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Effect of *Clostridium perfringens* Phospholipase C (Alpha-toxin) on the Human Diploid Fibroblast Membrane

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Summary. Human diploid embryonic lung fibroblasts were cultivated in Eagle's Minimum Essential Medium and labeled with ^{3}H-uridine. The release of soluble radioactive substances into the medium was used as an indicator of damage to the cell membrane. The assay method described is simple, sensitive and rapid and allows quantitative estimation of changes in membrane permeability before any morphological damage is observed microscopically. Crude commercial preparations of phospholipase C (E.C. 3.1.4.3) (40 μg/ml) were highly active on the cell membrane but most of the membrane damaging activity was found to be due to contaminating theta-toxin. However, also highly purified phospholipase C caused a membrane damage as measured by release of isotope through the plasma membrane. The release could be increased by including an optimal concentration of calcium ions in the incubation buffer, by treating the cells in a hypotonic medium and by simultaneous treatment with sublytic concentrations of Triton X-100. To our knowledge this is the first report of membrane damage on a live, intact, metabolizing human diploid cell caused by a highly purified phospholipase C. The results are in agreement with a dynamic membrane structure with the polar groups of a part of the phospholipids accessible at the membrane surface.

Highly purified phospholipase C (phosphatidylcholine cholinephosphohydrolase, E.C. 3.1.4.3) from *Bacillus cereus* is nonhaemolytic for erythrocytes of many different species [13, 45]. On the other hand, phospholipase C from *Clostridium perfringens* was shown three decades ago to be haemolytic, dermonecrotic and lethal [20], activities which were later found also in purified preparations [33, 37]. However, since this organism also produces other cytolytic proteins such as theta-toxin, it was not clear until recently whether the haemolytic activity was due to contaminating theta-toxin. Commercial, partially purified preparations of this phospholipase C contain this cytolytic factor, as well as about ten other toxins and enzymes [25].

However, highly purified clostridial phospholipase was still found to be haemolytic but with a much reduced specific activity, indicating that theta-toxin probably is the main cytolytic protein in crude and partially purified phospholipase C preparations (C. J. Smyth, Ph.D. Thesis, University of Glasgow, 1972 and ref. [26]).

Clostridial phospholipase C has been shown to interact with a variety of different biological membranes, such as the plasma membrane of fat cells [6] and leucocytes [14], mitochondrial membranes [3], myelin membranes [22] and viral membranes [24], as well as interfere with cell metabolism in several cases [6, 14, 29]. It is also agreed upon that phospholipase C from different sources liberates about 70 % of the phosphorus from membranes of erythrocyte ghosts [35, 45]. However, whole cells have been shown to be quite resistant to phospholipase C from *B. cereus* [45].

In the present investigation, the interaction of highly purified clostridial phospholipase C with plasma membrane of human diploid fibroblasts was studied. Release of soluble radioactive substances from prelabeled fibroblast monolayers was taken as an indicator of damage to the plasma membrane, according to a test system recently developed for the study of membrane-damaging effects of four staphylococcal cytolysins [39].

Materials and Methods

Materials

Phospholipase C was purified from culture supernatants of *Clostridium perfringens* type A (strain ATCC 13124). The strain was grown in a prereduced proteose peptone medium in a fermentor with controlled pH, temperature and redox-potential (C.-E. Nord, R. Möllby, & T. Wadström, *unpublished*). The toxin was purified by DEAE-Sephadex A-25 and Sephadex G-75 chromatography and by isoelectric focusing [26]. The following methods for analysis of homogeneity of the purified phospholipase were used: (1) acrylamide electrophoresis in different buffer systems [40, 41, 42]; (2) one precipitin band in immunoelectrophoresis; (3) devoid of any of the 18 different enzyme and toxin activities assayed for [25]. Commercial preparations of partially purified phospholipase C (Lot No. 101C6880) were purchased from Sigma Chemical Company, St. Louis, Missouri.

^{3}H-uridine (specific activity 20 C/mole) was obtained from The Radiochemical Centre, Amersham, England. Choline chloride (methyl-^{3}H) (specific activity 1 to 5 C/mmole) and Aquasol TM Universal L.S.C. Cocktail were obtained from New England Nuclear Chemicals GmbH, Frankfurt, W. Germany. Polystyrene multi-dish disposable trays (FB-6-TC, 3 cm) from Linbro Chemical Co. Inc., New Haven, Connecticut, were used as culture vessels. The components of Eagle's Minimum Essential Medium [8], Hank's balanced salt solution as well as calf serum were obtained from Flow Laboratories, Ltd., Irvine, Scotland. Triton X-100 was obtained from Rohm and Haas Co., Philadelphia, Pa. Boric acid, sodium borate, and NaCl were purchased from KEBO, Stockholm, Sweden. All chemicals except Triton X-100 were of an analytical grade.

Cultivation and Maintenance of Cell Cultures

Diploid fibroblasts from human embryonic lung tissue in the 5th to 10th passage after 15 to 30 cell divisions were used for the toxicity tests. In addition, two cloned HeLa lines were used. Cells were seeded at a density of 90,000 cells per culture (13,000 cells/cm^2) in Linbro trays and incubated with 3 ml of Eagle's Minimum Essential Medium containing 10% (v/v) calf serum, 4 mM glutamine, 1 mM sodium pyruvate and penicillin (100 IU/ml) and streptomycin (100 µg/ml). The cells were incubated at 37 °C, in a humid atmosphere containing 5% (v/v) CO_2. The medium was changed after 5 to 7 days and the cultures were used when complete monolayers had been developed after another 2 to 7 days.

Toxicity Testing

Monolayers of approximately 0.7×10^6 cells per culture were incubated for 2 hr at 37 °C with medium containing 1 µC/ml ^{3}H-uridine. After further incubation for 2 hr with fresh medium lacking ^{3}H-uridine the monolayers were washed with Hank's balanced salt solution three times to remove extracellular radioactivity before the enzyme was applied. One milliliter of enzyme diluted in the appropriate solution was added per culture, and the cultures were incubated for 30 to 180 min. Control cultures were incubated with the diluent alone. The solution was then removed and centrifuged ($1{,}000 \times g$ for 10 min at 4 °C). A sample of 0.1 ml of the supernatant was transferred to a scintillation vial containing 10 ml of Aquasol. Samples were counted in a Nuclear Chicago Liquid Scintillator for 1 min. All tests were performed in duplicate or triplicate.

If otherwise not stated, the incubation was performed for 30 min at 37 °C in 0.15 M NaCl buffered with 0.02 M Tris HCl, pH 7.0, containing 1 mM $CaCl_2$. The spontaneous release of isotope in the control cultures never exceeded 3 to 6% and was subtracted in the data presented.

All experimental results were expressed as per cent of a maximum release obtained after lysis of the plasma membrane with 0.06 M sodium borate buffer (pH 7.8) as described earlier [18, 39]. Standard deviation of the maximum release when measured in six parallel samples was ± 3 to 7%. This closely paralleled the standard deviation in number of cells per culture, which was also determined for six cultures in each experiment. This standard deviation was considered as acceptable in this biological assay system.

Measurement of Hydrolysis of Membrane Phospholipids

Cell cultures in logarithmic growth phase were incubated for 2.5 hr at 37 °C in Hank's balanced salt solution containing 5 µC/ml of ^{3}H-choline. After further incubation for 3 days in fresh medium resulting in confluent cultures, the cells were incubated for 30 min with enzyme as described above. The enzyme solution was removed and the remaining cells were treated with 1 ml of chloroform/methanol (2:1) for 1 min. Radioactivity in enzyme solution and lipid extract was determined in a liquid scintillation counter as described above.

As measured by lipid extraction of untreated cells according to Bligh and Dyer [2], 93% of the total radioactivity was found in the lipid phase. This fact indicates that the ^{3}H-choline had been efficiently incorporated into the phospholipids of the cell.

Measurement of Phospholipase Enzymatic Activity

Phospholipase activity was assayed on an egg yolk suspension and the release of acid was measured by continuous titration with 0.01 M sodium hydroxide in a pH-stat [45].

This was performed in a semimicro scale in a final incubation volume of 2.1 ml [26]. Calcium (10 mM) and zinc (0.1 mM) ions as well as sodium deoxycholate (5 mM) were included in the incubation mixture as recommended for the *B. cereus* phospholipase [45]. One unit of enzymatic activity (U) was defined as the amount of enzyme which liberated 1 μmole titrable H^+ per min. Commercial phospholipase C contained 25 U/mg (dry weight) and the highly purified phospholipase 370 U/mg protein.

Measurement of Haemolytic Activity

Haemolytic activity was assayed by incubating washed sheep erythrocytes (final concentration 0.5%, v/v) with twofold dilutions of the enzyme in 0.15 M NaCl buffered with 0.02 M Tris HCl, pH 7.0, containing 1 mM $CaCl_2$. Haemolysis was estimated visually after 1 hr at 37 °C and finally determined after 2 hr at 4 °C. The reciprocal of the dilution of the enzyme haemolyzing 50% of the erythrocytes indicated the number of haemolytic units (HU) per ml of undiluted sample [40].

Other Methods and Assays

Isoelectric focusing was performed in an LKB 110 ml column as earlier described [40]. Lethal activity was estimated by intravenous injection into Swiss white mice and dermonecrotic activity by intracutaneous injection into shaved rabbits [41]. Protein content was determined according to Lowry *et al.* [19] after thorough dialysis [40] and expressed as μg/ml.

Results

Effect of Commercial Phospholipase C

Fig. 1 shows the effect on the fibroblast membrane of crude commercial phospholipase C at a concentration of 40 μg/ml. This concentration corresponds to 1 U/ml of phospholipase activity. When a more purified enzyme preparation with equal enzymatic activity was applied to the cells, a marked reduction in effect was observed.

Crude commercial phospholipase C was fractionated on an isoelectric column by focusing in a shallow pH-gradient 5 to 8. The fractions were carefully dialyzed against 0.15 M NaCl buffered with 0.02 M Tris HCl, pH 7.0, and tested for phospholipase C, haemolytic and lethal activity and for fibroblast membrane damaging effect. The results show (Fig. 2) that the fractions containing phospholipase C are lethal but they are not very active on the fibroblast membrane as compared to the fractions containing haemolytic activity. The latter fractions contain the haemolytic theta-toxin [25], which has been purified and shown to exert a membrane-damaging effect in very small amounts (R. Möllby, *unpublished*).

These experiments indicate that membrane effects caused by crude commercial phospholipase C, previously attributed to the action of phospho-

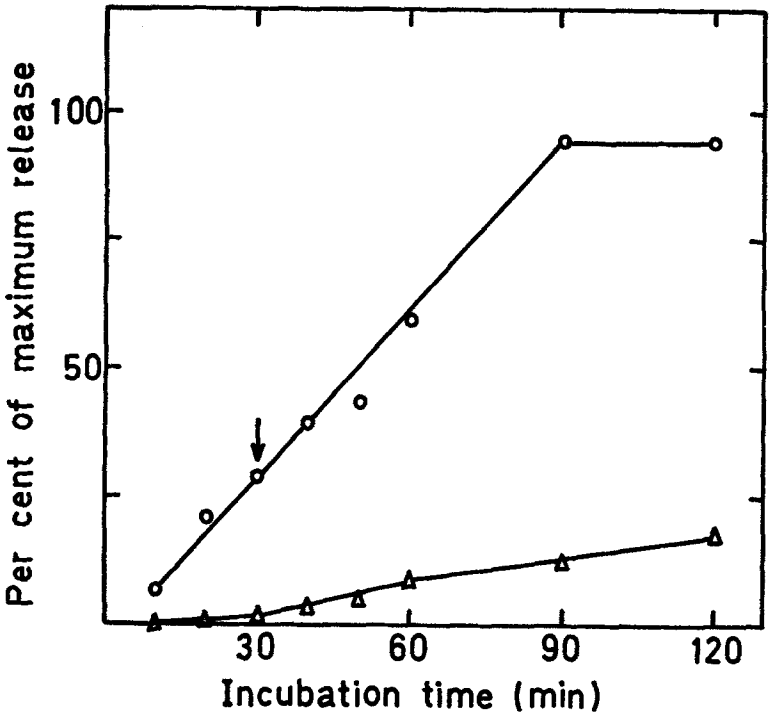

Fig. 1. Effect of crude and purified phospholipase C. Release of radioactive compounds from ^{3}H-uridine-labeled fibroblasts after incubation at 37 °C with commercial phospholipase C (Sigma, 40 μg/ml) o—o, and with purified phospholipase C (peak fraction of activity after isoelectric focusing, Fig. 2) △—△. Both preparations were diluted in Eagle's Minimum Essential Medium and contained 1 U/ml of phospholipase C activity. The arrow indicates when morphological changes, as seen in the light microscope, first appeared

lipase C enzyme activity, might be caused by contaminating theta-toxin and perhaps other biologically active proteins present in such preparations.

Effect of Phospholipase C in Combination with Theta-Toxin

Fibroblasts were incubated with highly purified phospholipase C (2 U/ml) together with highly purified theta-toxin (5 HU/ml). No difference in membrane-damaging activity was observed between the effect of theta-toxin alone and in combination with phospholipase C.

Effect of Divalent Cations

Table 1 shows the release of radioactive compounds after incubation with 6 U/ml of phospholipase C in 0.15 M NaCl buffered with 0.02 M Tris

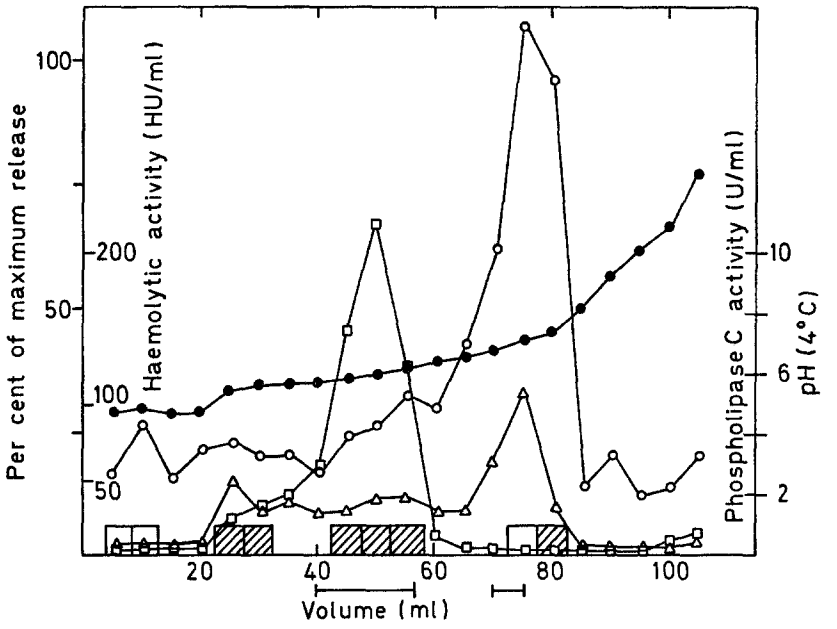

Fig. 2. Effect of crude phospholipase C after isoelectric focusing. Isoelectric focusing of 50 mg of commercial phospholipase C (Sigma, Lot No. 101 C 6880) in a shallow pH gradient, pH 5 to 7 (•—•), for 48 hr at 4 °C [25]. 2.5-ml fractions were collected, dialyzed and assayed for the following activities: ▫—▫, phospholipase C activity; △—△, haemolytic activity; ○—○, cell-damaging activity on human fibroblasts measured as release of radioactive compounds after five times dilution in Eagle's Minimum Essential Medium and incubation for 30 min at 37 °C. The squares at the bottom of the figure denote lethal activity for mice upon intravenous injection of 2.0 ml of each fraction diluted four times. Cross-hatched areas denote lethality within 2 hr and open squares within 24 hr. Dermonecrotic activity in shaved rabbit skin upon intracutaneous injection of 0.05 ml of each fraction is shown below the figure (⊢—⊣). Protein determination on each fraction by the Lowry method did not reveal any distinct protein peaks

Table 1. The effect of calcium and zinc ions on the release of isotope caused by phospholipase C

Salt added	Per cent of maximum release
None	33
1 mM $CaCl_2$	41
10 mM $CaCl_2$	10
0.1 mM $ZnCl_2$	18
1 mM $ZnCl_2$	28
1 mM $CaCl_2$ + 0.1 mM $ZnCl_2$	24

Highly purified phospholipase was diluted to 6 U/ml (16 µg/ml) in 0.15 M NaCl buffered with 0.02 M Tris HCl, pH 7.0, containing calcium or zinc ions. ^{3}H-uridine-labeled human diploid fibroblasts were incubated with the respective dilutions at 37 °C for 30 min. The membrane damage was measured after the incubation period as release of isotope into the medium. The observed values are expressed as per cent of a maximum release obtained by lysing the cytoplasmic membrane by scraping with a rubber policeman after incubating the cells on 0.06 M sodium borate buffer, pH 7.8, at 37 °C for 15 min.

HCl, pH 7.0, containing different concentrations of Ca^{++} and Zn^{++}. One millimolar of Ca^{++} is optimal for nucleotide release, while 10 mM of calcium ions seem to have an inhibitory effect on the release. This finding is in contrast to the egg yolk assay system, where 10 mM $CaCl_2$+0.1 mM $ZnCl_2$ were found to be optimal [26].

Effect of Increasing Concentrations of Phospholipase C

Fibroblasts were incubated with increasing concentrations of highly purified phospholipase C diluted in Eagle's Minimum Essential Medium or in 0.15 M NaCl buffered with 0.02 M Tris HCl, pH 7.0, containing 1 mM $CaCl_2$. The results are shown in Fig. 3. At phospholipase C concentrations above 10 U/ml there was little further increase in leakage of radioactive compounds through the membrane. Identical results were found when the incubation was performed in 0.15 M NaCl+1 mM of $CaCl_2$ with or without the Tris HCl buffer. The Eagle's Minimum Essential Medium seems to have a slightly inhibitory effect on the phospholipase C activity on

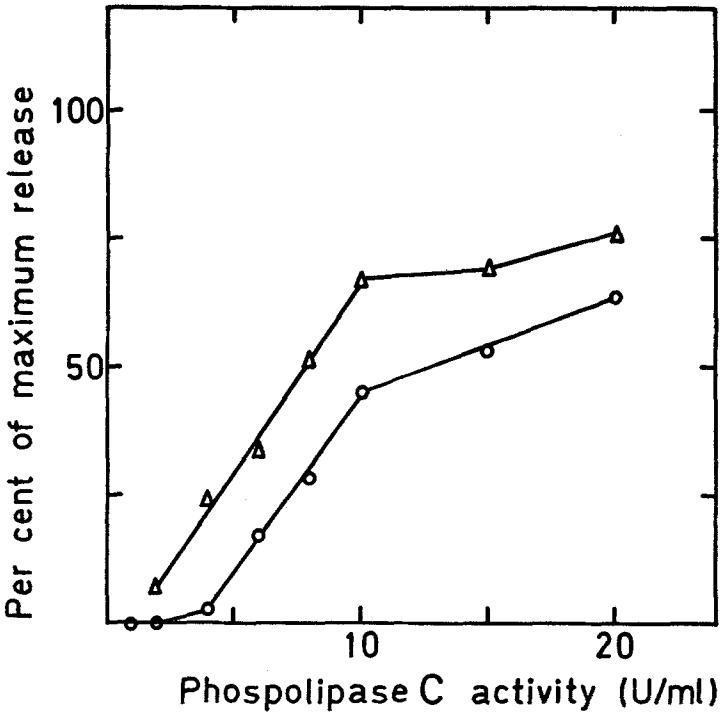

Fig. 3. Effect of increasing concentrations of phospholipase C. Release of radioactive compounds from ^3H-uridine-labeled fibroblasts after incubation for 30 min with increasing concentrations of highly purified phospholipase C. o—o, phospholipase C diluted in Eagle's Minimum Essential Medium; △—△, diluted in 0.15 M NaCl buffered with 0.02 M Tris HCl, pH 7.0, +1 mM $CaCl_2$

the membrane, as compared to the Tris-buffered NaCl, although no corresponding difference in enzymatic activity was noted in samples from each dilution series.

In experiments using HeLa cells it was found that corresponding concentrations of purified phospholipase C caused the release of equal amounts of isotope from these heteroploid tumor cells as from the fibroblasts.

Effect of Phospholipase C at Different Incubation Times

Fig. 4 illustrates the typical time curve found when cultures were incubated with 6 U/ml of phospholipase C in 0.15 M NaCl buffered with 0.02 M Tris HCl, pH 7.0, +1 mM $CaCl_2$ for different time periods. A maximum release is obtained after 60 min of incubation.

Hydrolysis of Membrane Phospholipids

About 46 % of the choline-containing phospholipids in the cell membrane were degraded by 8 U/ml of phospholipase C in 30 min, as shown in Table 2.

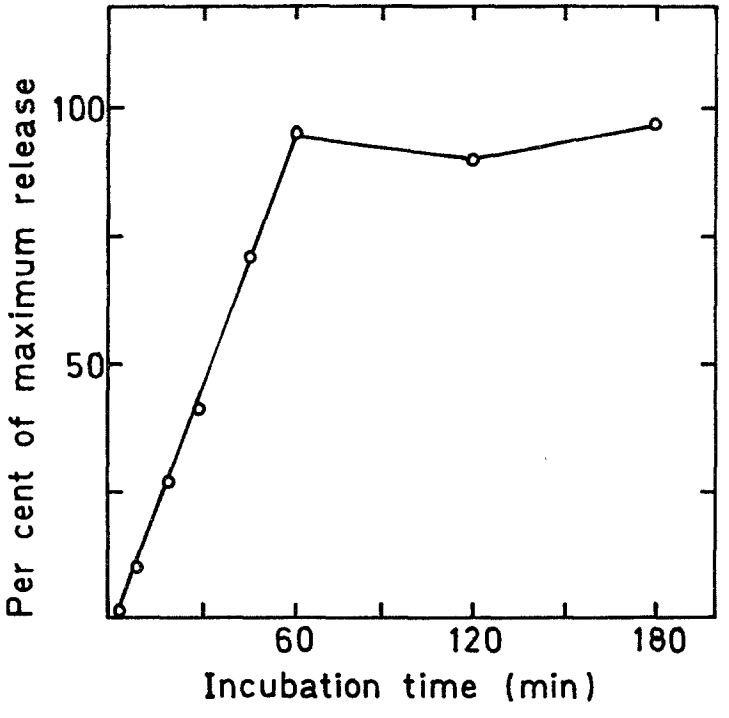

Fig. 4. Time curve for phospholipase C. Release of radioactive compounds from ^3H-uridine-labeled fibroblasts after incubation with highly purified phospholipase C at a concentration of 6 U/ml in 0.15 M NaCl buffered with 0.02 M Tris HCl + 1 mM $CaCl_2$. Incubation at 37 °C

Table 2. Hydrolysis of membrane phospholipids by phospholipase C

	Lipid extract (cpm/ml)	Enzyme solution (cpm/ml)	Per cent choline released
Control	2,655	0	0
Theta-toxin	2,456	51	2
Phospholipase C	1,240	1,059	46

Highly purified phospholipase C was diluted to 8 U/ml (22 µg/ml) in 0.15 M NaCl buffered with 0.02 M Tris HCl, pH 7.0, containing 1 mM $CaCl_2$. ^{3}H-choline-labeled fibroblasts were treated with the enzyme solution at 37 °C for 30 min. The enzyme solution was sucked off and the cell lipid extracted by chloroform/methanol (2:1) for 1 min. The results are compared with those obtained with a highly purified theta-toxin preparation, which caused the same release (42%) of radioactive nucleotides from ^{3}H-uridine-labeled fibroblasts as the phospholipase C concentration used.

Forty-two per cent of the radioactive compounds were released from similarly treated ^{3}H-uridine labeled cells, whereas a corresponding nucleotide release caused by highly purified theta-toxin (R. Möllby, *unpublished*) was not accompanied by a concomitant phospholipid hydrolysis (Table 2). This result strongly indicates, that the observed release of nucleotide following the action of phospholipase C on the fibroblast membrane was actually associated with enzymatic splitting of the phospholipids.

Effect of Phospholipase C on Hypotonically Swollen Cells

Fibroblasts were incubated in hypotonic solutions, which caused swelling of the cells as observed by the light microscope and a certain release of isotope (Fig. 5). Sodium borate buffer, 0.06 M (pH 7.8), caused a more obvious swelling and a slightly higher release of isotope than 8.5 mM NaCl, pH 6.5.

Table 3 shows the effect of 4 U/ml of phospholipase C (11 µg/ml) on the fibroblast cells in isotonic and hypotonic media. The hypotonic treatment of the cells evidently renders the membrane more sensible to the damaging activity of phospholipase C. The lower increase observed in sodium borate buffer than in NaCl may be explained by the ability of borate ions to chelate calcium ions.

When the cells were preincubated in a hypotonic buffer for 10 min at 22 °C before treatment with phospholipase C, no additional increase in isotope release was observed (Table 4). If calcium ions (1 mM) were included in the preincubation buffer, the effect of phospholipase C was significantly lowered.

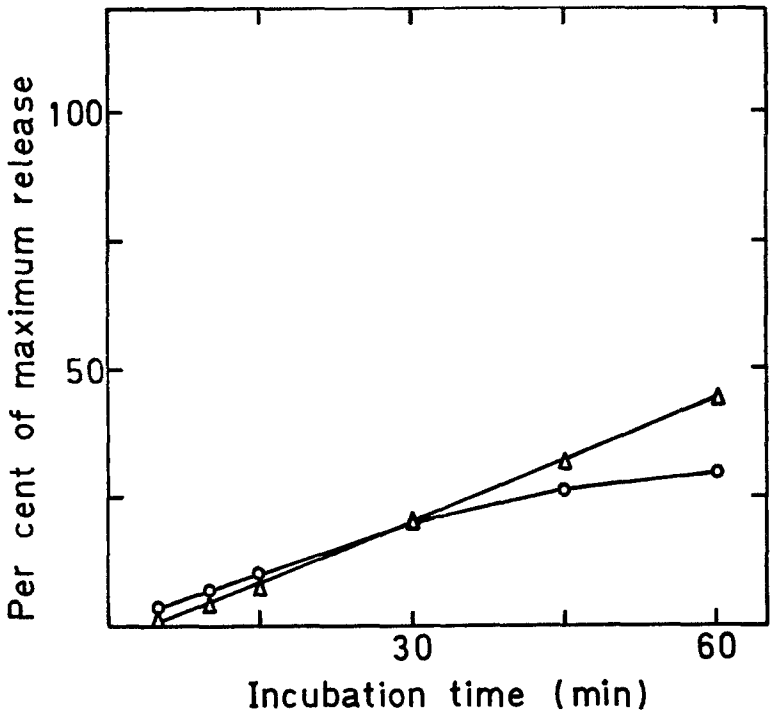

Fig. 5. Effect of hypotonic swelling. Release of radioactive compounds from ^{3}H-uridine-labeled fibroblasts after incubation in a hypotonic medium at 22 °C. ○—○, 8.6 mM NaCl, pH 6.5; △—△, 0.06 M sodium borate buffer, pH 7.8

Table 3. The effect of hypotonic environment on the release of isotope caused by phospholipase C

Incubation solution	Per cent of maximum release	
	Total release	Net release
Eagle's MEM (isotonic control)	2	
PhlC in Eagle's MEM	17	15
0.15 M NaCl, pH 6.5 (isotonic control)	0	
PhlC in 0.15 M NaCl, pH 6.5	23	23
8.6 mM NaCl, pH 6.5 (hypotonic control)	20	
PhlC in 8.6 mM NaCl, pH 6.5	84	64
0.06 M sodium borate, pH 7.8 (hypotonic control)	20	
PhlC in 0.06 M sodium borate, pH 7.8	64	44

Highly purified phospholipase C (PhlC) was diluted to 4 U/ml (11 μg/ml) in the respective solutions, and incubated at 37 °C for 30 min with ^{3}H-uridine-labeled fibroblasts. Except for Eagle's Minimum Essential Medium (MEM) all solutions contained 1 mM $CaCl_2$. For further details, *see* Table 1.

Table 4. The effect of preincubation in hypotonic environment and 1 mM Ca^{++} on the release of isotope caused by phospholipase C [a]

Preincubation 22 °C 10 min	Incubation with enzyme 37 °C 30 min	Per cent of maximum release	
		Total release	Net release
0.06 M sodium borate	0.06 M sodium borate (control)	20	
0.06 M sodium borate	PhlC in 0.06 M sodium borate	63	43
0.06 M sodium borate	0.06 M sodium borate+Ca^{++} (control)	30	
0.06 M sodium borate	PhlC in 0.06 M sodium borate+Ca^{++}	67	37
0.06 M sodium borate+Ca^{++}	0.06 M sodium borate+Ca^{++} (control)	26	
0.06 M sodium borate+Ca^{++}	PhlC in 0.06 M sodium borate+Ca^{++}	51	25
0.06 M sodium borate	Eagle's MEM (isotonic control)	2	
0.06 M sodium borate	PhlC in Eagle's MEM	19	17 [b]

[a] ^{3}H-uridine-labeled fibroblasts were preincubated in 0.06 M sodium borate buffer, pH 7.8, with or without 1 mM $CaCl_2$ at 22 °C for 10 min. After preincubation the buffer was removed and the fibroblasts were incubated in the same buffer, with or without 1 mM $CaCl_2$, or in Eagle's Minimum Essential Medium (MEM) containing phospholipase 4 U/ml (11 µg/ml) at 37 °C for 30 min. The membrane damage was measured as the release of isotope into the medium during the second incubation period and expressed as per cent of maximum release obtained by disrupting the cytoplasmic membrane after the preincubation period. Control experiments without preincubation gave identical values for isotope release as in Table 3.

[b] Compare with the effect of PhlC in Eagle's MEM in Table 3.

Preincubation in a hypotonic solution followed by treatment with phospholipase C in an isotonic medium gave no additional increase in isotope release as compared to a control without preincubation.

Effect of Phospholipase C in Combination with Triton X-100

The nonionic detergent Triton X-100 caused a release of almost 100% of the isotope in a concentration of 0.02% (v/v), which corresponds to about 3.1 mM. At lower concentrations, a very slight effect on the cell membrane was observed (Fig. 6).

Simultaneous treatment of fibroblasts with a sublytic dose of Triton X-100 (0.005%, v/v) and 4 U/ml of phospholipase C caused a significantly greater release of radioactive compounds from the fibroblasts than phospho-

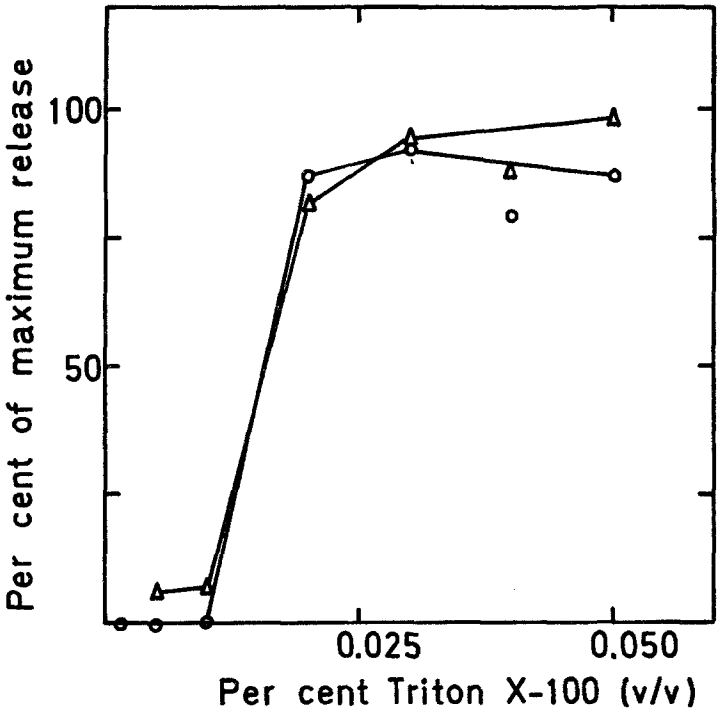

Fig. 6. Effect of Triton X-100. Release of radioactive compounds from ^{3}H-uridine-labeled fibroblasts after incubation with Triton X-100 at different concentrations. Incubation at 37 °C for 30 min. o—o, diluted in Eagle's Minimum Essential Medium; △—△, diluted in 0.15 M NaCl buffered with 0.02 M Tris HCl, pH 7.0, + 1 mM $CaCl_2$

Table 5. The effect of Triton X-100 on the release of isotope caused by different concentrations of phospholipase C

Phospholipase (U/ml)	Per cent of maximum release	
	in TBS	in TBS+Triton
2	8	14
4	25	36
6	33	55
10	65	81

Highly purified phospholipase was diluted to the concentrations indicated in 0.15 M NaCl buffered with 0.02 M Tris HCl, pH 7.0 (TBS), containing 1 mM of $CaCl_2$ with and without 0.005% (v/v) solution of Triton X-100 in the same buffer. ^{3}H-uridine-labeled human diploid fibroblasts were incubated with the respective dilutions at 37 °C for 30 min and membrane damage was measured as described in Table 1.

lipase C alone as shown in Table 5. By contrast, preincubation of the cells with Triton X-100 did not alter their reactivity to subsequent treatment with phospholipase C.

Effect of a Phospholipase C from Staphylococcus aureus

A highly purified phospholipase C from *S. aureus* (sphingomyelinase; β-haemolysin, ref. [40, 41]) has recently been shown to be without effect on the fibroblast membrane in this system [39]. Combination treatments with clostridial phospholipase C, Triton X-100 or hypotonic swelling did not make the membrane susceptible to this phospholipase, which has a more narrow substrate specificity than clostridial phospholipase C [41].

Discussion

Treatment of various membranes with enzymes capable of hydrolyzing specific bonds in phospholipids has often been used in studies on membrane structure and function [35]. Unfortunately, only a few cases have presented any criteria for the purity of the enzyme used. In the case of phospholipase C, the hydrolysis of the phospholipids has been estimated by measuring liberated water-soluble phosphorus and by phospholipid analysis of the membrane after treatment.

Several authors have reported hydrolysis of 70 to 90% of the phospholipids in microsomal or erythrocyte membranes by a crude phospholipase C from *Cl. perfringens* [4, 10, 17, 21] and by a purified enzyme from *B. cereus* [11, 27, 28]. In spite of this extensive breakdown of the phospholipids, gross morphological changes were not observed and some physical characteristics of the membrane proteins remained unchanged [11, 17]. Coleman *et al.* [4] also reported a shrinkage of erythrocyte ghosts after phospholipase C treatment, calculated to correspond to a 45% decrease of the total surface area of the ghosts.

On the other hand, it was recently shown by Zwaal *et al.* [45], Roelofsen *et al.* [32] and Woodward and Zwaal [44] that intact human erythrocytes were resistant to the action of highly purified phospholipase C from *B. cereus*. The phospholipase was, however, shown to hydrolyze most of the phospholipids in erythrocyte ghosts. Furthermore, in the presence of sublytic concentrations of detergents it caused haemolysis. Osmotically swollen erythrocytes and resealed ghosts were also susceptible to the enzyme [44].

Thus it was still uncertain, whether a highly purified phospholipase C, devoid of other toxins and enzymes [25], would affect the intact membrane of viable human diploid fibroblasts in monolayers. It should also be noted that these cells had not been subjected to various treatments, which could alter the membrane structure, such as trypsinization or removal from the solid surface by chelating agents or other methods.

Earlier observations with commercial partially purified preparations of phospholipase C from *Cl. perfringens* [4, 9, 10, 17, 21] may have provided conflicting results because of different degrees of contamination with proteins interacting with the membrane [25]. In this study it was found that the main membrane-damaging activity of such preparations resided in contaminating theta-toxin (Figs. 1 and 2), although no synergistic effect between theta-toxin and phospholipase was observed, as earlier proposed for erythrocytes [43].

The present study shows that about 4 U/ml of highly purified phospholipase is necessary to cause significant leakage of radioactive nucleotides through the fibroblast membrane in 30 min. Increasing the concentration of phospholipase could not induce more than 70 % release but prolongation of the incubation period with 6 U/ml caused 100 % release after 60 min. Observations made by light microscopy indicated that monolayers releasing up to 100 % of maximum release were not destroyed; i.e., the cells were swollen but the cytoplasmic membranes were not disintegrated. Thus, it is possible that the time curve simply reflects a continuous leakage through the plasma membrane.

The results of the present investigation indicate that the effect of *Cl. perfringens* phospholipase C on the fibroblast membrane can be significantly increased by performing the incubation in a hypotonic environment.

The effect of hypotonic swelling on the sensitivity of erythrocytes to purified phospholipase A from bee venom, purified phospholipase A from porcine pancreas and purified phospholipase C from *B. cereus* was previously investigated [16, 44]. In these cases, the nonhaemolytic phospholipases were shown to lyse hypotonically swollen cells and degrade the phospholipids in the membranes, while the enzymes were without effect on the erythrocytes in isotonic solution.

The hypotonic solution stretches the membrane, thus probably making the polar head-groups of the phospholipids more available to the enzyme. However, the possible structural reorganization of the membrane that this swelling induces seems to be entirely reversible, as preincubation in a hypotonic solution followed by enzyme treatment in an isotonic solution does not increase the isotope release as compared to enzyme treatment in an isotonic solution alone (Table 4). These results are well in accordance with the dynamic model of the membrane structure which has been proposed by Singer and Nicolson [35, 36]. As pointed out by Woodward and Zwaal [44], it is not clear whether the increased membrane degradation by phospholipase C by hypotonic swelling is caused by actual "stretching" of the lipid

bilayer or by alteration in the tertiary structure of the outer membrane proteins covering the lipids.

Sublytic doses of the nonionic detergent Triton X-100 caused an increase in the membrane-damaging effect of phospholipase C in this study. The enhancing effect of detergents or surface active peptides or proteins has been specially studied with phospholipase A and the direct lytic factor in snake venom or mellittin in bee venom [5, 15, 16].

The mechanism for the stimulating effect of Triton is not known. The possibility that it simply protected the phospholipase C against inactivation upon dilution in the incubation buffer, as shown with β-amylase [38] was investigated. No such effect was observed upon measuring the enzymatic activity after dilution with Triton. On the other hand, it is well known that detergents like Triton X-100 at higher concentrations solubilize biological membranes [1, 12, 23, 30, 34], which is also the case with human fibroblasts (Fig. 6). At sublytic concentrations the detergent binds to the membrane [34] and in these concentrations it may even cause activation of certain membrane enzymes [1, 7]. It is thus probable that the membrane architecture is somehow changed during such treatments, possibly making the phospholipids more easily accessible to the action of phospholipase C or perhaps simply making the damaged membrane leak to a greater degree than normally.

It has been reported elsewhere [39] that human diploid fibroblasts are resistant to the staphylococcal phospholipase C, which is a specific sphingomyelinase [40, 41]. In this study it was also shown that under more favorable conditions such as hypotonic media or simultaneous treatment with sublytic concentrations of Triton X-100, still no effect was found in concentrations 10^5 times those needed to haemolyze sheep erythrocytes. Thus, a degradation of sphingomyelin alone may not cause sufficient damage to the membrane to allow nucleotide leakage out of the cell.

In conclusion, the effect of phospholipase C on the human diploid fibroblast membrane was studied. It was not possible to study the effect of this enzyme before contaminating toxins and enzymes were removed, especially since the haemolytic theta-toxin was highly active on the fibroblast membrane.

To our knowledge this is the first report of membrane damage on a live, intact, metabolizing diploid cell caused by a highly purified phospholipase C. The results are in agreement with a dynamic, mosaic membrane structure [36] with the polar groups of a part of the phospholipids accessible at the membrane surface and not hidden inside the protein bilayers [31].

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References

1. Banerjee, S. P., Dwosh, I. L., Khanna, V. K., Sen, A. K. 1970. Solubilization of guinea pig kidney ($Na^+ + K^+$)-ATPase with Lubrol W and Triton X-100. *Biochim. Biophys. Acta* **211**:345
2. Bligh, E. G., Dyer, W. J. 1959. A rapid method of total lipid extraction and purification. *Canad. J. Biochem. Physiol.* **37**:911
3. Burnstein, C., Loyter, A., Racker, E. 1971. Effect of phospholipases on the structure and function of mitochondria. *J. Biol. Chem.* **246**:4075
4. Coleman, R., Finean, J. B., Knutton, S., Limbrick, A. R. 1970. A structural study of the modification of erythrocyte ghosts by phospholipase C. *Biochim. Biophys. Acta* **219**:81
5. Condrea, E., deVries, A. 1964. Hemolysis and splitting of human erythrocyte phospholipids by snake venoms. *Biochim. Biophys. Acta* **84**:60
6. Cuatrecasas, P. 1971. Perturbation of the insulin receptor of isolated fat cells with proteolytic enzymes. *J. Biol. Chem.* **246**:6522
7. Dunnick, J. K., Marinetti, G. V., Greenland, P. 1972. Effects of detergents and sodium fluoride on the enzyme activities of the rat liver plasma membrane. *Biochim. Biophys. Acta* **266**:684
8. Eagle, H. 1959. Amino acid metabolism in mammalian cell cultures. *Science* **130**:432
9. Finean, J. B., Coleman, R. 1970. Integration of structural and biochemical approaches in the study of cell membranes. *In:* Membranes, Structure and Function (FEBS symposium). J. R. Villanueva and F. Ponz, editors. Vol. 20, p. 9. Academic Press Inc., New York-London
10. Finean, J. B., Martonosi, A. 1965. The action of phospholipase C on muscle microsomes. A correlation of electron microscope and biochemical data. *Biochim. Biophys. Acta* **98**:547
11. Glaser, M., Simpkins, H., Singer, S. J., Sheetz, M., Chan, S. I. 1970. On the interactions of lipids and proteins in the red blood cell membrane. *Proc. Nat. Acad. Sci.* **65**:721
12. Helenius, A., Simons, K. 1972. The binding of detergents to lipophilic and hydrophilic proteins. *J. Biol. Chem.* **247**:3656
13. Johnson, C. E., Bonventre, P. F. 1967. Lethal toxins of *Bacillus cereus*. I. Relationships and nature of toxin, hemolysin and phospholipase. *J. Bacteriol.* **94**:306
14. Kaplan, S. S., Finch, S. C., Basford, R. E. 1972. Polymorphonuclear leukocyte activation: Effects of phospholipase C. *Proc. Soc. Exp. Biol. N.Y.* **140**:540
15. Klibansky, C., London, Y., Frenkel, A., deVries, A. 1968. Enhancing action of synthetic and natural basic polypeptides on erythrocyte ghost phospholipid hydrolysis by phospholipase A. *Biochim. Biophys. Acta* **150**:15
16. Lankisch, P. G., Vogt, W. 1972. Direct haemolytic activity of phospholipase A. *Biochim. Biophys. Acta* **270**:241
17. Lenard, J., Singer, S. J. 1968. Structure of membranes: Reaction of red blood cell membranes with phospholipase C. *Science* **159**:738

18. Litwin, J., Thelestam, M. 1972. The lysis of human diploid fibroblasts with borate buffer. *Acta Path. Microbiol. Scand.* **80B**:115
19. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265
20. MacFarlane, M. G., Knight, B. C. J. G. 1941. The biochemistry of bacterial toxins. I. Lecithinase activity of *Cl. welchii* toxins. *Biochem. J.* **35**:884
21. Martonosi, A. 1968. Sarcoplasmic reticulum. IV. Solubilization of microsomal adenosine triphosphatase. *J. Biol. Chem.* **243**:71
22. McIlwain, D. L., Rapport, M. M. 1971. The effects of phospholipase C (*Clostridium perfringens*) on purified myelin. *Biochim. Biophys. Acta* **239**:71
23. Miller, D. M. 1970. Total solubilization of erythrocyte membranes by nonionic detergents. *Biochem. Biophys. Res. Commun.* **40**:716
24. Mitzutani, H., Mitzutani, H. 1964. Action of phospholipase on influenza virus. *Nature* **204**:718
25. Möllby, R., Nord, C.-E., Wadström, T. 1973. Biological activities contaminating preparations of phospholipase C (alpha-toxin) from *Clostridium perfringens. Toxicon* **11**:139
26. Möllby, R., Wadström, T. 1973. Purification of phospholipase C (alpha-toxin) from *Clostridium perfringens. Biochim. Biophys. Acta* **321**:569
27. Op den Kamp, J. A. F., Kauerz, M. T., van Deenen, L. L. M. 1972. Action of phospholipase A_2 and phospholipase C on *Bacillus subtilis* protoplasts. *J. Bacteriol.* **112**:1090
28. Ottolenghi, A. C., Bowman, M. H. 1970. Membrane structure: Morphological and chemical alterations in phospholipase-C-treated mitochondria and red cell ghosts. *J. Membrane Biol.* **2**:180
29. Patriarca, P., Zatti, M., Cramer, R., Rossi, F. 1970. Stimulation of the respiration of polymorphonuclear leukocytes by phospholipase C. *Life Sci.* **9**:841
30. Razin, S., Barash, V. 1969. Solubilization of mycoplasma membranes by the nonionic detergent Triton X-100. *FEBS Lett.* **3**:217
31. Robertson, J. D. 1964. Unit membranes: A review with recent new studies of experimental alterations and a new subunit structure in synaptic membranes. *In:* Cellular Membranes in Development. M. Locke, editor. p. 1. Academic Press Inc., New York-London
32. Roelofsen, B., Zwaal, R. F. A., Comfurius, P., Woodward, C. B., van Deenen, L. L. M. 1971. Action of pure phospholipase A_2 and phospholipase C on human erythrocytes and ghosts. *Biochim. Biophys. Acta* **241**:925
33. Roth, F. B., Pillemer, L. 1953. The separation of alpha-toxin (lecithinase) from filtrates of *Clostridium welchii. J. Immunol.* **70**:533
34. Simons, K., Helenius, A., Garoff, H. 1973. Solubilization of the membrane proteins from Semliki Forest virus with Triton X-100. *J. Mol. Biol.* **80**:119
35. Singer, S. J. 1971. Molecular organisation of biological membranes. *In:* Structure and Function of Biological Membranes. L. J. Rothfield, editor. p. 192. Academic Press Inc., New York-London
36. Singer, S. J., Nicolson, G. L. 1972. The fluid mosaic model of the structure of cell membranes. *Science* **175**:720
37. Sugahara, T., Ohsaka, A. 1970. Two molecular forms of *Clostridium perfringens* alpha-toxin associated with lethal, hemolytic and enzymatic activities. *Jap. J. Med. Sci. Biol.* **23**:61
38. Takeda, Y., Hizukuri, S. 1972. Effect of Triton X-100 on sweet potato beta-amylase. *Biochim. Biophys. Acta* **268**:175

39. Thelestam, M., Möllby, R., Wadström, T. 1973. Effects of staphylococcal alpha-, beta-, delta-, and gamma-hemolysins on human diploid fibroblasts and HeLa cells: Evaluation of a new quantitative assay for measuring cell damage. *Infect. Immunity* **8**:938
40. Wadström, T., Möllby, R. 1971. Studies on extracellular proteins from *Staphylococcus aureus*. VI. Production and purification of beta-haemolysin in large scale. *Biochim. Biophys. Acta* **242**:288
41. Wadström, T., Möllby, R. 1971. Studies on extracellular proteins from *Staphylococcus aureus*. VII. Studies on beta-haemolysin. *Biochim. Biophys. Acta* **242**:308
42. Wadström, T., Möllby, R. 1972. Some biological properties of purified staphylococcal haemolysins. *Toxicon* **10**:511
43. van Heyningen, W. E. 1941. The biochemistry of gas gangrene toxins. 2. Partial purification of the toxins of *Cl. welchii* type A. *Biochem. J.* **35**:1257
44. Woodward, C. B., Zwaal, R. F. A. 1972. The lytic behavior of pure phospholipases A_2 and C towards osmotically swollen erythrocytes and resealed ghosts. *Biochim. Biophys. Acta* **274**:272
45. Zwaal, R. F. A., Roelofsen, B., Comfurius, P., van Deenen, L. L. M. 1971. Complete purification and some properties of phospholipase C from *Bacillus cereus*. *Biochim. Biophys. Acta* **233**:474