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Effect of perfringolysin O on the lateral diffusion constant of membrane proteins of hepatocytes as revealed by fluorescence recovery after photobleaching

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Perfringolysin O is a thiol-activated cytolytic exotoxin the primary receptor of which is the membrane cholesterol on the cell surface. The effect of perfringolysin O was tested in various hepatocyte preparations. (i) Smears of fresh liver exposed to a mild H_2O_2 (1.0 mM) injury for 10 min at 37°C, develop a 'peroxide-induced autofluorescence' (PIAF) on the membrane proteins. PIAF is suitable for measuring the average lateral diffusion constant (D) of the membrane proteins by means of fluorescence recovery after photobleaching technique (FRAP). Incubation for 5 min with 600 or 2000 units/ml of the perfringolysin O resulted in a significant increase (32 and 46%, respectively) of D as compared to the controls of the same age group (13–14 months). Various tests like heat denaturation of cholesterol saturation of perfringolysin O before its application as well as thiol-activation of the smears with dithiothreitol revealed that the increase of D is a specific toxin effect due mot probably to the reaction of perfringolysin O with cholesterol. (ii) Isolated hepatocytes were exposed to perfringolysin O and their viability as well as the release of two cytosolic enzymes (lactate dehydrogenase and glutamic-pyruvic transaminase) were measured; 40–60 units/ml of perfringolysin O in 30 min reduced the viability of the hepatocytes to zero and caused a release of about 70% of both cytosolic enzymes. The significance of the results is discussed from the points of view of both the toxin-effect and the FRAP method.

Introduction

The physicochemical properties of the cell plasma membrane and their functional implications have been in the focus of interest during the last years [1–3]. For example, the lateral diffusion velocity of the membrane proteins has been measured using the technique called fluorescence recovery after photobleaching (FRAP) or fluorescence photobleaching recovery (FPR) [4–6]. A crucial problem still unresolved is that, according to some theoretical expectations, the proteins and lipids should display nearly identical lateral diffusion velocity [7,8]. However, this is not the case: in the reality the measured values of the diffusion constants for lipids and proteins differ by at least

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Abbreviations: FRAP, fluorescence recovery after photobleaching; PIAF, peroxide-induced autofluorescence.

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two orders of magnitude. This difference was attributed to some cytoskeleton-membrane interactions, however, experimental data resulted in a controversy on this issue [9–14]. Therefore, it is of interest to extend the studies to new cellular models in order to gain more detailed information about the factors influencing the membrane protein mobility.

A possibility of studying the effect of membrane cholesterol on the protein mobility emerged from the available knowledge regarding the toxin called perfringolysin O. This toxin (called also theta-toxin [15]) is produced by *Clostridium perfringens* type A; it belongs to the class of oxygen-labile, thiol-activated exotoxins the primary receptor of which is the membrane cholesterol on the cell surface and exert a non-osmotic, temperature-dependent hemolysis [15,16–21]. Apart from the erythrocytes, the cytolytic effect of perfringolysin O has been reported also for human fibroblasts [22], HeLa [23] and for heart muscle cells [24]. It is well established that the perfringolysin O is first bound to the membrane cholesterol (see for references, Ref. 15), and several further events occur, e.g., an increase of the membrane permeability for calcium [21,25], an activation of phosphatidic acid metabolism [26], before the cytolysis takes place. However, to best of our knowledge, no experiments have been carried out to reveal an eventual influence of perfringolysin O on the lateral mobility of membrane proteins.

Since 1982 we have been dealing with the development of a preparative technique rendering the cells of compact tissues like the liver suitable for the FRAP studies. During this work an automated FRAP instrument has been constructed [27] and a properly standardized smearing technique has been elaborated for the liver [14]. Transmission electron microscopic studies performed on perpendicular sections of the liver smear have shown that most of the hepatocytes remain intact on the smear surface [28]. It turned out also when using liver smears for FRAP measurements that the hepatocytes exposed to the atmospheric oxygen pressure develop a yellowish-green autofluorescence in their plasma membrane which can be enhanced and stabilized by a mild (1 mM) H_2O_2 treatment [14]. Studies performed recently on isolated plasma membranes of hepatocytes [29] revealed that this peroxide-induced autofluorescence (PIAF) is strongly bound to the membrane proteins, and is absent on the chloroform-soluble components of the membrane; it has an excitation maximum at 468 nm and a broad emission between 500 and 620 nm. this autofluorescence bears a close resemblance to that of oxidized flavin compounds (riboflavin, lumiflavin, FAD, FMN) [29]. The PIAF could be found in numerous protein fractions of the SDS-solubilized isolated hepatocyte membranes, i.e., the diffusion constant measured by this fluorescence represents an 'average' diffusion velocity of the membrane proteins. Other experimental findings indicate tht our FRAP method as applied to the PIAF of the liver smear reveals physiologically meaningful changes in the lateral diffusion velocity of the plasma membrane proteins during aging or experimental interventions [14,28,30].

The aim of the present work was on one hand to establish whether the addition of perfringolysin O to the liver smears exerts any influence on the lateral diffusion constant of proteins measured using the PIAF and FRAP method. On the other hand, it was also aimed to study the effect of perfringolysin O on isolated hepatocytes, in order to establish the cytolytic effect of this toxin on the liver cells.

Materials and Methods

Experimental animals. The experiments were performed on the liver of male Fischer 344 of 14 months of age.

Preparation of the toxin. Detailed description of the purification of perfringolysin O from a *Clostridium perfringens* strain PB6K N5 can be found elsewhere [31]. This procedure yields a perfringolysin O showing a single protein band on SDS-polyacrylamide gel electrophoresis; it is free from alfa-toxin (phospholipase C) activity [16]. The toxin was activated before its application as follows. Proper quantities of perfringolysin O were incubated at 37 °C for 15 min in phosphate buffered saline (10 mM sodium phosphate +0.9% NaCl, pH 7.0) containing 10 mM dithiothreitol and 1 mg/ml bovine serum albumin (BSA, crystallized and lyophilized, fatty acid-free, Cat. No. A7511 of Sigma). The hemolytic activity of the

activated toxin was assayed by using a standardized method as described by Saito et al. [32] and expressed in hemolytic units (HU/ml).

FRAP measurements. The instrumentation and procedure measuring the average protein lateral mobility in hepatocyte plasma membrane by using the peroxide-induced autofluorescence (PIAF) has been described elsewhere [14,28]. Here we list only the main steps involved.

(i) Preparation of the liver smear of about 50 μm thickness (two cell layers) by a standardized method.

(ii) Incubation of the smears for 10 min at 37°C with Krebs-Henseleit bicarbonate Ringer solution of pH 7.4 [33], containing 1 mM H_2O_2.

(iii) 3×1 min washing with H_2O_2-free Ringer solution.

(iv) Incubation for 5 min at 37°C with the same Ringer solution containing 600 or 2000 HU/ml of perfringolysin O. 50 μl aliquotes of the incubation medium were taken 1, 3 and 5 min subsequent to the start of incubation in which the toxin activity was tested with the standardized hemolytic method [32]. In one experiment the sequence of addition of H_2O_2 and the toxin to the smear was reversed: in that case the toxin was completely inactivated by the H_2O_2.

(v) Covering with cover slide and sealing with melted paraffin in order to avoid drying.

The FRAP measurement took place by using a laser beam of 476.5 nm being the nearest to the excitation maximum of PIAF (468 nm [29]) among the available wavelengths of an NEC argon-ion laser. An Olympus FLPL objective ($\times 40$, NA = 0.75) was used in epi-illumination system. The effective size of the measured area is characterized by the spot half diameter (W) at an intensity of Ie^{-2}, being in our case 1.5 μm in average [27]. The procedure is computer-monitored as shown by Fig. 1. The lateral diffusion constant (D) is calculated by the computer program using two different mathematical methods, and in order to minimize the error of the results obtained, we use the average of these two results which is symbolized by DD on Fig. 1 [14].

The results were checked first for normal distribution by a suitable computer program as explained in our previous paper [14]. This procedure was necessary in order to avoid from consideration the cells heavily damaged by the smearing method and therefore, early autolyzed, which give unusually high values for DD out of the normal distribution of the intact hepatocytes. This correction procedure eliminates usually not more than 10% of the measured cells.

Apart from the toxin-treated liver smears (seven rats), the following control experiments were also carried out.

(i) Untreated control smears (five rats);

(ii) Smears incubated as in the toxin experiments, however, the incubation medium contained only bovine serum albumin plus dithiothreitol, in order to check the eventual direct effect of these components of toxin activation on the protein mobility (three rats).

(iii) Smears incubated with activated toxin which had been heat-denatured (boiling for 5 min) before its application (one rat).

(iv) Smears incubated with activated toxin which had been saturated before use with cholesterol (one rat).

Electron microscopy. Representative control and perfringolysin O-treated smears were processed for transmission electron microscopy as described previously [14,28], in order to reveal the qualitative nature of eventual structural changes caused by the perfringolysin O on the hepatocytes. The smears were processed first as for the FRAP studies, however, they were fixed and embedded so that ultrathin sections perpendicular to the surface of the smear could be obtained. The sections were double contrasted by uranyl-acetate and lead citrate as usual.

Studies on isolated hepatocytes. These studies were aimed to establish the effect of perfringolysin O on three relevant parameters of the isolated hepatocytes, namely, the viability, as well as the leakage of two cytosolic enzymes, lactate dehydrogenase and alanine aminotransferase (called also glutamic-pyruvic transaminase).

The isolation procedure of hepatocytes was essentially identical with that elaborated and described in detail by Van Bezooijen et al. [34]. According to their method, the intercellular structures are disrupted by perfusing the liver for 20 min with a medium containing $7.5 \cdot 10^4$ units/l collagenase and with $4.6 \cdot 10^5$ units/l hyaluronidase. In our experiments, however, the hy-

aluronidase treatment was omitted. The isolated hepatocytes were finally suspended (10^6 cells/ml) in Krebs-Henseleit bicarbonate Ringer solution of pH 7.4 [33].

The effect of perfringolysin O on the isolated hepatocytes was studied as follows. To a 5 ml aliquot of hepatocyte suspension the necessary amount of activated toxin in a volume of 0.15 ml was added and the cells were incubated at 37 °C under an 5% CO_2/95% O_2 stream with gentle shaking. Parallel samples were run where before the addition of perfringolysin O a pretreatment of the hepatocytes with 1 mM H_2O_2 for 10 min at 37 °C was performed. In such experiments, H_2O_2 was washed out from the cells 3×1 min and the cells were resuspended in the original volume. At certain time intervals (indicated on Figs. 3–5) 0.5-ml aliquotes were taken and the viability as well as the release of cytosolic enzymes (lactate dehydrogenase and glutamic-pyruvic transaminase) were determined. Viable cells were counted using a hemocytometer after addition of equal volume of 0.5% Trypan-blue solution. In case of the cytosolic enzymes the cells were centrifuged from the suspension (10000 rpm, for 10 s) and the enzyme activities were assayed in the supernatants using specific assay kits (Shino-test of Code No. 3080 for lactate dehydrogenase and No. 40 for glutamic-pyruvic transaminase, respectively, produced by Shoji Co. Ltd. Tokyo). Total enzyme activities (100%) were obtained in supernatants of hypotonically disrupted cells. At least three parallel experiments were done for each test; and the experiments were repeated several times. Figs. 3–5 demonstrate typical results of the three assays.

Isolated hepatocytes were studied as morphologically by using a phase contrast microscope in normal conditions as well as after the addition of perfringolysin O.

Results

Since neither the type of specimen nor the method of FRAP measurement used by us are widely known, we demonstrate the computer plot of a typical experiment in Fig. 1. The intensity of the fluorescence is recorded for 100 s (observation period) measuring for 1 s every 4 seconds, in order

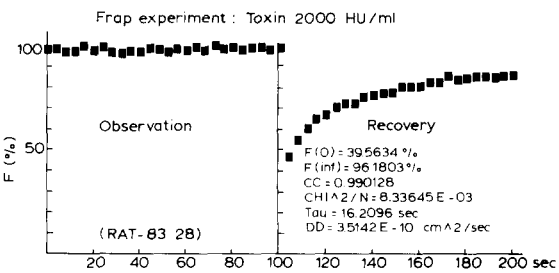

Fig. 1. The computer plot of a real FRAP experiment when adding 2000 HU/ml perfringolysin O to hepatocytes in liver smear. Meaning of abbreviations used, $F(0)$, fluorescence intensity at zero post-bleach time; $F(\text{inf})$, fluorescence intensity when the maximum recovery is reached; CC, correlation coefficient of the fit to the reciprocal equation of Yguerabide et al. [46]; chi^2/N, a figure indicating the goodness of the same fit; tau, average characteristic diffusion time; DD, the average diffusion constant derived from two different ways of calculation. Other explanations in the text.

to be sure that neither mechanical (movement of the cell or liquid streams under the cover slip) nor chemical instability (photodecomposition at the low exciting beam intensity) will disturb the measurement. The fluorescence is then bleached in the very same area of measurement by a 200 ms long, 10^4-times more intense laser impulse so that a considerable drop of the intensity comes into being, however, the membrane itself becomes not 'perforated'. (The simplest proof for that is the fact that no regular fluorescence recovery can be recorded from perforated membranes). The fluorescence intensity is further recorded after bleaching for another period of 100 s (Fig. 1) during which the fluorescence intensity gradually recovers due to the diffusion of the non-bleached molecules from outside into the bleached area. A computer assisted mathematical method helps then to get the values of all important parameters like the fractional recovery, the maximum recovery, and, or course, the diffusion constant (DD) of the fluorescent molecules, i.e., proteins.

Evidence has been presented elsewhere that this FRAP technique reveals a significant, sex-dependent, negative linear age-correlation of DD in hepatocyte membranes of Fischer 344 [14,35] as well as Wistar rats [30]. Other, yet unpublished observations in our laboratory showed that the negative linear age-correlation of DD exists also in C57BL/6 mice, the slopes of the regression

TABLE I

SUMMARY OF THE RESULTS ON THE LATERAL DIFFUSION CONSTANT (D) OF PROTEINS WITH PERFRINGOLYSIN O AND THE RESPECTIVE CONTROL MEASUREMENTS IN RAT LIVER SMEARS USING THE FRAP METHOD

D is presented as mean ± S.E. of n cells measured from N rats
BSA, bovine serum albumin; DTT, dithiothreitol.

Group of rats (N)	n	D (10^{-10} cm$^2 \cdot$s^{-1})	Significance $P < \%$ [a]
A: controls (5)	162	2.30 ± 0.04	A/B: 0.1
B: Toxin 600 HU/ml (3)	82	3.03 ± 0.08	B/D: 1.0
C: Toxin 2000 HU/ml (4)	111	3.37 ± 0.09	C/D: 0.1
D: BSA + DTT (3)	35	2.61 ± 0.09	A/D: 0.5
E: Heat-denat. (1)	23	2.44 ± 0.13	A/E: n.s. D/E: n.s.
F: Cholest. saturation (1)	20	2.36 ± 0.10	A/F: n.s. D/F: n.s.

[a] Note: Significance is given on the basis of the unpaired Student's t-test. n.s., not significant.

lines are, however, significantly different (smaller in males, larger in females) from those of the rats.

Table I summarizes the results obtained. It should be mentioned that the total number of cells measured was 484, and as a result of checking the normal distribution, 10.5% of them must have been omitted, i.e., the total number of cells shown in Table I amounts to 433.

Five control rats displayed an average value of $DD = 2.3 \cdot 10^{-10}$ cm$^2 \cdot$ s^{-1} in good agreement with the established age-dependent regression line of this parameter for male Fischer 344 rats [14].

The toxin treatment resulted in a considerable increase of the protein mobility in the hepatocyte membranes: the values of DD were 31.9 and 46.2% higher after the addition of 600 or 2000 HU/ml toxin, respectively. In both cases a strong statistical significance ($P < 0.1\%$) could be established (Table I). It should be pointed out, however, that when the liver smears were exposed to the effect of bovine serum albumin plus dithiothreitol (BSA + DTT) only, a small increase of

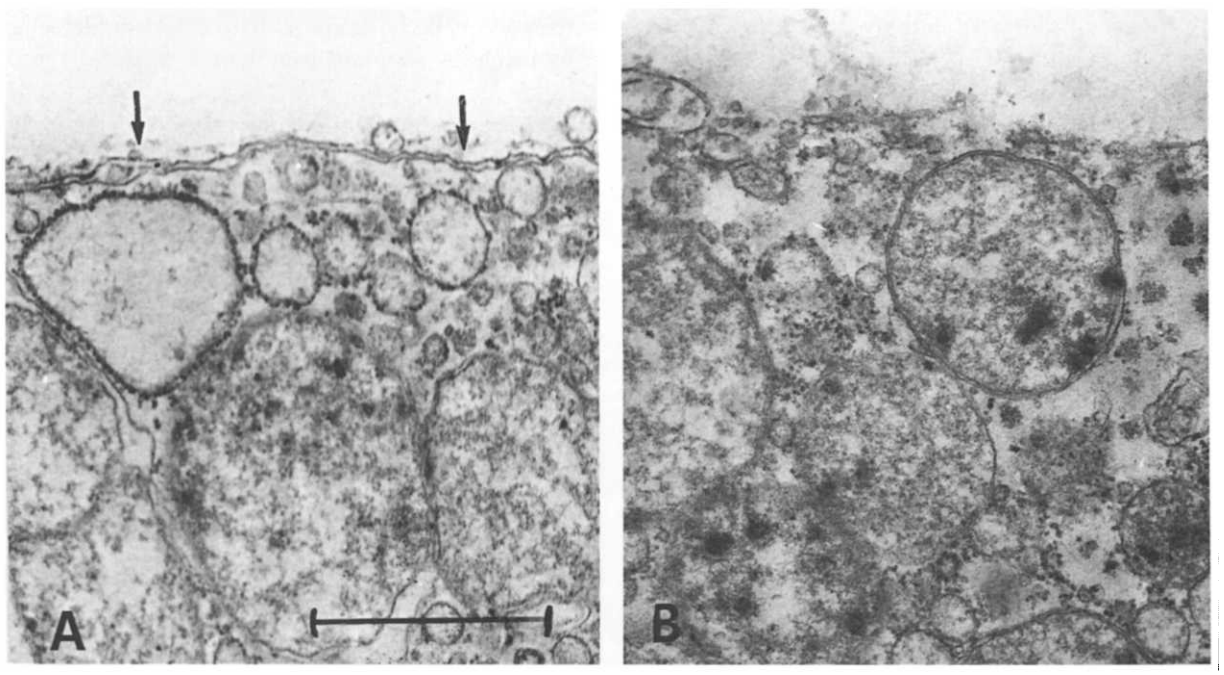

Fig. 2. Transmission electron micrographs of perfringolysin O-treated hepatocytes in the liver smears at the beginning (A) and at the end of the 3rd hour (B) of the FRAP experiment. Note the continuous plasma membrane at the cell surface in A (arrows), whereas in B this membrane is absent from the cell surface. The bar represents 1 μM for both parts of the figure.

DD was also measured (+13.4%), however, the difference proved to be significant at a $P < 0.5\%$ level. The experiments in this respect were performed with the amount of BSA + DTT belonging to the higher toxin concentration, and obviously, the 600 HU/m toxin brings into the system proportionally less BSA + DTT. Nevertheless, both toxin-induced results are strongly different not only from the control value but also from that of the BSA + DTT experiments in statistical terms ($P < 0.1\%$). When applying only 100 HU/ml perfringolysin O, the value of DD remained at the same level as found after BSA + DTT treatment only (data not shown).

Since perfringolysin O is heat-sensitive, the effect of activated then heat-denatured toxin was also tested on the hepatocytes. This resulted in only a 6.1% increase of DD, however, this difference proved to be insignificant as compared to the control value (Table I).

The binding sites of the activated toxin can be saturated with cholesterol before its application: performing this experiment, we obtained only a negligible, statistically insignificant increase (2.7%) of DD (Table I).

Checking the toxin activity during the incubation revealed that perfringolysin O maintains practically its original activity above the smear during the whole incubation period, i.e., it is present in high abundance as compared to the binding capacity of hepatocytes. It is necessary, however, to wash out completely the H_2O_2, since even the rests of it may inactivate the perfringolysin O. When the toxin and the H_2O_2 were applied together, or the addition of H_2O_2 followed the perfringolysin O, no toxin activity could be detected by using the standard hemolytic test.

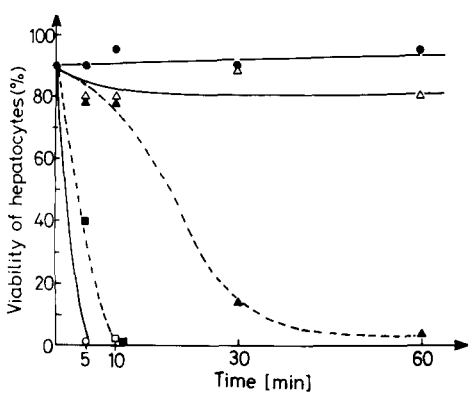

Fig. 3. The effect of perfringolysin O as well as H_2O_2 + perfringolysin O treatment on the viability of isolated hepatocytes using Trypan blue uptake test. ●———●, control cells; △———△, 6 HU/ml of perfringolysin O; ▲— — —▲, 1 mM H_2O_2 + 6 HU/ml perfringolysin O; □— — —□, 60 HU/ml of perfringolysin O; ■— — —■, 1 mM H_2O_2 + 60 HU/ml perfringolysin O; ○———○, 600 HU/ml of perfringolysin O.

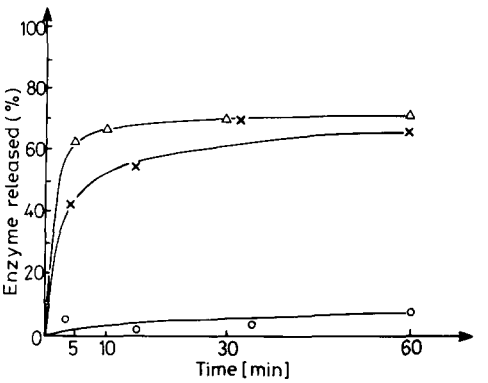

Fig. 4. The effect of perfringolysin O on the release of the cytoplasmic enzyme lactate dehydrogenase from isolated hepatocytes. ○———○, control cells; ×———×, 40 HU/ml toxin; △———△, 2000 HU/ml toxin.

Electron microscopy of the liver smears

This method revealed that most hepatocytes in the liver smear maintain their structural integrity

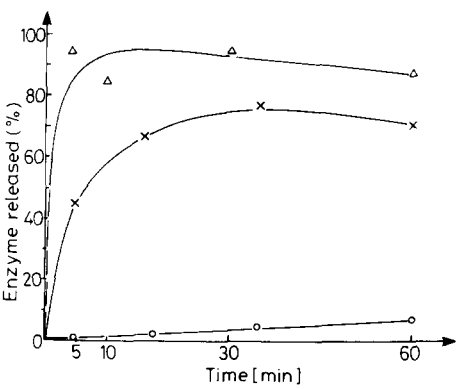

Fig. 5. The effect of perfringolysin O on the release on the cytoplasmic enzyme glutamic-pyruvic transaminase from isolated hepatocytes. ○———○, control cells; ×———×, 40 HU/ml toxin; △———△, 2000 HU/ml toxin.

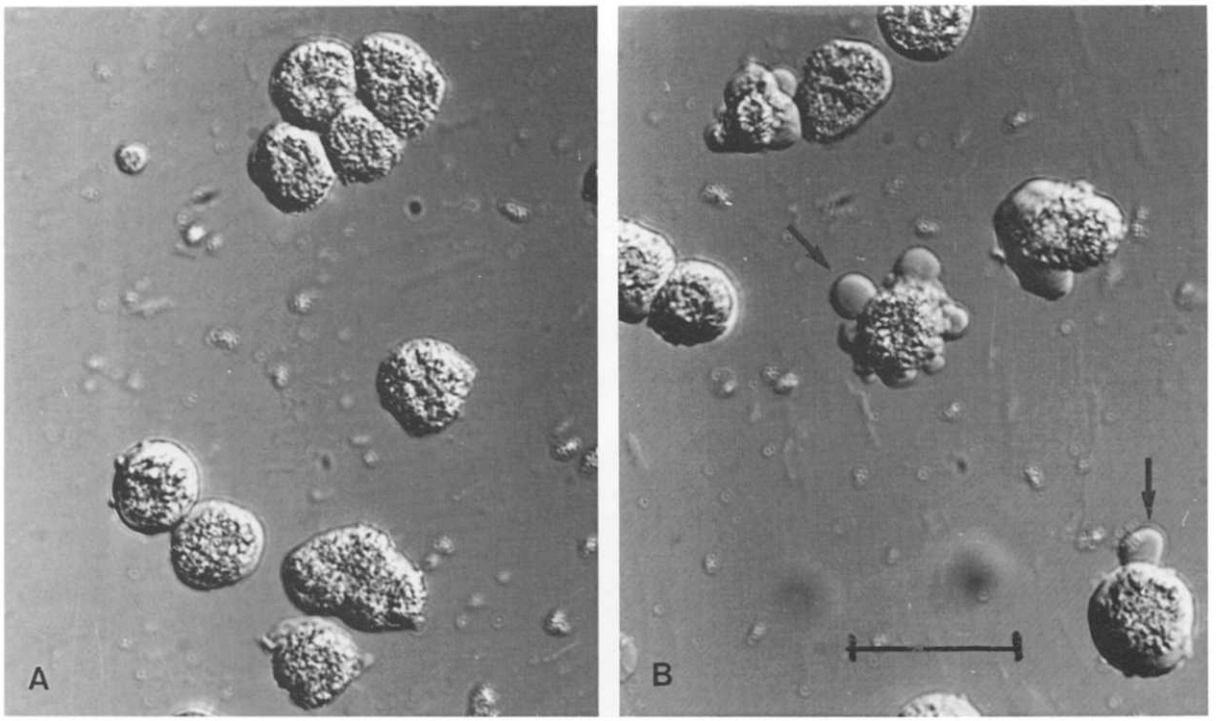

Fig. 6. Phase contrast light micrographs of the isolated hepatocytes before (A) and after (B) perfringolysin O treatment. Note the intense bleb formation in B (arrows) which is absent in A. The bar represents 50 μm for both parts of the figure.

and their upper surface is covered by a continuous plasma membrane [28]. On the other hand, the treatment with perfringolysin O causes an increased frequency of membrane ruptures on the smear surface; by the end of the 4th hour subsequent to the removal of the liver from the animal, practically all cells are devoid of their plasma membrane and show the morphological signs of an intense cytolysis (Fig. 2).

Observations on the isolated hepatocytes

Typical results of these experiments are shown in Figs. 3–5. It is evident from the viability studies that the isolated hepatocytes lose very quickly their membrane integrity after getting in contact with the perfringolysin O (Fig. 3). Already 6 HU/ml perfringolysin O affects the viability to a slight extent, whereas 60 HU/ml was sufficient to reduce the viability of practically all cells to zero in 10 min; 600 HU/ml (or 2000 HU/ml, not shown) performed the same effect in an even shorter time. When the effect of perfringolysin O on the viability test was studied after the incubation of the hepatocytes with 1 mM H_2O_2 for 10 min, an acceleration of the cell destruction was observed with 6 HU/ml of perfringolysin O (Fig. 3). Higher perfringolysin O concentrations decreased the viability of cells so fast that addition of H_2O_2 did not show any apparent influence on this parameter.

Lactate dehydrogenase and glutamic-pyruvic transaminase studies revealed that the release of these two cytosolic enzymes is going parallel with the loss of viability of the hepatocytes (Figs. 4 and 5). Pretreatment of the hepatocytes with 1 mM H_2O_2 resulted in even faster release of these enzymes into the extracellular space.

Light microscopic morphological studies showed that the perfringolysin O effect manifests itself in a bleb-formation on the surface of the isolated hepatocytes, whereas the non-treated cells show such blebs only sporadically (Fig. 6).

Discussion

The validity of the measurement of the average D of the proteins in hepatocyte plasma membrane

using liver smears and of the PIAF as a fluorescent label has been discussed in detail elsewhere [14,27–30,35]. We would like to stress here only several points: (i) The development of PIAF is apparently damaging the conformation of the membrane proteins of hepatocytes to a lower extent than the application of concanavalin A-FITC as a label. This latter compound causes a considerable, concentration-dependent reduction of the mobile fraction of the proteins [36], which is explained by chemical [37] or light-induced [38] cross-linking effect, whereas in the PIAF-FRAP experiments the mobile fraction remains fairly high [14]. The fact that the main chromophore of PIAF was found to be an oxidized form of flavin compound(s) [29] agrees quite well with such an interpretation, since in this case the chromophore can be assumed a priori bound to proteins in reduced form and only its oxidation is causing the appearance of the fluorescence. (ii) In the liver smears prepared by our method one can safely study the hepatocytes by the FRAP instrument for about 2–3 h, since the autolysis of the cells comes into being in an explosion-like manner only after this time (most probably when the majority of the lysosomes 'pour out' their enzymes) [14,27].

The main finding of our experiments was that the perfringolysin O treatment of 5 min significantly increased the value of D, and on the other hand, perfringolysin O exerted a cytolytic effect on the isolated hepatocytes. This latter effect has been observed in numerous other cell types [15,16,18–24].

The control experiments (heat-denaturation of toxin, or saturation of it with cholesterol before application) performed on the liver smears showed unanimously that (like in other cellular models), the effect of perfringolysin O on D should be attributed to the binding of perfringolysin O to cholesterol. The observed phenomena can be interpreted from various points of view as follows.

(i) The results of the present experiments strengthen the other arguments [14,29] according to which PIAF is localized to the cell membrane, otherwise it is difficult to find any reason why perfringolysin O treatment increased the measured lateral diffusion of proteins.

(ii) The effect of perfringolysin O on the value of D allows us to discuss the role cholesterol may play in the determination of the lateral mobility of membrane proteins. It is well established that cholesterol contributes to the rigidization of the membrane lipid bilayer [39,40]. On the other hand, it is also known that the cytolytic activity of perfringolysin O (being dependent on its essential thiol group) is strongly inhibited by cholesterol [41]. Although the available data suggest only indirectly that cholesterol is removed from the erythrocyte ghosts by this toxin [15], there seems to be no doubt that the properties of cholesterol (and the membrane lipid bilayer) are strongly altered during the 'sequestration' of cholesterol by perfringolysin O [15,41]. As a matter of fact, changes in the cholesterol content and lipid composition of reconstituted lipid vesicles were reported to cause serious alterations in the rotational diffusion of human band 3 proteins [42]. On the other hand, experiments with artificial membranes and reconstituted acetylcholine receptors from *Torpedo marmorata* revealed no difference in the translational diffusion constant of the receptor protein, if it was tested in cholesterol-free dimyristoylphosphatidylcholine or in the presence of 45% cholesteryl hemisuccinate [43]. Since the system used by the latter authors is by no means comparable to the real cell membrane just due to the high dilution, and to the fact that the receptor moved there with $D = (1–3) \cdot 10^{-8}\,cm^2 \cdot s^{-1}$, which is higher by two orders of magnitude than our values, these results can hardly constitute an evidence against the role of cholesterol in the determination of the lateral diffusion of proteins in the cell membrane. As regards the theoretically emerging possibility of some reactions of perfringolysin O with membrane components other than cholesterol, the inhibitory effect of cholesterol saturation on the activated enzyme seems to exclude it, although not absolutely. In any case, further experimental proofs are necessary to clarify the mechanism of action of this toxin in more detail.

In our opinion, the fact that the highest concentration of perfringolysin O used in our experiments (2000 HU/ml) caused 46% increase of D, demonstrates that the composition (and the fluidity) of the lipid bilayer in itself is an important factor influencing the average protein mobility in the hepatocyte membrane. If we consider the fact

that in male Fischer 344 rats, D decreased linearly about 41% from the newborn to 30 months of age (and even smaller decrease was found in females) [14], the extent of the change of D under perfringolysin O cannot be neglected. Nonetheless, one should also consider that perfringolysin O was shown to influence the Ca^{2+} permeability of the cell membrane [21,25], as well as to alter the phosphatidic acid metabolism [26], i.e., there exists the possibility that apart from the 'sequestration' of cholesterol from the membrane, D can also be altered through these influences.

It is obvious that perfringolysin O exerts a strong cytolytic effect even in our cellular model, and this effect is still more enhanced by a mild pretreatment of the cells with H_2O_2. There is, however, one point to be discussed, namely, the cytolysis as measured by the viability test as well as by the release of two cytoplasmic enzymes occurred already at low concentrations of perfringolysin O (e.g., 60 HU/ml) which were well below the toxin concentrations resulting in significant changes of D in the liver smears (600 HU/ml). Several arguments can be listed to explain this problem as follows.

(i) The cell membrane of isolated hepatocytes is exposed to a preparative injury during the collagenase digestion, the mechanical disruption of the tissue organization, etc. These interventions were shown to cause serious alterations in the membrane functions of cultured fibroblasts [44]. In addition, the toxin has a free access to 100% of the surface of the isolated hepatocytes and the intense bleb formation (Fig. 6) further increases the accessible membrane surface area. In the smears we have a considerably different situation, namely, the cells have not been exposed to enzyme digestion, and they remain in a quasi-tissue organization in which the perfringolysin O can reach only the upper surface of the hepatocytes.

(ii) When human erythrocytes membranes were exposed to perfringolysin O and studied by freeze-fracture electron microscopy [45], fine structural alterations (random aggregation of the intramembranous particles) were observed on the protoplasmic half of the membrane already under the effect of 40 HU/ml toxin concentrations, whereas the exoplasmic half of the membrane displayed similar structural alterations only after the addition of as much as 3300 HU/ml perfringolysin O. Since even 40 HU/ml is well above the hemolytic concentration of this toxin, one can assume that cytolysis comes into being already much before the complete destruction of the cell membrane. This interpretation is consistent with the observation that D can be measured also in isolated cell membranes which are disrupted at certain places [13].

As a final conclusion, one can see from our results that hepatocytes in liver smear may represent a suitable model for further studies of the toxin effect, and also of the functional significance of D in the cellular metabolism.

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