lifetimes. Apparently the channel adopts two states of the same zero conductance. Consequently closed and resting events cannot be unequivocally identified. However, the respective dissimilar rate constants are computable. *Experimental conditions: Lipid:* 1,3-SMPC/1,2-DOPS/cholesterol (7/2/1) Phallolysin (apparent activity 4,800 hemolytic units/mg protein; to cis side only): 80 n*M*. *Electrolyte:* 1 *M* NaCl, 10 m*M* Hepes, pH 7.0. *Applied voltage:* −50 mV. Note that channel openings at negative voltage are drawn upwards in this particular trace only

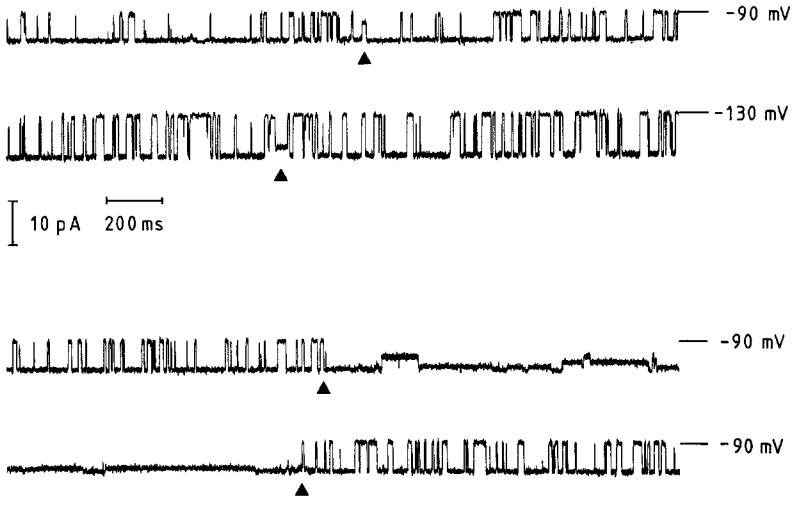

Fig. 2. Substate conductance levels of the phallolysin channel. Quite rare events of lower conductances (*arrows in the two upper traces*) are observed during periods of channel activity. No preference in transition sequence or sub-conductance level could be detected. At pH 6.0 and even more markedly at pH 5.5, open events of another open state with lifetimes significantly longer than those of the R_0 state appear. During such a period a few irregular conductance levels are occasionally adopted as demonstrated in the two lower traces. Arrows mark beginning and end of a particular long open event. *Experimental conditions* are the same as in Fig. 1 except for pH 6.0 and the use of a highly purifed sample of 16,000 hemolytic units/mg protein activity. Channel openings at negative voltage are drawn downwards

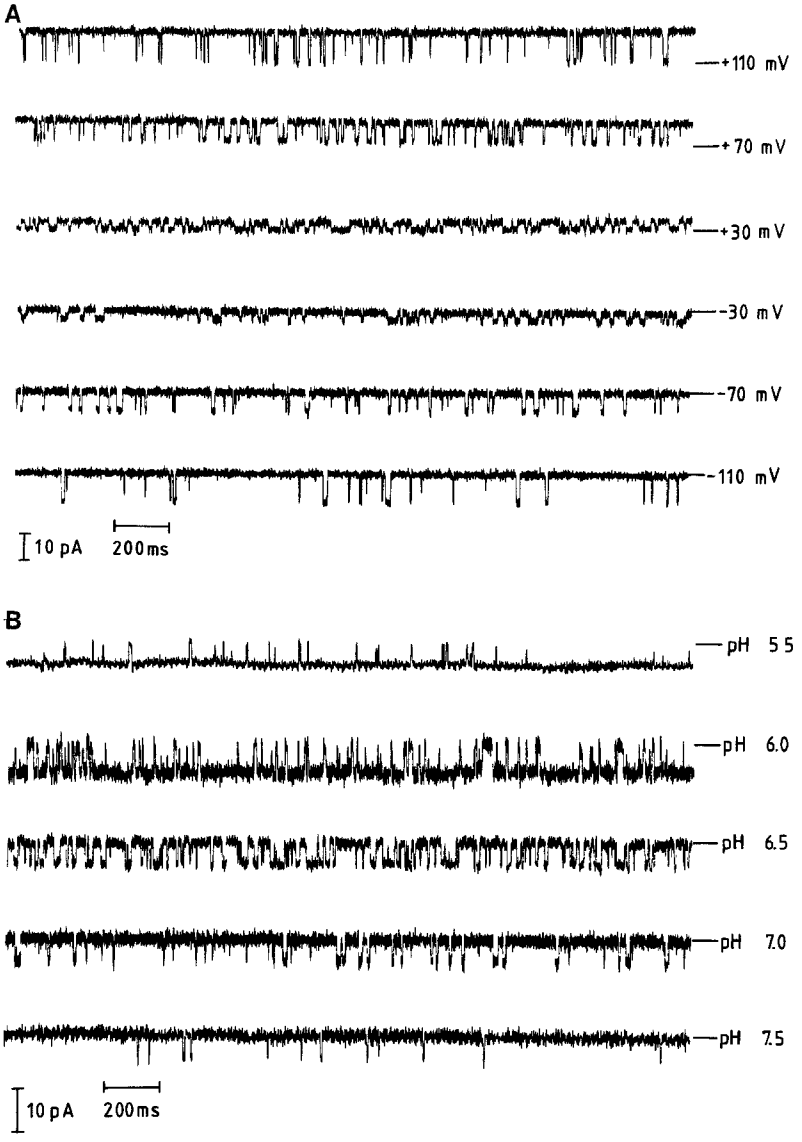

Fig. 3 A and B. Voltage dependence at constant pH (**A**) and pH dependence at constant voltage (**B**) of intraburst fluctuations of single phallolysin channels. Bars on the right indicate closed state current levels, i.e. channels open upwards at positive and downwards at negative voltages. **A** The probability of finding the channel in the open state is high at large positive and low at large negative voltages. Experimental conditions are the same as in Fig. 1. Constant pH: 7.0. **B** The probability of finding the channel in the open state is high at low pH and low at high pH. Phallolysin concentration varied between 16 and 80 n*M*. Membrane voltage at pH 5.5, 6.5 and 7.0: − 50 mV; at pH 6.0: − 60 mV and at pH 7.5: − 52 mV. Otherwise same conditions as in **A**

voltage curve in a 1.0/0.2 *M* KCl gradient, buffered at pH 7.0. From ion activities, a permeability ratio of $P_{K+}/P_{Cl-} = 4/1$ was calculated. Thus phallolysin forms a "cationic channel".

Besides the predominant conductance level, a few events of lower substate conductances are observed. No fixed substate level seems to exist. The two upper traces in Fig. 2 show examples at the arrow marks. Transitions to the substates occur by starting from the closed as well as from the open state.

At pH 6.0 and with increasing probability towards lower pH values another open state appears, and this exhibits significantly longer lifetimes. An example is recorded between the arrows on the lower two current traces of Fig. 2. The particular event passes through various different current levels in addition to the open state level. This is rarely seen with similar events.

Voltage and pH dependence of channel gating

A conspicuous feature of the phallolysin channel is its bursting behavior. In this report we restrict ourselves to a quantitative description of the intraburst kinetics, i.e. transitions between the open and closed states. As mentioned above this reaction constitutes the predominant voltage- and pH-dependent process.

A series of current fluctuation traces at different applied voltages and constant pH 7.0 is presented in Fig. 3A. Bars on the right side indicate closed state levels, i.e. channels open upwards at positive and downwards at negative voltages, respectively. It is clearly seen that the channel state changes from being mainly open at higher positive to preferably being closed at more negative voltages. A similar shift in state distribution is shown in Fig. 3B. Here the membrane voltage is kept constant and the pH of the compartment solutions is changed. At high pH the probability is large for the closed and small for the open state. Towards decreasing pH values this state distribution is inverted.

Collecting all the experimental data together into one graph, a series of curves is obtained in a p_0/V-plot with the pH as shift-inducing parameter (Fig. 4A). p_0 is defined as open state probability, i.e. the mean of the time a channel spends in its open state during bursting periods divided by the mean burst lifetime. In order to compare quantitatively the voltage and pH-induced changes of channel state distributions, we define a reference voltage V_0 by $V = V_0$ at $p_0 = 1/2$. The plot of V_0 versus pH reveals a linear relationship between the two variables (Fig. 4B). The slope of the straight line obtained

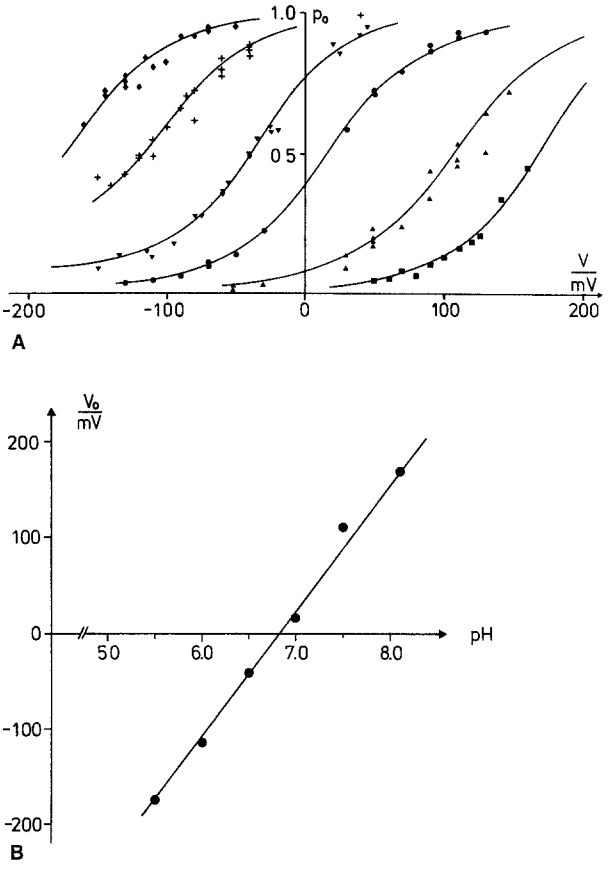

Fig. 4. **A** Plot of the open state probability p_0 as a function of membrane voltage and pH parameter under the conditions of Fig. 3: (◆) pH 5.5, (+) pH 6.0, (▼) pH 6.5, (●) pH 7.0, (▲) pH 7.5 and (■) pH 8.1. **B** Plot of the reference voltage V_0 ($p_0 = 1/2$) from *A* versus the pH parameter. Data points are well fitted by a straight line which reflects an equivalence relation between changes in voltage and pH. The slope of the straight line yields a change of 130 mV per pH unit

yields the result that a 130 mV voltage change is equivalent to a pH shift of 1 unit. This means that channel state distributions are very sensitive to pH, e.g. a scatter of 0.1 unit in pH corresponds to an inaccuracy of 13 mV in the reference voltage V_0 ($p_0 = 1/2$). A more detailed analysis of the voltage and pH dependence of the mean lifetimes of the open and closed states will be presented in a separate report which will also include kinetic model considerations.

Since membrane voltage affects channel gating asymmetrically, we asked if pH-induced changes are observed from both sides or if this effect exhibits sidedness. Figure 5 shows that only a cis side pH change leads to the expected shift in channel state distribution. Altering trans side pH has virtually no effect.

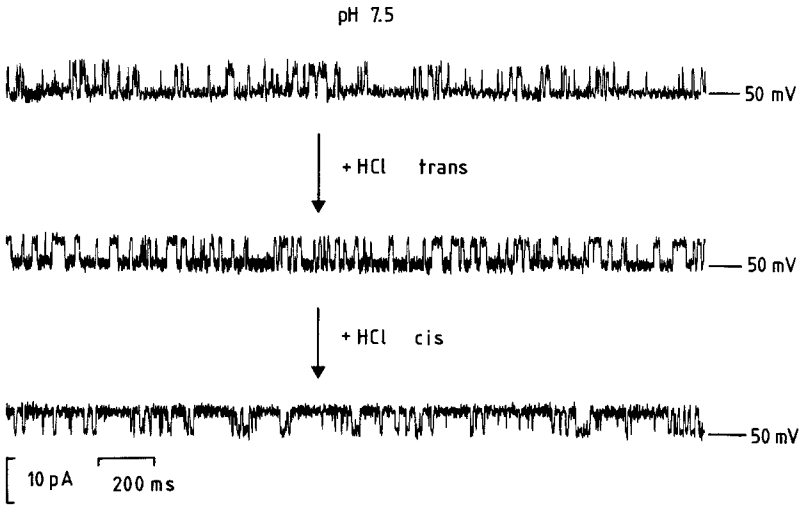

Fig. 5. Sidedness of the sensitivity of the phallolysin channel to protons. Only cis side pH changes are sensed by the channel gate. Starting with symmetrical pH 7.5, first trans side pH was lowered to pH 6.6 by the addition of 50 µl 0.1 *M* HCl. The open state probability remained virtually constant, i.e. $p_0 = 0.23$ (*upper trace*) only changed to $p_0 = 0.27$ (*middle trace*). Then cis side pH was reduced to the same pH 6.6. The lower current fluctuation trace reveals that the open state probability has significantly altered to $p_0 = 0.82$. *Experimental conditions* are the same as in Fig. 1 except of the pH value. Membrane voltage: + 50 mV. Horizontal bars indicate closed state level

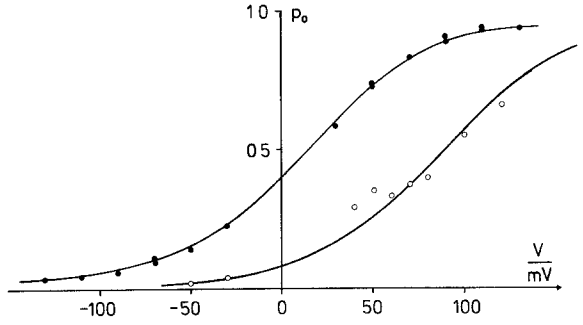

Fig. 6. Lipid dependence of open state probability, p_0. p_0 is plotted as a function of the applied voltage for membranes of two different compositions: (●) charged membranes formed from 1,3-SMPC, 1,2-DOPS, cholesterol (7/2/1) and (○) neutral membranes from 1,3-SMPC, cholesterol (9/1) solutions, respectively. The two curves are similar apart from a shift of approximately 75 mV along the voltage axis. This voltage shift corresponds to an 0.6 unit change in pH according to Fig. 4B. Experimental conditions are the same as in Fig. 1 (pH 7.0)

Lipid charge dependence

From experiments on membane damage of liposomes by phallolysin (Bühring et al. 1983) it was concluded that negatively charged membrane components seem to be necessary for its lysing effect. However, applying the technique of voltage-induced phallolysin incorporation, we observed channel activity independent of membrane composition. Figure 6 shows that the p_0/V characteristic of the open and closed channel states is simply shifted along the voltage axis after removal of the charged lipid component. This shift is indistinguishable from a corresponding pH change of the compartment solution (see Fig. 4A, B). For example, the presence of 20% 1,2-DOPS caused a change of the reference voltage, V_0, from 90 mV to 15 mV, i.e. a 75 mV shift. According to Fig. 4B this equals a pH change from 7.6 to 7.0 or from 7.0 to 6.4. The membrane which contains the larger amount of negatively charged lipids senses the lower pH value. This would be consistent with a surface charge effect. We would like to emphasize the observation that a pH change does not influence open channel conductance but does influence channel gating kinetics.

Discussion

The mushroom toxin phallolysin forms transmembrane ion channels in lipid bilayers. In order to achieve a classification of this channel type with respect to well described classes of channel-forming proteins, we will focus our discussion mainly on two subjects: (1). the comparison of the channel properties with those of other water soluble proteins and of integral membrane proteins and (2). the problem of channel formation from single-chained proteins or cooperatively interacting clusters of protein units (subunits) of comparable molecular size. Hemolysis activity is compared with that of other hemolysing agents.

Channel conductance and selectivity

Open state conductance and selectivity of the phallolysin channel are comparable to those of the acetylcholine receptor channel but quite different from those of the Ca^{++}-dependent K^+ channel. The acetylcholine activated channel has a conductance of 90–95 pS in 1 *M* NaCl (Boheim et al. 1981) and 19 pS for the extrasynaptic or 31 pS for the synaptic channel modification, respectively, in 150 m*M* NaCl

(Hamill and Sakmann 1981). It does not discriminate between alkali cations and is only slightly permeable to anions (Adams et al. 1980). In complete contrast, the Ca^{++}-dependent K^+ channel exhibits a comparatively large conductance: 240 pS in 150 m*M* KCl (Methfessel and Boheim 1982), with very high selectivity for K^+ over Na^+.

Channels which turn out to be only weakly selective for small ions are formed by a variety of water soluble proteins, e.g. the colicins including the *C*-terminal tryptic fragment of colicin E1 (Schein et al. 1978; Davidson et al. 1984), diphtheria and tetanus toxin and their corresponding fragments (Kagan et al. 1981; Boquet and Duflot 1982), sea anemone toxin from *Stoichactis helianthus* (Michaels 1979; Varanda and Finkelstein 1980), the porin family (Benz 1984) and others. Table 1 indicates that although the molecular size and channel selectivity of these proteins are comparable, channel conductances and aggregation state of protein units seem to be different. This may be explained by differences in effective size of channel lumen diameters. Since the elegant work on the 3-dimensional structure of the hydrophobic membrane protein bacteriorhodopsin (Hendersen and Unwin 1975), membrane spanning sequences of L-amino acids are thought to adopt α-helical structure. Amino acid sequence determination of colicin E1 (Davidson et al. 1984) and of the four acetylcholine receptor subunits (Noda et al. 1983) indeed reveal potentially amphipathic helical segments. This led to the proposal of channel structures being built up by a circular array of α-helical rods (Davidson et al. 1984; Guy 1984).

Table 1. Comparison of various non-selective protein channels with respect to conductance (A), molecular mass (M_r) of the protein unit, and aggregation state (number of units per channel, N). Conductance values are given for 1 *M* KCl solution; diphtheria toxin conductance at 1 *M* was extrapolated from the measured value at 0.1 *M* KCl.

Toxin	pH	A [pS]	M_r	N
Porins (Benz 1984):	indep.	1,500–3,000	ca.40,000	3
Sea anemone toxin (Varanda and Finkelstein 1980):	7.0	1,000–2,000	16,000	4
Phallolysin:	indep.	83	34,000	?
Diphtheria toxin fragment (Kagan et al. 1981):	5.5	ca. 50	24.000	2
Colicin E1 fragment (Cleveland et al. 1983):	4.5	20	20,000	1
Acetylcholine receptor (Boheim et al. 1981):	indep.	79	250,000	5
– receptor subunits			40,000–65,000	
Alamethicin (Hanke and Boheim 1980):	indep		ca. 2,000	
– channel level 1		19		3–4
level 2		280		4–5
level 3		1,300		5–6
level 4		2,700		6–7

Such channel structures are described by the "barrel stave model" proposed for monazomycin (Finkelstein and Holz 1973) and alamethicin (Boheim et al. 1983) channels. Alamethicin, a polypeptide of 20 amino acids, forms a single α-helical rod with large intrinsic dipole moment. A multitude of channel states is established by variation of the number of α-helices arranged in a circular array. Conformational transitions between the various states occur by a voltage-dependent flip-flop of the monomer rods due to their high mobility (Boheim et al. 1983). This leads to the sequence of non-integral conductance levels characteristic of alamethicin. For comparison the conductance values of level 1 to 4 are listed in Table 1. In addition the estimated numbers of helices which constitute the channel are given (Hanke and Boheim 1980). Because alamethicin consists of amino acids with primarily hydrophobic side chains, it possesses only a poor ionic selectivity. Considering the data of Table 1, a crude estimate of the number of helices lining the interior of the phallolysin channel would yield a number of 4–5 helices, comparable to the model for the acetylcholine-activated channel ($N = 5$, Guy 1984).

Generally, we have to consider a more complex situation. Figure 2 shows the occurrence of several substate conductances, especially at low pH values. This may be interpreted in two ways: (1). orientational changes of amino acid side chains near the channel interior could affect the translocation rate of ions through the channel (Urry et al. 1984), or (2). a cluster of low conductance channels which interact with high cooperativity may display a gating behavior indistinguishable from that of a single channel. This type of cluster gating has been reported for a highly purified and reconstituted Na^+ channel protein (Boheim et al. 1985). Cooperative turn-on steps but independent open-closed state fluctuations of the corresponding monomer channels have been described for a dimeric Cl^- channel detected in native vesicles from *Torpedo californica* electroplax (Hanke and Miller 1983). According to Fig. 2 the smallest conductance unit of the phallolysin channel would amount to approximately 7–8 pS. Interestingly, another toxin, rubescenslysin, from the related mushroom *Amanita rubescens* (Seeger et al. 1973) exhibits a defined sequence of non-integral conduc-

tance levels somewhat comparable to that of alamethicin, as well as a pronounced variety of sublevels indicating protein cluster formation (Wilmsen HU, Faulstich H, and Boheim G, unpublished results).

Channel gating kinetics

Gating kinetics of the phallolysin channel are very similar to those of the acetylcholine receptor channel (Sakmann et al. 1980), the Ca^{++}-dependent K^+ channel (Methfessel and Boheim 1982) and many other membrane channels (Sakmann and Neher 1983). At least three different states are discriminated: the closed state (C), the open state (O) and the resting (inactivated, desensitized) state (R). Two of these states have zero conductance (C and R). In contrast to the behavior of the Ca^{++}-dependent K^+ channel, which seems to follow the linear reaction scheme $C \rightleftharpoons O \rightleftharpoons R$, interburst kinetics of the phallolysin channel do not depend on voltage and pH value. This would mean that transitions to the resting state occur from both the open and the closed state (Methfessel and Boheim 1982). On the other hand, intraburst kinetics ($C \rightleftharpoons O$ transitions) are described by a reaction sequence comparable to that of the Ca^{++}-dependent K^+ channel with respect to voltage and ion gating (Methfessel and Boheim 1982).

Both the phallolysin and the Ca^{++}-dependent K^+ channel, prefer the open state at positive voltage. Both are activated by a specific ion, i.e. at constant voltage an increase in H^+ or Ca^{++} concentration, respectively, increases the probability that the channel adopts the open state. An equivalence relation was deduced for each of the two channels which leads to the concept that two ions are involved in the modulation of the gating processes. A 58 mV change in reference voltage per decade of Ca^{++} concentration at 20 °C (Methfessel and Boheim 1982), and a 130 mV change per decade of H^+ concentration at 37 °C, indicate this fact. In both cases the specific ion is effective from only one side. Details of the phallolysin channel kinetics will be presented in a separate paper. Another channel which is modulated by protons is the Cl^- channel from *Torpedo* electroplax (Hanke and Miller 1983). Very similar effects of voltage dependence, pH dependence and sidedness of channel gating have been observed there.

Whereas the phallolysin channel behaves like a "real" ion- and voltage-gated channel of an excitable membrane, this is different for diphtheria toxin (Kagan et al. 1981), the colicins (Cleveland et al. 1983) and the porins (Benz 1984). In these cases the channel activity pattern is not characterized by the appearance of bursts; in fact, long lived open events are reported. Whereas the gating behavior of the diphtheria toxin channel depends significantly on the trans side pH value (Kagan et al. 1981), a pH dependence of the open channel conductance is observed with colicin A (Pattus et al. 1983). A similar pH-dependent conductance is seen with the mushroom toxin rubescenslysin, i.e. large values at neutral pH and small ones at low pH (Wilmsen HU, Faulstich H, and Boheim G, unpublished results).

Channel incorporation

Phallolysin channels are incorporated into planar bilayers by the application of a negative voltage. A similar prerequisite, but of opposite sign (positive voltage), is reported for the colicins (Davidson et al. 1984) and diphtheria toxin (Kagan et al. 1981). No significant voltage requirement is needed by sea anemone toxin from *Stoichactis helianthus* (Varanda and Finkelstein 1980). With phallolysin, voltage-induced incorporation was independent of membrane composition and pH. We therefore believe that the decrease in hemolytic activity towards basic pH (Seeger and Burkhardt 1976) does not result from a lack of incorporation into the bilayer but from the fact that the phallolysin channel preferentially adopts the non-conducting closed state at high pH. Channel insertion into the membrane seems to be virtually irreversible, because current fluctuations are persistently observed for several hours. This property of larger water-soluble proteins to irreversibly form channels after incorporation into a lipid bilayer phase has been known since the detection of the first discrete current fluctuations induced by the protein called EIM (Mueller and Rudin 1968) in 1969 (Bean et al. 1969).

Almost complete orientation of the phallolysin channels across the bilayer suggests the existence of a preferred interaction site of phallolysin with the bilayer. The observed increase in channel incorporation rate into vesicles (in the absence of an applied voltage) with the amount of negatively charged lipids (Bühring et al. 1983) may indicate electrostatic interaction of this type of lipid with the basic phallolysin molecule. The isoelectric points of phallolysin A, B and C are 8.1, 7.5–7.6 and 7.0, respectively (Seeger 1975; Faulstich et al. 1983). Because of the direction of the membrane potential gradient which induces channel incorporation, whereby electrical potential is more negative on the protein addition (*cis*) side, formally a negative gating charge has to be translocated from the cis to the trans side during the protein insertion process. This would mean that after channel incorporation the hydro-

philic interfacial parts of the channel protein are more positively charged on the cis than on the trans side. However, because of the sidedness of channel activation by protons, negative charges must also be exposed to the cis side. The simplest explanation would imply that the gating charge for protein incorporation and channel state transitions may be the same. In the closed state the negative gating charge may be exposed to the trans side (situation after protein incorporation). Application of a positive voltage would lead to gating charge movement to the cis side and simultaneously to channel opening. If protons are available on the cis side, the gating charge is neutralized and the channel stays open. This mechanism is exactly the same as that proposed for the Ca^{++}-dependent K^+ channel (Methfessel and Boheim 1982). The semi-logarithmic plot $-\log\{p_0/(1-p_0)\}$ versus $V-$ of the data presented in Fig. 4A yields a mean formal gating charge of 0.71 e (e: elementary charge). The sign of this gating charge would be negative if the charge moves from *trans* to *cis* during the process of channel opening, consistent with the considerations given above. Complications in gating charge movement are expected to occur at lower pH. Possibly this may be an explanation for the second long living open state at pH $\leqq$ 6.0.

The high rate of phallolysin incorporation into human erythrocytes compared to that with cattle erythrocytes and lipid vesicles has been attributed to specific receptors, i.e. glycoproteins or glycolipids (Bühring et al. 1983). In view of our experimental data on single channel behavior it is conceivable that an asymmetric charge distribution on such a peculiar receptor molecule initiates phallolysin insertion into the bilayer by electrostatic interaction. Asymmetric charge distribution on the two electrolyte-facing sides of a membrane-spanning integral protein has recently been proposed for the Na^+ channel protein on the basis of its amino acid sequence (Noda et al. 1984). Interfacial adsorption of phallolysin onto the membrane would lead to channel incorporation, only if the gating charge actually senses the local electric field gradient. Because this gradient mainly resides within the hydrophobic membrane core, the adsorbed molecule has to be partly embedded in it. We would like to emphasize that polarity of membrane voltage for the incorporation of phallolysin channels into planar lipid bilayers is strongly unfavorable for living cells. A negative resting potential means that under physiological conditions phallolysin is added to the more positive potential side (outer membrane surface). However, once inserted at neutral pH, the channel stays open most of the time. The detection of specific receptors which facilitate phallolysin incorporation into human erythrocytes (Bühring et al. 1983) explains the much higher insertion rate observed with these cells as compared to the extremely low chance of spontaneously finding phallolysin channels in planar lipid bilayer membranes.

Lipid specificity and mechanism of hemolysis

An effect of surface charges, due to a negatively charged lipid component in artificial membranes, on open channel conductances has been reported for the gramicidin A channel (Apell et al. 1979) and the K^+ channel from sarcoplasmic reticulum (Bell and Miller 1984). However, in both cases the effect is negligible at 1 *M* salt concentration. There is no report of lipid charge effects on channel gating, not even for the pH-dependent Cl^- channel from *Torpedo* electroplax above 100 m*M* salt (Hanke W, unpublished results). In 1 *M* alkali chloride the Debye length is approximately 0.3 nm. This means for the phallolysin channel, where the charged gating particle senses the lipid charge, that both have to be located closely together. Alternatively, negatively charged lipids could neutralize positive charges on the phallolysin molecule (*cis* side) which may have modified interfacial pH due to the Gouy-Chapman effect.

Experimental results reveal that phallolysin forms channels of fixed conductance and gating properties. There is no indication of unlimited aggregation of protein units leading to large channel clusters as is found with alamethicin, melittin and their analogs (Boheim et al. 1983) which are highly active in lysing cells (Jung et al. 1981). We believe that the underlying mechanism of cell lysis by phallolysin is different from that of the alamethicin-type polypeptides. At concentrations higher than those used for single channel measurements, alamethicin destroys membrane lipid structure, similar to the action of detergents (Jung et al. 1981). In contrast, phallolysin induces the formation of protrusions on the cell surface and subsequent bursting of these membrane patches (Petzinger and Seeger 1976). This confirms our interpretation that phallolysin causes cytolysis by an osmotic effect, which mainly results from the formation of ion-permeable channels (Bühring et al. 1983).

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