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Pore-forming protein from *Entamoeba histolytica* forms voltage- and pH-controlled multi-state channels with properties similar to those of the barrel-stave aggregates

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Pore-forming protein from *Entamoeba histolytica* forms cation-selective channels in planar bilayers. With increasing potentials, the open-state probability of these channels decreases, and channel aggregates collapse (Young, J.D.-E. and Cohn, Z.A. (1985) J. Cell. Biochem. 29, 299–308). In this communication we report the following observations: (i) incorporation of the pore in black-lipid membranes was stimulated by membrane potential, (ii) pores were rectifying, (iii) breakdown of pores resulted in a continuous spectrum of subconductance states, (iv) the open-state probability increased strongly with pH. This pattern of behaviour is similar to that of the barrel-stave aggregates (alamethicin and related toxins). We therefore conclude that the amebal pores, like those of the barrel-stave class, may consist of complexes involving variable numbers of membrane-spanning subunits.

Introduction

The protozoan parasite *Entamoeba histolytica* is capable of destructing host tissue by a process involving contact-dependent cytolysis (for a review, see Ref. 1). One of the factors that may contribute to this process is a toxin (amoebapore [1–3], pore-forming protein (PFP) [4,5]), which has pore-forming activity in planar lipid bilayers and depolarizes intact cells [2,4]. The pores formed in black lipid membranes are selective towards monovalent cations, and exhibit multi-state behaviour in a voltage-dependent manner [4,5]. Pores are homo-oligomers of a 14000 M_r subunit [2]. Interestingly, whereas both nonpathogenic and pathogenic Entamoebae contain pore-forming protein, the properties of the respective pores are different [6].

In the present communication we report additional details on the effects of both voltage and pH on incorporation and channel properties of PFP. We infer from our results that PFP probably has a mechanism of action similar to that of barrel-stave forming toxins such as alamethacin [7].

Abbreviation: PFP, pore-forming protein.

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Methods and Materials

Entamoeba histolytica, strain HMI IMMS [8], was grown axenically as described by Diamond [9], in the presence of penicillin G (100 U/ml) and streptomycin (100 μg/ml). Amoebae were harvested at the end of the exponential growth phase and washed twice in saline A (see below). Cells were broken up by two freeze-thaw cycles. The homogenate was spun at 1500 × *g* for 10 min to remove remaining intact cells and large debris, then spun again at 100000 × *g* for 1 h. The supernatant of this spin, which contained up to 70% of the PFP present in the homogenate, was directly used in the experiments.

Protein was determined according to Lowry et al. [10] as modified by Markwell et al. [11].

Planar lipid bilayers were formed according to Montal and Mueller [12] on Teflon partitions with holes of approx. 150 μm diameter. To this end, asolectin was dissolved in hexane at a concentration of 10 mg/ml, and 20 to 50 μl of this solution was spread on the air/water interfaces, which on both sides of the partition were positioned below the hole. The solvent was allowed to evaporate for at least 5 min. Then the bilayer was formed by raising the level of the aqueous solutions in the two compartments. The partition was pretreated with hexadecane/hexane (1.9, v/v) for better membrane stability. The PFP was added directly to the aqueous solution on the cis-side of the bilayer. Potential

was applied to the cis-side, the trans-side was virtually grounded Details of the mechanical and electrical setup have been described previously [13]

Pore parameters were calculated from long (65 s) traces of single-channel recordings as described previously [14,15], multichannel data were directly plotted on a strip-chart recorder or an *X-Y* recorder

All salts and solvents used were at least p a. grade, and the water was double quartz distilled All experiments were performed at room temperature (20 °C) in Saline A, unless indicated otherwise Saline A was composed of (mM) KCl, 100, $CaCl_2$, 1, Tris, 10, brought to pH 7 5 with HCl

Results

We first investigated the kinetics of incorporation of PFP into planar lipid bilayers Fig 1, main figure, shows the increase in conductance after addition of a relatively high concentration of PFP at t-0 It can be seen that PFP was incorporated on a minute time scale into the bilayer Incorporation was a complex process the curve of Fig 1 could only be fitted by a combination of three exponentials, with associated half-times of 0 3 min 3 6 min and 22 min, respectively (results not shown) We interpret this complex time course as being due to a fast initial binding phase followed by a slow reorientation phase A similar time dependence of incorporation has been found for other channels, including both barrel-stave aggregates (alamethicin [16,17]) and fixed pores (bacterial porins [22]) The inset in Fig 1, recorded at reduced PFP concentration, shows that at fixed potential one functional pore after the other was incorporated The multi-pore current at fixed potential levelled off after approx. 30 min (results not shown)

In Fig. 2 we show the voltage dependence of channel incorporation Traces as presented in Fig 1 were recorded at different potentials. The inset shows the current after 4 min as a function of potential Clearly, at higher potentials the incorporation of functional pores was more efficient This implies that the rate-limiting step (possibly the reorientation step, see above) involves electrostatic interactions In agreement with this notion, PFP bears a net positive charge at neutral pH [1] Physiologically, the direction of the membrane potential in target cells (positive outside) is the same as that in our experimental setup, and thus would favour incorporation

Fig 3 shows an *I-V* curve for a multi-pore system recorded 45 min after addition of PFP to the bath solution The system was slightly rectifying between −50 mV and +35 mV, then a region of negative resistance followed up to approx 75 mV At higher potentials pore activity increased steeply In another set of experiments, performed at a much lower protein concentration, we evaluated the open-state probability of the single channels as a function of potential (Fig 3,

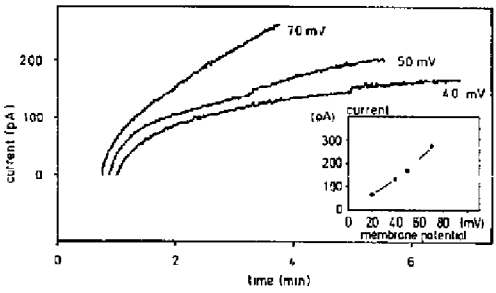

Fig 2 Dependence of PFP incorporation on the membrane potential After addition of amebal extract (protein concentration, 50 μg/ml) to bilayers, traces as shown in Fig. 1 were recorded at different potentials In the inset the current values after 4 min are plotted as a function of membrane potential

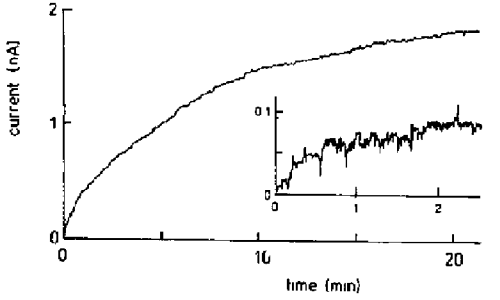

Fig 1 Kinetics of incorporation of PFP into planar lipid membranes At $t = 0$ amebal extract (protein concentration 200 μg/ml for the main figure, 20 μg/ml for the lower inset) was added to the aqueous solution at the cis-side of the bilayer A potential of 20 mV was applied to the membrane and the transmembrane current was recorded as a function of time

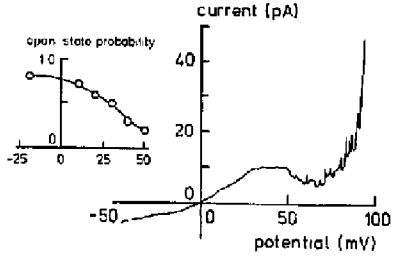

Fig 3 Dependence of transmembrane current and open-state probability on transmembrane potential The main figure shows an *I-V* curve recorded 45 min after addition of amebal extract (protein concentration, 50 μg/ml) to the bilayer The inset shows the open-state probability as a function of membrane potential, it was calculated [14,15] from traces of single pore currents such as those presented in Fig 4A

inset) Of the three characteristics of the *I-V* curve, the region of negative resistance has also been reported by Young et al [4,5], similarly to these authors, we explain this behaviour by a decrease in the probability of the PFP channel to be open at higher potentials (Fig 3, inset) The other two peculiarities of our *I-V* curve, the rectification at negative potentials and the steep increase in conductance at high positive potentials, have not been observed so far The rectification at negative potentials was clearly not due to a decrease in the open-state probability (Fig. 3, inset) Thus, it must have been caused by a decrease in the number of pores that was incorporated This implies that the effect of membrane potential on incorporation was asymmetric (compare Fig 2, inset) We noticed that after long ($\geq$ 1 h) preincubation times, the *I-V* curve gradually became more symmetrical, this indicates that PFP slowly equilibrated across both aqueous compartments (results not shown) A similar phenomenon has been observed with alamethicin [18] As to the steep increase in conductance above 75 mV the reason this has not been observed before is probably that membranes become very unstable under these conditions In view of the fact that the tendency of the single pores is to close up at higher potentials (Refs 4 and 5 and Fig 3, inset), the increase in conductance must reflect an even steeper increase in incorporation of PFP into the bilayer This again is very reminiscent of the behaviour of alamethicin [16,17]

On the single-pore level, we generally obtained results similar to those reported by Young et al [4,5] Specifically, up to 50 mV we observed a single open state with a conductance of approx 1 ns (Figs 4A,

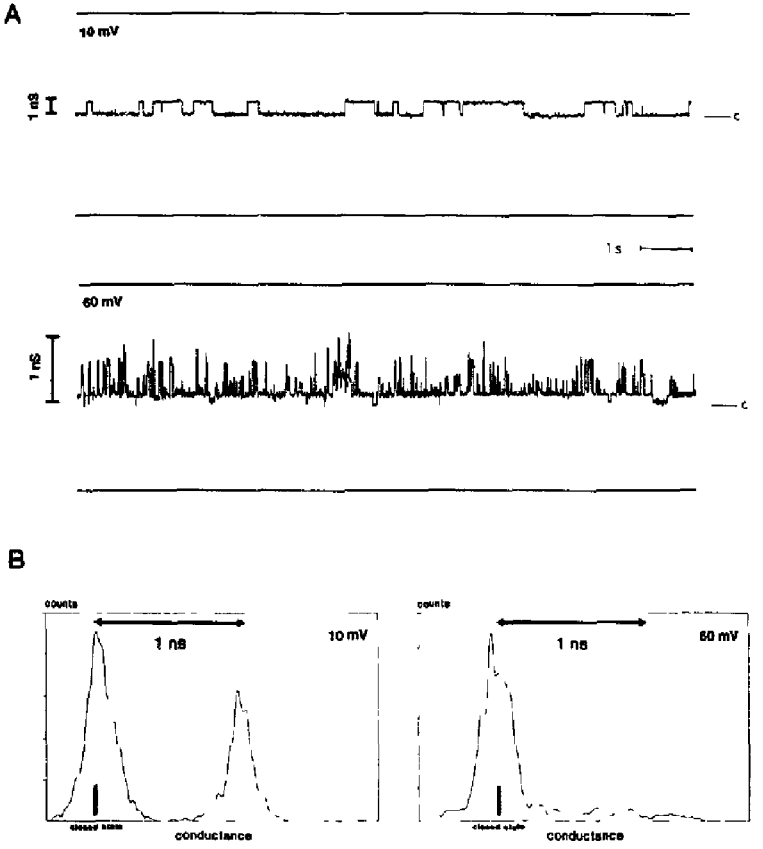

Fig. 4 Single pore behaviour at low and high potential (A) Traces recorded at transmembrane potentials of 10 mV and 60 mV respectively (B) The corresponding amplitude histograms (evaluated from 16 kB data) for the distribution of the different conducting states The peaks at baseline indicate the probability that the channel is closed

upper trace and 4B, left histogram) Whereas the probability of the channel to be open decreased towards higher potentials (Fig 3, inset, see above), the conductance of this pore state was purely ohmic (results not shown) At 60 mV, not a single pore state but a fluctuation between a large number of substates was observed (Fig 4A, lower trace) As is clear from the amplitude histogram (Fig 4B, right side), most of the conductance levels at the higher potential were below that at the lower potential Similar to Young et al [5], we interpret this finding in terms of the breakdown of a multi-subunit pore at higher potentials However, in contrast to these authors we found that breakdown did not result in the appearance of discrete conductance steps rather, there was a virtually uniform distribution of subconductance states (right-side histogram of Fig 4) Some pores in the barrel-stave class behave like PFP in this respect [19], alamethicin itself behaves differently in exhibiting defined, non-stoichiometric conductance states, associated with larger channels at higher potentials [7] In essence, both kinds of behaviour exclude a simple model in which aggregates are composed of identical parallel pores [20]

Finally, we investigated the dependence of pore incorporation and of pore behaviour on pH We were unable to find a consistent effect of pH on incorporation By contrast, single-pore parameters were strikingly pH-dependent Fig 5A shows three traces, recorded at different pH values Obviously, the activity of the channels increased with increasing pH Fig 5B shows how this effect was related to the pH dependence of the single-pore parameters the open-state probability increased strongly towards higher pH values (Fig. 5B, left), as a result of an increase in the open-state lifetime coupled to a simultaneous decrease in the closed-state lifetime (Fig 5B, right)

Discussion

As shown in this communication, membrane potential and aqueous pH affected both the rate of incorporation and the subsequent action of PFP in planar lipid

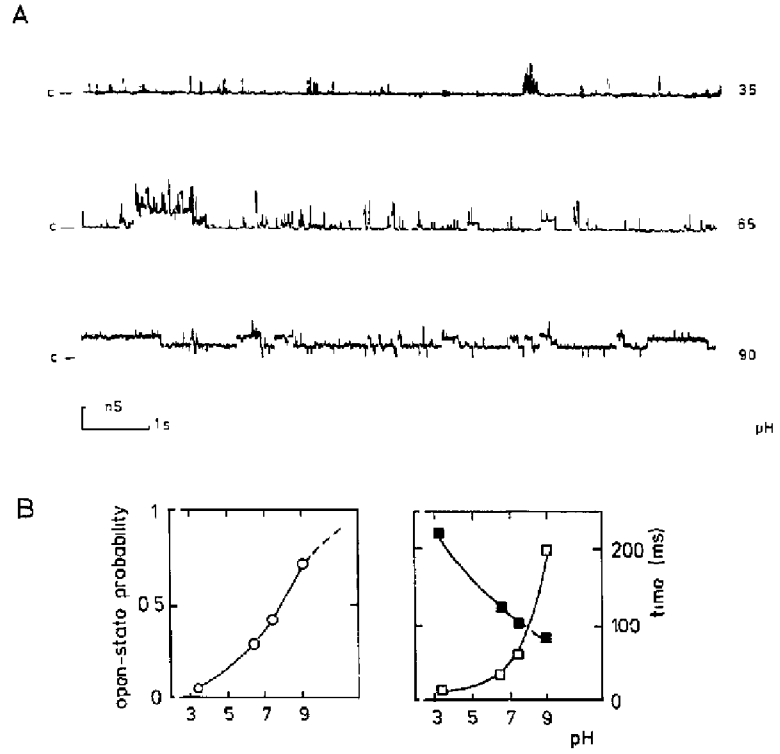

Fig 5 pH dependence of single pore parameters (A) Current fluctuations at different pH values (indicated next to each trace) of a bilayer containing three PFP pores (B) Dependence of open state probability (left, ○) and state lifetimes (right, □, open state lifetime ,a, closed state lifetime) on pH

bilayers Details of this control point to a strong functional relationship of PFP with the barrel-stave class of channel formers Basically, the ionic pore of PFP is a homooligomer [1,5] Oligomers are already present in the aqueous solution, and incorporate as such into the bilayer [5] In our experiments, incorporation reached a steady state in which pores equilibrated across both fluid compartments (results not shown) At high positive potentials, incorporation was dramatically increased (Fig 3 and text) Incorporation was asymmetric and pores were rectifying (Fig 3, [4,5]) At high potentials the oligomers were degraded and formed a continuous spectrum of voltage-dependent multi-state pores, the maximum conductance of which was that of the initial aggregate (Fig 4B) High pH values increased the open-state probability (Fig 5) All these characteristics are typical for the barrel-stave class of pores [7,16,17,19,20] They contrast with those of, for instance, the acetylcholine receptor pore [21] or the bacterial porins [22] in the latter, the interaction between the subunits is fixed and static Moreover, the aggregates are basically hydrophobic after assembly, the complex does not equilibrate with the fluid phase anymore, and has to be extracted by detergents The similarity in overall behaviour of PFP with that of the barrel-stave class leads us to suggest that also the molecular mechanism of PFP pore formation may be similar, in that aggregates of variable diameter are formed from membrane-spanning stretches of polypeptide contained in different subunits

Starting from alamethicin over melittin and certain synthetic analogs [16,17,20], those members of the barrel-stave class with molecular weights ranging from approx 500 to 4000 have been well characterized, on the higher end of the molecular weight scale, a novel group of mushroom toxins ($M_r \simeq 34000$) has been shown to fall into the same category [19] Within this series, further study of PFP with its M_r of 14000 [2] would meaningfully complement the existing knowledge about pore formation by homooligomeric aggregates

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