Complement pore genesis observed in erythrocyte membranes by fluorescence microscopic single-channel recording

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The formation and opening of single complement pores could be directly observed in erythrocyte ghosts by confocal laser-scanning microscopy employing the recently introduced method of fluorescence microscopic single-channel recording. Resealed sheep erythrocyte ghosts were incubated with human complement. By limiting the concentration of C8, the eighth component of complement, the fraction of cells rendered permeable for the small polar fluorescent probe Lucifer Yellow was varied between 0.50 and 0.90. Under each condition the flux rate, k , of Lucifer Yellow was determined for a substantial number of ghosts. By analysing the sample population distribution of k the flux rate k_1 of ghosts with a single pore was found to be $(4.8 \pm 1.1) \times 10^{-3} \text{ s}^{-1}$ consistent with a pore radius of about 3.5 nm (35 Å). The genesis of single complement pores was studied by continuous influx measurements while triggering pore formation by a temperature shift. Pore genesis was found to be a very slow process, proceeding on a time scale of several minutes. During pore genesis the influx curves had a sigmoid shape, which excluded the possibility that the pore was preformed on the membrane surface and subsequently inserted. However, the influx curves could be well simulated by a model which assumed that pores grow stepwise by sequential incorporation of C9 monomers. The model predicts conditions under which the incorporation of single monomers can be directly revealed.

INTRODUCTION

Immunological reactions frequently involve proteins which are able to insert into the plasma membrane of target cells, forming large transmembrane pores [for a review, see Bhakdi & Tranum-Jensen (1987)]. Such pores are permeable to water and electrolytes, but not to macromolecules, and therefore can induce the swelling and lysis of the target cell by colloid osmotic forces. At least in the case of erythrocytes, a single transmembrane pore is sufficient to effect lysis (Mayer, 1972). The classical example of such a pore-former is complement [for a review, see Müller-Eberhard (1988)]. It consists, however, not of a single component, but is a complex reaction system involving about 15 different proteins. At the beginning of the complement reaction an antibody which is bound to the target-cell membrane is recognized and marked by complement components C1–4. Then a membrane anchor consisting of complement components C5b–8 is generated. This enables complement component C9 to be integrated into the C5b–8 complex to yield the final transmembrane pore, also referred to as the ‘membrane-attack complex’.

Functional aspects of complement pores have been extensively studied in the past by a variety of techniques, including electron microscopy (Tranum-Jensen & Bhakdi, 1983), osmotic-protection experiments (Green *et al.*, 1959; Sears *et al.*, 1964), marker release (Giavedoni *et al.*, 1979; Ramm & Mayer, 1980), and electrical single-channel recording (Jackson *et al.*, 1981; Young *et al.*, 1986). In spite of the large body of experimental material, widely differing estimates of the pore size have been reported. Extreme values for the pore radius are 0.4 nm (4 Å) (Jackson *et al.*, 1981) and 5–6 nm (50–60 Å) (Young *et al.*, 1986; Malinski & Nelsestuen, 1989). It is generally believed, however, that pore composition and size depend on the concentration of C9 and possibly also on that of C8 and that the pore radius has an upper limiting value of about 5–6 nm (50–60 Å).

Conventional methods, such as osmotic protection or marker release, which have been used in the past to characterize functional properties of complement pores yield average parameters of large channel populations. In contrast, the analysis of single channels by electrical measurements of inorganic-ion fluxes (Neher & Sackmann, 1976) yields direct information, for instance, on the opening and closing of channels, their homo- or heterogeneity, and their subcellular distribution. We have recently introduced a new type of single-channel recording (Peters *et al.*, 1990) in which the flux of fluorescent tracers is measured by fluorescence microphotolysis (fluorescence recovery after photobleaching). The new method is applicable to classes of molecules, cellular systems and time scales different from those applicable to electrical single-channel recording and thus may yield complementary information. Fluorescence microscopic single-channel recording has been worked out (Peters *et al.*, 1990) for transmembrane pores formed by the cytolytic lymphocyte protein perforin. Perforin is a 66 kDa protein (Masson & Tschopp, 1985; Podack *et al.*, 1985) which, without mediation of other proteins, inserts into the plasma membrane of target cells and forms large pores by oligomerization. C9 is closely related to perforin [Shinkai *et al.*, 1988; Lichtenheld *et al.*, 1988; for reviews, see Stanley (1988) and Tschopp (1990)].

The present study applied fluorescence microscopic single-channel recording to the complement pore. Thus it was possible for the first time to measure the flux of a fluorescent tracer through single complement pores in erythrocyte membranes, to derive a functional pore radius for the presently employed experimental conditions, and to monitor pore genesis directly. The opening of newly formed pores was found to proceed on a time scale of several minutes. This is very slow, especially in comparison with the formation of complement pores in artificial lipid membranes (Young *et al.*, 1986), which could not be followed at a time resolution of 2 ms. The flux curves obtained during pore formation had a sigmoid shape which excluded the

Abbreviations used: LY, Lucifer Yellow; CLSM, confocal laser-scanning microscope (microscopy).

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possibility that the pore was assembled on the membrane surface and then inserted into the membrane. The data were consistent, however, with a mechanism in which pores grow stepwise by sequential incorporation of C9 monomers.

MATERIALS AND METHODS

Sheep blood was obtained from Flow Laboratories, Meckenheim, Germany. Resealed ghosts were prepared by the method of Schwach & Passow (1973) as modified by Ramm & Mayer (1980).

Complement pores were generated by incubating 10 ml of resealed sheep erythrocyte ghosts (5×10^8 cells/ml in Hepes buffer containing 137 mM-NaCl, 10 mM-glucose, 5.3 mM-KCl, 0.39 mM- K_2HPO_4 , 1.3 mM- $CaCl_2$, 0.3 mM- $MgCl_2$, pH 7.4) with anti-Forsman antibodies (800-fold dilution). Purified C8 (0.2 mg/ml) was diluted with Hepes buffer to yield a series of 50 μ l samples in which the C8 concentration was 1/100, 1/200, 1/400 etc. of that in the stock. To each sample 50 μ l of a 10-fold diluted C8-depleted serum, prepared as described by Bhakdi & Trantum-Jensen (1986) and 50 μ l of antibody-coated ghosts were added. The 10-fold diluted C8-depleted serum contained C9 in large excess (Bhakdi & Trantum-Jensen, 1986). After 30 min at 37 °C ghosts were pelleted and resuspended in 50 μ l of Hepes buffer. To each sample the hydrophilic fluorescent probe Lucifer Yellow (LY) was added to a final concentration of 100 μ M. By microscopic fluorescence measurements in the photobleaching apparatus the fraction of ghosts which had become permeable for LY was determined. Samples in which this fraction, hereinafter referred to as the 'degree of lysis', amounted to 50, 70 or 90 % were used for flux measurements. To obtain 90 or 70 % lysis, the C8 concentration was 1/2400 or 1/4800 respectively.

The confocal laser-scanning microscope manufactured by Wild-Leitz Instruments, Heidelberg, Germany, was used without modifications. The fluorescence microphotolysis apparatus was described by Peters & Scholz (1990). Flux measurements were performed as described previously for perforin (Peters *et al.*, 1990), using LY as tracer.

The mathematical methods used in the single-channel analysis and the computer routine for fitting the mathematical model to the experimental data were described previously (Peters *et al.*, 1990).

RESULTS AND DISCUSSION

Single-channel recording after photolysis

The confocal laser scanning microscope (CLSM) employed a laser beam which was focused down to the diffraction limit. The focused beam was scanned across the object point by point. At each point a fluorescence measurement was made. The image was finally reconstructed from those measurements. With regard to the present application, two features of the CLSM manufactured by Wild-Leitz Instruments were of particular importance: (i) the diameter of the confocal aperture could be varied continuously to adjust the effective thickness of the optical section. Sheep erythrocyte ghosts are rather small cells with a thickness of only 1–2 μ m. By optimizing the effective thickness of the optical section (lower limit $\sim$ 400 nm; Brackenhoff *et al.*, 1989) the spatial selectivity of intracellular fluorescence measurements could be significantly improved; (ii) the so-called 'zoom' function allowed the size of the scanned area to be varied within wide limits. With a 100-fold objective, for instance, the scanned area could be reduced from 50 μ m $\times$ 50 μ m (zoom 1) to 6 μ m $\times$ 6 μ m (zoom 8). For photobleaching purposes the laser beam could thus be concentrated on an area little larger than that

of a ghost, increasing, at the same time, the intensity 70-fold. Since the power of the laser beam could be varied in addition by a factor of $\sim$ 10, intensity ratios of $\sim$ 700 could be obtained by simple means.

A flux measurement performed with the CLSM is shown in Fig. 1. Resealed sheep erythrocyte ghosts were treated with human complement. The C8 concentration was limited (see the Materials and methods section) in such a manner that the fraction of ghosts which had become permeable for LY was $\sim$ 0.5, i.e. about 50 % of ghosts had acquired one or more pores. The single-channel analysis described below strongly supports the idea that the distribution of pores among ghosts is correctly described by the Poisson distribution:

$$P_n = (\lambda^n/n!)e^{-\lambda}$$

where P_n is the fraction of ghosts with n pores per ghost and λ is the mean value of pores per ghost. Thus, for $P_0 = 0.5$, λ is 0.7 and P_1 , P_2 and P_3 amount to 0.34, 0.12 and 0.03 respectively, i.e. among permeable ghosts the majority (68 %) have only one pore. Ghosts were incubated with LY, filled into a flattened glass capillary (microslide), and inspected in the CLSM. A first scan of a permeable ghost was taken at 'zoom 1' or 'zoom 2' and a small laser-beam power (Fig. 1A). The ghost was then subjected to photobleaching by increasing the zoom and the laser-beam power and taking several scans in immediate succession. Subsequent scans (Figs. 1B–1D) were taken at the initial zoom factor and beam power. The relative intra- and extra-cellular fluorescence intensities were read from scans, plotted versus time after photobleaching (Fig. 1E), and fitted by (Peters, 1983):

$$f = e^{-kt} \quad (1)$$

where f , the fractional fluorescence recovery, equals $[F(t) - F(\infty)]/[F(0) - F(\infty)]$; $F(t)$, $F(-)$, $F(0)$ and $F(\infty)$ are fluorescence at time t , before, immediately after, and very long after, photolysis respectively, and k is the flux rate. This yielded

Fig. 1. Single-channel recording of fluorescent tracer flux through complement pores by CLSM

Resealed erythrocyte ghosts were incubated with complement in such a manner that the majority of permeable ghosts had one pore. Ghosts were incubated with the small hydrophilic fluorescent tracer LY, and CLSM scans were taken before (A) and at different times after (B–E) photobleaching. In the diagram the ratio $I(is)/I(os)$ of the fluorescence intensity inside and outside a ghost, as derived from CLSM scans, is plotted versus time. Points and bars represent means $\pm$ S.D. of ten measurements. The experimental data were fitted by eqn. (1) (continuous line), which yielded a flux rate, k , of $4.0 \cdot 10^{-3} s^{-1}$. Arrows indicate the times at which the scans A–E were taken.

Fig. 2. Sample population distribution of the flux rate, k at (a) 90 % and (b) 70 % lysis

In the histograms the probability density $D(k)$, i.e. the fraction of k values within a k interval of 10^{-3} s^{-1} , is plotted versus k . The occurrence of distinct peaks which are equidistantly spaced on the k -axis suggest that subpopulations of ghosts with no, one, two and three pores per ghost were resolved. The experimental data were simulated by a mathematical model (see the text) in which the broken-line curves represent the subpopulations of ghosts, whereas the continuous line is the sum of the broken-line curves.

$k = (4.0 \pm 1.2) \times 10^{-3} \text{ s}^{-1}$ (mean $\pm$ S.D. of ten measurements), in good agreement with the results obtained by the photobleaching measurements described below.

Single-channel analysis

Resealed sheep erythrocyte ghosts were treated with complement. The C8 concentration was limited so that ghost populations were obtained in which either 90 or 70 % of the cells were rendered permeable to LY. The flux rate was determined for 338 (90 % lysis) and 471 (70 % lysis) individual ghosts respectively, by the methods described previously (Peters *et al.*, 1990). The sample population distributions of k are plotted in Fig. 2. For both 90 and 70 % lysis the distributions showed distinct, equidistantly spaced peaks with maxima at approx. $k = 0 \times 10^{-3}$, 5×10^{-3} , 10×10^{-3} and $15 \times 10^{-3} \text{ s}^{-1}$. This strongly suggests that subpopulations of ghosts with no, one, two and three pores were resolved, whereas the k -values of ghosts with more than three pores merged into a noisy, slowly decaying, continuum. Although the position of the peaks on the k -axis was identical for 70 and 90 % lysis, the frequency of the subpopulations was quite different. This, however, is qualitatively

consistent with a Poisson distribution of pores among ghosts, predicting P_0 , P_1 , P_2 and P_3 to be 0.1, 0.22, 0.26 and 0.2 respectively at 90 % lysis, and 0.3, 0.35, 0.22 and 0.09 respectively at 70 % lysis.

The quantitative analysis of k -distributions followed previously established principles [for details, see Peters *et al.* (1990)]. The experimental data were fitted by a sum of Gaussian functions where each Gaussian, G_n , accounts for the k -distribution of a subpopulation of ghosts with n pores per ghost. The analysis is based on the assumption that (i) the mean k -value μ_n of G_n is a multiple of s_1 plus a constant s_0 , and (ii) the fraction of ghosts per subpopulation is given by the Poisson distribution. The least-squares fit of the theoretical model to the data is shown in Fig. 2. Broken lines represent the Gaussians, whereas continuous lines represent the sum of the Gaussians. Fig. 2 shows that the theoretical model simulates the experimental data fairly well and accounts, in particular, for the observation that the distributions become broader with increasing n , so that only the first three or four subpopulations were resolved. For the flux rate, k_1 , of one-pore ghosts, the fit yielded $(4.9 \pm 1.0) \times 10^{-3} \text{ s}^{-1}$ at 90 % lysis and $(4.7 \pm 1.1) \times 10^{-3} \text{ s}^{-1}$ at 70 % lysis respectively.

The analysis described implies that the size of the complement pore is quite homogeneous. Otherwise, it would have been impossible to resolve distinct peaks in the flux-rate distributions. In fact, the deviation of the k_1 -value (about ± 25 % of the mean) sets an upper limit to the variability of the pore area of about ± 25 % and thus to the pore radius of about ± 12 %.

Single-channel radius

The functional radius, r , of the complement pore can be derived from the flux rate, employing the hydrodynamic approach for restricted diffusion of hard spheres through cylindrical pores (Pappenheimer *et al.*, 1951; Renkin, 1953). If, as usual, the number of pores per cell was unknown, the pore radius could be calculated by combining the transport data of two or more differently sized tracers [Peters, 1984; for summary and discussion, see Peters (1986)]. The present case, however, is basically different, because k_1 , the flux rate of cells with a single pore, is known. This enables one to calculate r directly:

$$r = \left[\frac{k_1 V L}{\pi D R(q)} \right]^{\frac{1}{2}} \quad (2)$$

where V is the ghost volume, D is the diffusion coefficient of the tracer in the pore, and L is the pore length. $R(q)$ is equal to $(1-q)^2 (1 - 2.04 q + 2.09 q^3 - 0.95 q^5)$ where $q = a/r$ (the ratio of tracer radius, a , and the pore radius, r). We determined D for LY by photobleaching measurements to be $\sim 275 \cdot 10^{-8} \text{ cm}^2 \cdot \text{s}^{-1}$ (iso-osmotic buffer, pH 7.4, 23 °C). The Stokes radius of LY was derived from D to be 0.78 nm (7.8 Å). V was estimated from horizontal and vertical optical sections of individual ghosts, obtained with the CLSM, to be about 45 fl (cf. Blunt, 1975). Insertion of these values together with $L \sim 18 \text{ nm}$ (180 Å) (Tranum-Jensen & Bhakdi, 1983) into eqn. (2) yields $r \sim 3.5 \text{ nm}$ ($\sim 35 \text{ Å}$).

As mentioned above, the functional radius of the complement pore has been previously determined by a variety of methods, including osmotic protection, tracer release and electron microscopy [for reviews, see Bhakdi & Tranum-Jensen (1987) and Müller-Eberhard (1988)]. It has been concluded that the size of the complement pore depends on the C8 and C9 concentrations, with a maximum radius of 5–6 nm (50–60 Å). In the present study, C8 was limited to yield 50–90 % lysis. Our results are therefore consistent with previous measurements and, in addition, show that the pore radius is approximately constant over the studied lysis range.

Fig. 3. Formation of complement pores

The formation of complement pores was triggered off by a temperature-shift method. After the temperature shift, the influx of LY was monitored by measuring the intracellular fluorescence of single ghosts. After completion of pore formation a photobleaching measurement of the flux rate was performed to determine the number of pores per ghost. A complete measurement is shown in (a), where intracellular fluorescence is plotted versus time after the temperature shift. At time (t) ~ 2000 s a photobleaching pulse was applied and the flux rate, k_1 , was measured to be $\sim 4 \cdot 10^{-3} \text{ s}^{-1}$, indicating that the ghost had acquired one pore. (b–e) Different examples for the formation of single pores; ordinate and abscissa have the same scales and dimensions as in (a). (f and g) Examples of the sequential formation of two pores.

Single-channel genesis

At 70% lysis the distribution of k -values is such (Fig. 2b) that the one-pore-fraction is well separated from the no-pore- and two-pore-fractions respectively. We have used this circumstance to identify ghosts with single pores and to monitor, in real time, the formation of single new pores.

Resealed sheep erythrocyte ghosts were incubated with C8-free serum at 37 °C, washed, cooled down to 0 °C, and incubated with purified C8 and C9 (at concentrations yielding finally, after warming up, 70% lysis). By these means C5b-9 was bound to the membrane, but functional pores did not form (Boyle *et al.*, 1978; Bhakdi & Tranum-Jensen, 1986). Pore formation was triggered by warming ghosts quickly to room temperature (23 °C). Subsequently, intracellular fluorescence was continuously measured in single ghosts by using the photobleaching apparatus. The onset of pore formation was indicated by an increase of intracellular fluorescence. When the intracellular fluorescence had reached a constant value, a measurement of the flux rate was made and the k -value used to determine the number of pores carried by that particular ghost. If k was between $3 \times 10^{-3} \text{ s}^{-1}$ and $6 \times 10^{-3} \text{ s}^{-1}$, the ghost was assumed to have only one pore.

Representative examples of such pore-formation measurements are given in Fig. 3. First indications of pore formation were usually observed ~ 100 s after shifting the temperature from 0° to 23 °C. In 29 measurements on one-pore ghosts, the intracellular fluorescence usually had a more or less smooth sigmoidal time-dependence, with a turning point at about 500–1000 s after the temperature shift (Figs. 3a–e). The sequential

Fig. 4. Simulation of complement pore formation

The mathematical model, described in the text, assumes that pores grow stepwise by sequential incorporation of C8 and/or C9 monomers. The diagrams plot the normalized intracellular concentration of the fluorescent probe versus normalized time of pore formation where τ is the interval between monomer incorporation. (a) Effect of a variation of the maximum number, M , of monomers per pore. Assuming a pore radius of 0.35 nm (35 Å) and a fluorescent probe radius of 0.78 nm (7.8 Å) (i.e. LY) flux curves were simulated for $M = 5$ (a), 8 (b), 12 (c) and 15 (d) respectively. (b) Effect of the size of the fluorescent probe. Assuming $M = 12$ and a maximum pore radius of 0.35 nm (35 Å) flux curves were simulated for probe molecules with a radius of 0.2 nm (2 Å) (a), 0.78 nm (7.8 Å) (b), and 0.16 nm (16 Å) (c) respectively.

formation of two pores was clearly apparent (Figs. 3f and 3g) in two out of 31 measurements.

The observation that the flux curves recorded during pore formation have a sigmoid shape excludes the possibility that the complement pore is first assembled in closed form and then undergoes a sudden transition to an open state. Sigmoid flux curves are consistent, however, with either a gradual opening of preformed closed pores or with the growth of small open pores by sequential incorporation of C8 and/or C9 monomers.

We have simulated the sequential incorporation mechanism of pore genesis by a simple mathematical model. It assumes that pores are staved from monomers of constant width w . Thus the cross-section of the pores are regular polygons with an inscribed circle radius, r , equal to $[w/2 \tan(180/m)]$, where m is the number of monomers per pore. Pores form by sequential assembly of $m = 1, 2, 3, \dots, M$ monomers. We assume that the addition of a monomer to an existing nascent pore is a fast event occurring at regular intervals, namely at time $t = 0, 1\tau, 2\tau, \dots, M\tau$. For simplicity, but without restriction of generality, τ was taken here to be constant, whereas in reality τ might have a distribution and might also be time-dependent. It may be noted that τ incorporates the time requirements of several processes, e.g. the diffusion of C9 monomers in the aqueous phase, the insertion of C9 monomers into the lipid bilayer phase, the diffusion of C9 in the lipoprotein bilayer of the erythrocyte membrane, the capture of C9 monomers by nascent pores, and also the incorporation of the monomer into the nascent pore. The model, at the present stage, does not differentiate between these processes. The flux rates $k(m)$ of pores consisting of m monomers are calculated from eqn. (2). The time course of the normalized intracellular

tracer concentration $C_i(t)$ during pore formation is then calculated piecewise for the time intervals $[n\tau - (n-1)\tau]$ according to:

$$C_i(t) = \sum_{n=0}^{\infty} C_i(t = n\tau) [1 - e^{-k(n+1)(t-n\tau)}] \Bigg|_{t=n\tau}^{t=(n+1)\tau} \quad (3)$$

In the present study a fluorescent tracer with 0.78 nm (7.8 Å) radius (i.e. LY) was employed. The radius of the final pore was determined to be 3.5 nm (35 Å), which, taking into account current ideas about the molecular dimensions of C9 (Tschopp, 1990), suggests a composition of about eight C9 monomers. These conditions are simulated in Fig. 4(a), curve b. On a qualitative level the similarity between that theoretical curve and the experimental flux curves in Fig. 3 is striking: both display a relatively smooth, but not perfectly smooth, sigmoid shape. Verification of the model on a quantitative level would require further data. It would be particularly informative, for instance, if the lateral diffusion coefficient of C8 and C9 monomers in the aqueous phase and on or in the erythrocyte membrane could be determined.

Interestingly, the model predicts that the incorporation of monomers into nascent pores should be directly apparent under certain conditions. If either M , the maximum number of monomers, were particularly small (Fig. 4a, curve a) or if, at normal M , a tracer much smaller than LY could be employed (Fig. 4b, curve a) the flux curves are predicted to display distinct breaks at small τ values. If these breaks could be observed experimentally, the distribution of τ could be directly determined.

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