Streptolysin-O induces release of glycosylphosphatidylinositol-anchored alkaline phosphatase from ROS cells by vesiculation independently of phospholipase action

Mingsheng XIE* and Martin G. LOW†

Department of Physiology and Cellular Biophysics, College of Physicians and Surgeons of Columbia University, New York, NY 10032, U.S.A.

Streptolysin-O (SLO), a cholesterol-binding agent, was used for studies on the release of glycosylphosphatidylinositol (GPI)-anchored alkaline phosphatase (AP) from ROS cells. Treatment of cells with SLO resulted in a time- and concentration-dependent release of AP into the extracellular medium. This release was potentiated by Ca^{2+} and bovine serum, but not by GPI-specific phospholipase D (GPI-PLD) purified from bovine serum. The released AP distributed to the detergent phase after Triton X-114 phase separation. This result suggested that the released AP contained an intact GPI anchor, and thus both proteolysis and anchor degradation by anchor-specific hydrolases, including GPI-PLD, as the potential mechanisms for SLO-mediated AP release were ruled out. The released AP sedimented at 100000 *g*. A substantial amount of lipids was detected in the 100000 *g* pellet. Cholesterol and sphingomyelin were enriched in SLO-released material, compared with intact cells. These results were consistent with vesiculation as the mechanism for SLO induction of AP release. Two other cholesterol-binding agents, saponin and digitonin, were also able to release AP, possibly by a similar vesiculation mechanism, whereas others, including nystatin, filipin and β-escin, failed to elicit any AP release. Eight GPI-anchored proteins were identified in ROS cells, and all were substantially enriched in the vesicles released by SLO. Taken together, these results do not provide any support for the hypothesis that the clustering of GPI-anchored proteins in the plasma membrane is responsible for their resistance to GPI-PLD cleavage.

INTRODUCTION

An increasing number of cell-surface proteins (currently over 100) have been found to be anchored to the plasma membrane by glycosylphosphatidylinositol (GPI) [1–4]. GPI-anchored proteins are both functionally and evolutionarily diverse, and do not share any structural similarity except the C-terminal of the precursor, which comprises a stretch (ranging in length from 8 to 35) of hydrophobic amino acids and is cleaved upon GPI addition [1–4]. Furthermore, the role of this type of anchoring remains largely unknown [1–4]. One proposed role for the GPI anchor is that it facilitates the release of GPI-anchored proteins from the cell surface by serving as a target substrate for anchor-specific phospholipases [1,5].

Mammalian plasma has been found to contain a substantial amount of a GPI-specific phospholipase D (GPI-PLD) [6,7]. However, only a limited number of cell types, including keratinocytes [8], myeloid cells [9], mast cells [10,11] and pancreatic islets [12], have been shown to express this enzyme. Although its molecular properties have been well characterized, a definitive demonstration of cleavage of cell-associated GPI-anchored proteins by either crude or purified GPI-PLD has not been achieved [13–15]. However, two recent studies suggest that endogenous GPI-PLD in HeLa cells and bone marrow may release GPI-anchored proteins [16,17]. It is reasonable that GPI-PLD action is stringently regulated, since any cells in contact with serum continuously risk losing important GPI-anchored proteins (e.g. decay-accelerating factor and CD59, which are essential for inhibiting autologous complement-mediated cell lysis). One potential restriction of GPI-PLD action is suggested by several recent studies indicating that GPI-anchored proteins may not be distributed uniformly in the plane of the bilayer. Instead, they may cluster in or close to small invaginations of the cell surface previously identified as caveolae [18–20]. Both cholesterol and sphingolipids have been found to be enriched in these clusters and postulated to play an important role in both the formation and maintenance of these complexes [18,21–23]. For example, Rothberg et al. [18] have shown that several cholesterol-binding agents are able to disrupt the clustering of GPI-anchored proteins.

We have undertaken the present study to test the hypothesis that disruption of the clustering of GPI-anchored proteins by cholesterol-binding agents may lead to activation of GPI-PLD. Of the variety of agents tested, streptolysin-O (SLO) induced release of a substantial amount of GPI-anchored alkaline phosphatase (AP) without serious cell damage in a rat osteosarcoma (ROS) cell line. SLO is a 69 kDa water-soluble protein produced and secreted by the β-haemolytic group A streptococci [24]. SLO binds to and then permeabilizes specifically the plasma membrane through complex-formation with cholesterol and self-aggregation, while leaving intracellular membranes largely intact [24,25]. The results in the present paper show that SLO induced AP release by a mechanism independent of GPI-PLD action. The most likely mechanism for SLO induction of AP release is the formation of membrane vesicles enriched in GPI-anchored proteins.

Abbreviations used: SLO, streptolysin-O; HU, haemolytic unit; AP, alkaline phosphatase; GPI, glycosylphosphatidylinositol; GPI-PLD, GPI-specific phospholipase D; PI-PLC, phosphatidylinositol-specific phospholipase C; BSS, basal salt solution; sulpho-NHS-biotin, sulphosuccinimidobiotin.

* Present address: Laboratory of Biological Chemistry, National Cancer Institute, N.I.H., Bldg. 37, Room 5D-02, Bethesda, MD 20892, U.S.A.

† To whom correspondence should be addressed at: Department of Physiology and Cellular Biophysics, Columbia University, 630 W. 168th Street, New York, NY 10032, U.S.A.

MATERIALS AND METHODS

Materials

SLO was obtained from VWR Scientific (South Plainfield, NJ, U.S.A.). The haemolytic unit (HU) of SLO activity was defined by the manufacturer. Recombinant *Bacillus thuringiensis* phosphatidylinositol-specific phospholipase C (PI-PLC) was prepared and the unit of its activity defined as described previously [5]. GPI-PLD purified from bovine serum was kindly provided by Dr. Jau-Yi Li [5,26]. GPI-PLD activity was assayed as described previously [5,26], with [^{3}H]myristate-labelled trypanosome variant surface glycoprotein (VSG) in 0.1 % (w/v) Nonidet P-40 as substrate, and 1 unit of GPI-PLD activity was defined as the amount of enzyme required to hydrolyse 1 % of substrate in 1 min. Sulphosuccinimidobiotin (sulpho-NHS-biotin) biotinylating agent and AP-conjugated streptavidin were purchased from Pierce (Rockford, IL, U.S.A.) and Calbiochem (La Jolla, CA, U.S.A.) respectively. Culture medium and bovine serum were from GIBCO–BRL (Grand Island, NY, U.S.A.). Silica-gel plates were from Whatman (Clifton, NJ, U.S.A.). Ca^{2+} ionophore A23187 was from Boehringer–Mannheim (Indianapolis, IN, U.S.A.). All other agents and chemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Release of AP from ROS cells with SLO and other agents

ROS cells (strain 17/2.8) were cultured in F-12 medium supplemented with 10 % (v/v) fetal-bovine serum in Falcon multi-well cluster dishes until confluent (approx. 2×10^5 cells per well in a six-well dish). The cells were washed first with F-12 medium and then with basal salt solution (BSS: 125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 25 mM Hepes, pH 7.5). The washed cells were incubated with pre-chilled freshly reconstituted SLO at a concentration in the range 0.125–2 HU/ml at 4 °C for 20 min (0.5 ml per well in a 6-well dish). After this binding step, excess SLO along with its reconstituting buffer was removed and the cells were washed once with BSS. Then 0.5 ml of BSS supplemented with desired agents was added to each well and the dishes were transferred to 37 °C and incubated for the desired times. For all other cholesterol-binding agents and Ca^{2+} ionophore, the procedure was essentially the same as above, except that the washing step after agent addition was omitted. The incubation medium was collected and centrifuged at 10000 ***g*** for 5–10 min at 4 °C to remove any detached cells. The resulting supernatant was sampled for determination of AP activity as described previously [5]. The amount of AP released was expressed either as a percentage of total cellular AP activity (total activity determined by scraping control wells and assaying a portion) or as a percentage of the maximal SLO response.

Saponin (from *Saponaria* species), digitonin and nystatin were dissolved in BSS at stock concentrations of 0.6, 0.2 and 0.4 mg/ml respectively. Filipin (from *Streptomyces filipinensis*) and β-escin were dissolved in 95 % ethanol at stock concentrations of 100 and 5 mg/ml respectively. A23187 was dissolved in dimethyl sulphoxide at a stock concentration of 10 mM. A stock solution of 5.2 mM cholesterol was prepared as follows: 1.2 mg of cholesterol in chloroform was dried under N_2, and 0.17 ml of BSS supplemented with 0.1 % (w/v) BSA was added. The mixture was vortex-mixed and sonicated in a bath sonicator for 10 min. This procedure gave a homogeneous emulsion of cholesterol.

Since AP was completely and irreversibly inhibited in the presence of millimolar concentrations of metal-ion chelators, such as EGTA, EDTA and 1,10-phenanthroline, the use of these chelators to achieve desired metal ion concentrations (sub-micromolar to sub-millimolar range) was not feasible in this study. Consequently, we used serial dilution of $CaCl_2$ and $MgCl_2$ solutions to examine the effects of these two metal ions on SLO induction of AP release.

After treatment of cells with various agents, cell permeability was usually determined by the criterion of nuclear staining by Trypan Blue at a final concentration of 0.25 %.

Characterization of the GPI anchor of released AP

Two procedures were employed to examine the possibility that the release of AP was due to anchor degradation or proteolysis. First, Triton X-114 phase separation [5,27] was used to examine the anchor hydrophobicity of AP, based on the fact that the intact GPI-anchored AP would go into the detergent-rich phase and anchor-free AP would go into the aqueous phase. Briefly, 0.1 ml of the above 10000 ***g*** supernatant was mixed on ice with 0.15 ml of BSS and 0.25 ml of 2 % (w/v) Triton X-114. A 0.1 ml sample of the mixture was taken for determination of total AP activity. The rest of the mixture was incubated at 37 °C for 10 min to promote phase separation, centrifuged at 12000 ***g*** for 5 min, and 0.1 ml of the upper, detergent-poor, phase was removed for AP assay. Data were expressed as the relative distribution of AP between detergent-rich and -poor phases. Secondly, ultracentrifugation was used to examine the sedimentability of AP, based on the fact that anchor-free and dissociated AP would not sediment. Briefly, the supernatant from the 10000 ***g*** spin was further centrifuged at 100000 ***g*** for 30–60 min at 4 °C. Portions (0.1 ml) of the starting sample and the resulting supernatant were taken for determination of AP activity. Data were expressed as the relative distribution of AP between the supernatant and pellet fractions.

Analysis of lipids from SLO-released vesicles

The media of cells treated with SLO and 0.5–1 mM $CaCl_2$ from two to four dishes of the six-well type were combined and centrifuged at 10000 ***g*** for 10 min at 4 °C. The resulting supernatant was further centrifuged at 100000 ***g*** for 45 min at 4 °C. The resulting pellet was dissolved in 0.5 ml of BSS plus 2 ml of chloroform/methanol/conc. HCl (10:20:1, by vol). The phases were split by addition of 0.6 ml of chloroform and 0.6 ml of 2 M KCl [28,29]. The lower chloroform phase, containing most of the lipids, was collected and dried in a Speed-Vac concentrator. The dried lipid samples and standard lipids were resuspended in 20–100 μl of chloroform/methanol (2:1, v/v) and spotted on to silica-gel 60 A K6 plates. The plates were developed in chloroform/methanol/water (25:10:1, by vol.) and air-dried. The resolved lipids were located by staining with Coomassie Blue dye (0.03 g of Coomassie Blue/100 ml of 100 mM NaCl in 30 % methanol), followed by destaining with 100 mM NaCl in 30 % methanol [30].

Analysis of GPI-anchored proteins from SLO-released vesicles

To determine (1) whether other GPI-anchored proteins were also present in the SLO-released vesicles and (2) whether GPI-anchored proteins were enriched in these vesicles, we used a water-soluble, membrane-impermeant agent, sulpho-NHS-biotin, to label specifically cell-surface proteins and then we analysed these labelled proteins, using a protocol modified from Lisanti et al. [31,32]. In brief, well-washed cells in cluster dishes were labelled with 0.5 mg/ml sulpho-NHS-biotin in buffer L (PBS supplemented with 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$) at 4 °C for 30 min. The cells were washed twice with F-12 medium and three times with BSS, and then treated with 2 HU/ml SLO as described above; 1 mM $CaCl_2$ was used to potentiate SLO induction of

vesicle release. The reaction media were collected and centrifuged at 10000 *g* for 10 min at 4 °C. The resulting supernatant was further centrifuged at 100000 *g* for 45 min at 4 °C. The resulting pellet was extracted with 1 % Triton X-114 in buffer E (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5 mM phenylmethanesulphonyl fluoride, 5 μg/ml leupeptin and 10 μg/ml pepstatin). The extract was centrifuged at 13000 *g* for 10 min at 4 °C, and the supernatant subjected to temperature-induced phase separation. The aqueous phase containing soluble proteins was discarded. To decrease contamination by soluble proteins further, the detergent phase containing integral membrane proteins, including GPI-anchored proteins, was re-extracted with 10 vol. of buffer E containing 0.06 % Triton X-114. The resulting detergent phase was then diluted 5-fold with buffer E, divided into two aliquots, and incubated in the absence or presence of PI-PLC (2 units/ml) for 30 min at 37 °C with constant mixing. Subsequently, an equal volume of 2 % Triton X-114 was added to each tube and a phase separation was performed. Proteins in the detergent phase were precipitated with 5 vol. of acetone [33]. The precipitated proteins were dissolved in BSS. Any GPI-anchored and other membrane protein contaminants remaining in the aqueous phase were cleared away by incubation with 0.2 vol. of 75 % (w/v) phenyl-Sepharose (Pharmacia) at 4 °C for 48 h in a shaker. Proteins in the final aqueous phase were quantitatively precipitated by the deoxycholate/trichloroacetic acid method [33]. The precipitated proteins were dissolved in 0.1 M NaOH. GPI-anchored proteins in intact ROS cells were analysed in the same way as above. Both the detergent- and aqueous-phase proteins were separated by SDS/PAGE, and transferred to nitrocellulose. The sulpho-NHS-biotin-labelled proteins were revealed by first binding with AP-conjugated streptavidin, followed by AP-catalysed colour development.

RESULTS

In this study, we have chosen rat osteosarcoma (ROS) cells as our experimental model system to study release of GPI-anchored proteins, based on the following two properties of this cell line. First, they express abundant GPI-anchored AP, which can be readily assayed and quantified [15,34]. Second, the GPI anchor in ROS cells is not acylated on its inositol ring, so that cell-surface AP is completely sensitive to PI-PLC digestion, and consequently Triton X-114 phase-separation analysis of GPI-anchored proteins after GPI-PLD cleavage is feasible [15,34]. Thus the release of AP from cells into the extracellular medium was measured as the primary index for the effect of SLO on GPI-anchored proteins in ROS cells.

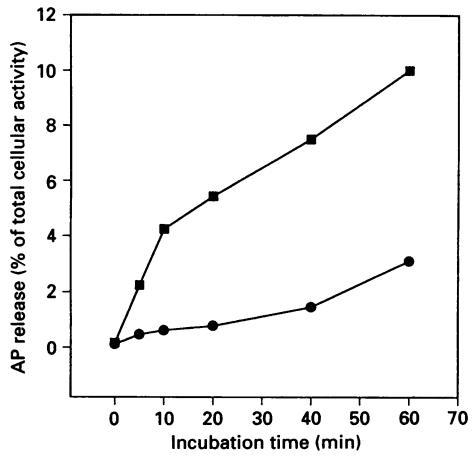

Figure 1 Time course of SLO induction of AP release

ROS cells were pretreated with either BSS (●) or SLO (2 HU/ml; ■) as described in the Materials and methods section. Cells were then incubated at 37 °C for various lengths of time (0, 5, 10, 20, 40 and 60 min) in the presence of 1 mM $CaCl_2$. The amount of AP released into media is expressed as a percentage of total cellular AP activity. Values are means of duplicate incubations from a single representative experiment, one of two giving similar results.

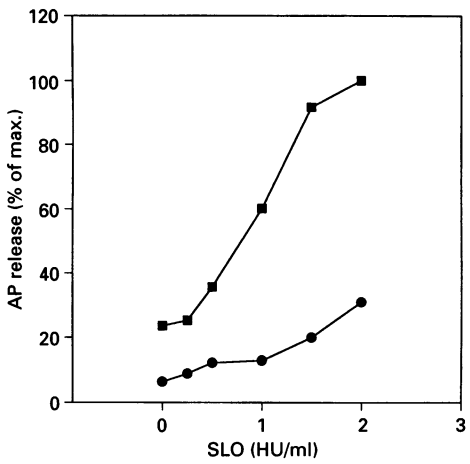

Figure 2 SLO induction of AP release from ROS cells

ROS cells were pretreated with various concentrations of SLO (0, 0.25, 0.5, 1, 1.5 and 2 HU/ml) as described in the Materials and methods section. Cells were then incubated at 37 °C for 60 min in the absence (●) or presence (■) of 1 mM $CaCl_2$. The amount of AP released into media is expressed as a percentage of the maximal response. Values are means of duplicate incubations from a single representative experiment, one of three giving similar results.

SLO induces release of AP from ROS cells

Previous studies have indicated that cholesterol plays a critical role in both the formation and the maintenance of the GPI-anchored protein clusters in the plasma membrane [18,21–23]. We therefore examined the possibility that disruption of such clustering with cholesterol-binding agents might lead to either shedding of GPI-anchored proteins or facilitation of anchor cleavage by GPI-PLD. During a preliminary survey of the effects of cholesterol-binding agents on release of GPI-anchored AP in ROS cells, SLO was found to have a large effect, without seriously damaging the cells. Consequently, the effect of SLO on AP release was thoroughly investigated. We first determined the time course of SLO-mediated AP release. As shown in Figure 1, AP was released without a lag, and proceeded linearly for the first 10 min and then at a slower rate during the rest of the experimental period (longest incubation examined: 60 min). This time course is compatible with the observation that the action of SLO is extremely rapid and permeabilization generally occurs within seconds [24,25]. In 60 min, the maximal release of AP amounted to a range of 8–15 % of total cellular AP activity. In the absence of SLO, there was a basal release of AP, with a magnitude of 1–2 % of total cellular activity during a 60 min incubation (Figure 1).

Incubation of ROS cells with increasing concentrations of SLO for 60 min resulted in release of increasing amounts of AP activity into the reaction medium (Figure 2). Maximal AP release induced by SLO was attained at a concentration of 2 HU/ml. The amount of AP released showed close correlation with the number of cells positively stained by Trypan Blue (an indication

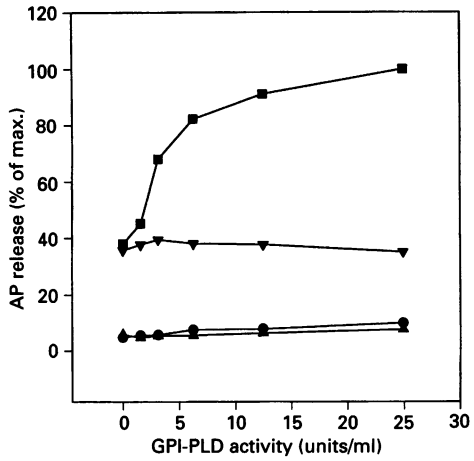

Figure 3 Effect of bovine serum and GPI-PLD on SLO-mediated AP release

ROS cells were pretreated with either BSS (●, ▲) or SLO (2 HU/ml; ■, ▼) as described in the Materials and methods section. Cells were then incubated at 37 °C for 60 min in the presence of various amounts of GPI-PLD activity (0, 1.56, 3.12, 6.24, 12.47 and 24.94 units/ml) from either bovine serum (●, ■) or pure GPI-PLD purified from the same bovine serum (▲, ▼). The amount of AP released into media is expressed as a percentage of the maximal response. Values are means of duplicate incubations from a single representative experiment, one of three giving similar results.

of cell permeabilization; results not shown). After the 60 min incubation with the highest concentration of SLO (2 HU/ml) used, most of the cells were permeabilized but still attached to the culture dishes. If the $CaCl_2$ present in the incubation mixture was omitted, the releasing effect of SLO was substantially decreased (Figure 2).

Is GPI-PLD activation responsible for the SLO-mediated release of AP?

In previous studies it was observed that detergent extracts of well-washed ROS cells contained a significant amount of GPI-PLD activity (M. Xie and M. G. Low, unpublished work). This raised the possibility that activation of endogenous GPI-PLD was the mechanism for SLO induction of AP release. Exogenously added GPI-PLD has previously been shown to be unable to release GPI-anchored proteins from intact cells [15]. One explanation for this observation is that the interaction of GPI-anchored proteins with cholesterol and sphingolipids prevents GPI-PLD access to its substrates. Thus sequestration of cholesterol with SLO could potentially relieve such hindrance to GPI-PLD action. To test this interesting hypothesis, we examined whether addition of exogenous GPI-PLD could release more AP in SLO-treated cells. In agreement with our earlier study [15], incubation of untreated ROS cells with either bovine serum or GPI-PLD purified from bovine serum at a concentration of up to 25 units/ml did not elicit any significant release of AP (Figure 3). In SLO-treated cells, bovine serum increased the basal SLO-mediated AP release in a dose-dependent fashion. The half-maximal and maximal potentiation effect was achieved with 0.25% and 2% (v/v) bovine serum, containing approx. 3 and 25 units/ml GPI-PLD activity respectively (Figure 3). In contrast, pure GPI-PLD with a concentration of up to 25 units/ml failed to exert any effect on the SLO-mediate AP release (Figure 3). These results thus do not support the suggestion that GPI-PLD activation is responsible for the SLO induction of AP release.

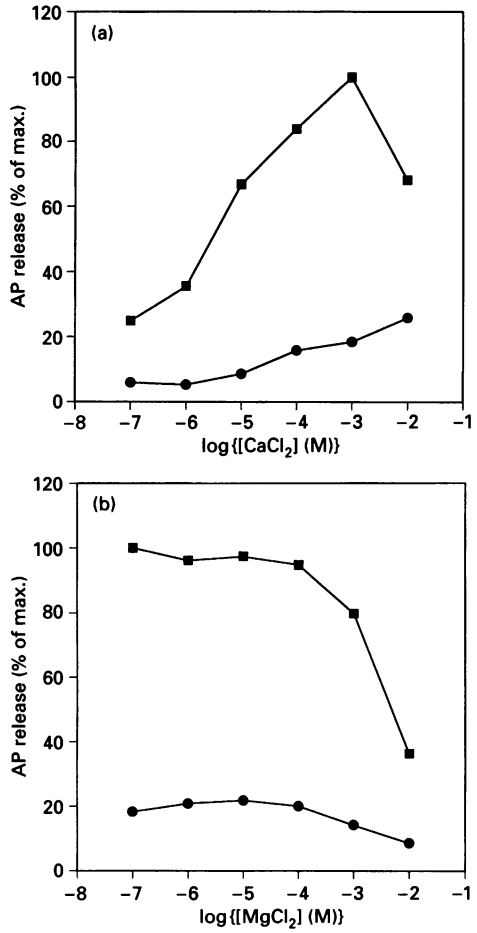

Figure 4 Effect of bivalent metal ions on AP release stimulated by SLO

ROS cells were pretreated with either BSS (●) or SLO (2 HU/ml; ■) as described in the Materials and methods section. Cells were then incubated at 37 °C for 60 min in the presence of various concentrations of either exogenously added $CaCl_2$ (**a**) or $MgCl_2$ (**b**). In (**a**), 1 mM $MgCl_2$ was also present in all reactions, whereas 0.2 mM $CaCl_2$ was also present in all reactions of (**b**). The desired concentrations of these two metal ions were obtained as described in the Materials and methods section. The amount of AP released into media is expressed as a percentage of the maximal response. Values are means of duplicate incubations from a single representative experiment, one of two giving similar results.

Ca^{2+} potentiates the effect of SLO

The above discrepancy between bovine serum and pure GPI-PLD in potentiating the effect of SLO did not automatically rule out the involvement of GPI-PLD action, since it was possible that a factor important in GPI-PLD action was present in bovine serum, but was lost during GPI-PLD purification. An alternative possibility is that a factor in bovine serum which is irrelevant to GPI-PLD action is responsible for the potentiating effect of bovine serum. As noted above (Figure 1), $CaCl_2$ could potentiate the SLO effect. Since bovine serum contains millimolar levels of $CaCl_2$, the possibility that $CaCl_2$ was the potentiating factor in bovine serum was investigated. The potentiating effect of $CaCl_2$ was first characterized in greater detail. As shown in Figure 4(a), externally added $CaCl_2$ potentiated the SLO-mediated AP release in a concentration-dependent manner, with an EC_{50} at 5–10 μM. Maximal potentiating effect (range: 2–4-fold increase over the basal SLO effect) was achieved with 1 mM $CaCl_2$. Higher concentrations of $CaCl_2$ exhibited a lesser potentiating effect (Figure 4a). In the absence of SLO treatment, $CaCl_2$ itself

Table 1 Triton X-114 phase separation and ultracentrifugation of AP released by PI-PLC, SLO, saponin and digitonin

ROS cells were pretreated with BSS, SLO (2 HU/ml), saponin (0.15 mg/ml) or digitonin (0.1 mg/ml) as described in the Materials and methods section. Cells were then incubated at 37 °C for 60 min in the presence of PI-PLC (10 m-units/ml), 1 mM $CaCl_2$ (Ca), or 2% (v/v) bovine serum (BS). The media were subjected to either Triton X-114 phase separation or ultracentrifugation. The relative distributions of AP between detergent-rich and -poor phases and that between supernatant and pellet fractions were determined. The percentage of released AP in the detergent-rich phase and that in the pellet fraction are shown. Values are means ± S.D. (two independent experiments).

Treatment	Triton X-114 phase separation (% of released AP in detergent phase)	Ultracentrifugation (% of released AP in pellet)
PI-PLC	4.5 ± 0.5	3 ± 1
SLO + Ca	87	97 ± 1
SLO + BS	80	96
Saponin + Ca	89.5 ± 0.5	94.5 ± 0.5
Saponin + BS	86.5 ± 4.5	93
Digitonin + Ca	81.5 ± 2.5	85 ± 1
Digitonin + BS	78	87.5 ± 0.5

induced a much slower rate of AP release in a concentration-dependent manner (Figure 4a). We then determined whether the effects of bovine serum and $CaCl_2$ were additive. Additivity would be predicted if bovine serum and $CaCl_2$ potentiated SLO action by separate mechanisms. The amount of AP released by SLO plus 1 mM $CaCl_2$ was not further increased by the addition of 2% (v/v) bovine serum (results not shown). This lack of additivity between effects of bovine serum and $CaCl_2$ thus suggests that the bovine serum effect is due to $CaCl_2$, but not due to GPI-PLD action.

In the above experiments, all incubations contained 1 mM $MgCl_2$. To determine the role of $MgCl_2$ in the SLO-mediated AP release, the effect of $MgCl_2$ was characterized in greater detail. As shown in Figure 4(b), up to 100 μM externally added $MgCl_2$ had no effect on SLO-mediated AP release. Higher concentrations of $MgCl_2$ had an inhibitory effect on both the basal and SLO-induced AP release. For example, 10 mM $MgCl_2$ inhibited the effect of SLO by 70% (Figure 4b). Thus cation potentiation of SLO action is relatively specific for Ca^{2+}. In routine experiments, 1 mM $MgCl_2$ was included for the purpose of maintaining a favourable condition for attachment of ROS cells to culture dishes.

Is the SLO-mediated release of AP due to anchor cleavage or proteolysis?

Although the involvement of GPI-PLD action was tentatively excluded in the mechanism of AP release induced by SLO treatment, other possibilities of enzyme cleavage, i.e. proteolysis close to the C-terminus of AP, or glycosidic or phospholipase C cleavage of the anchor of AP, still exist. For example, SLO preparations may contain contaminating protease [25]. To evaluate these possibilities, Triton X-114 phase separation was performed to determine the intactness of the GPI anchor. As a positive control, AP released by PI-PLC was subjected to Triton X-114 phase separation. As expected, more than 95% of PI-PLC-released AP went into the aqueous phase as a result of loss of the detergent-binding domain, i.e. diacylglycerol (Table 1). In striking contrast, most (80–87%) of the AP released by either SLO plus $CaCl_2$ or SLO plus bovine serum went into the detergent phase after phase separation. Since the aqueous phase still contains a significant amount of Triton X-114 (approx. 0.7 mM) [5,31], contamination of the aqueous phase by intact GPI-anchored proteins could account for the remaining 13–20% of the released AP. Indeed, treatment of the aqueous phase with phenyl-Sepharose almost completely depleted AP in the aqueous phase (results not shown; see below). Thus it is concluded that the GPI anchor of AP released by SLO action is intact. This result rules out the possibility of either proteolysis or anchor degradation as the mechanism for the SLO-mediated AP release.

Vesiculation is the most likely mechanism for the SLO-mediated release of AP

To study further the mechanism responsible for the SLO induction of AP release, the sedimentation characteristics of released AP were examined by ultracentrifugation. As shown in Table 1, more than 96% of AP released by SLO (potentiated by either 1 mM $CaCl_2$ or 2% bovine serum) was recovered in the pellet fraction after centrifugation at 100000 *g*, indicating that essentially all the SLO-released AP is sedimentable. As a control, AP was released from ROS cells with PI-PLC, and then subjected to the same analysis. Less than 5% of PI-PLC-released AP was recovered in the pellet fraction (Table 1). The physical property of AP released by SLO suggests that its molecules either self-aggregate into sedimentable complexes or associate with other proteins and lipids to form vesicles. Such association is predicted by its intact GPI anchor if lipids are available. In order to explore this potential association of the SLO-released AP with lipids, a chloroform/methanol extract of the 100000 *g* pellet was separated by t.l.c., and lipids were identified by Coomassie Blue staining. Individual lipid species were identified by comparison with pure lipid standards. As shown in Figure 5, the pellet contained a significant amount of lipids, with a profile similar to that exhibited by intact ROS cells. Simple neutral lipids (SL), including triacylglycerols, diacylglycerols and fatty acids, were the most intensely stained. In comparison with intact cells, SLO-released material exhibited some differences in the lipid composition: (i) relatively high concentrations of cholesterol, sphingomyelin, and an unidentified species running below cholesterol in Figure 5; and (ii) relatively low concentrations of phosphatidylcholine (PC) and phosphatidylethanolamine (PE). This lipid composition is reminiscent of the well-established lipid profile of mammalian plasma membrane, and could support formation of lipid vesicles. Taken together, these results are consistent with the suggestion that SLO releases AP by inducing generation of vesicles from the plasma membrane.

Erythrocytes can be induced to form two types of vesicles: (i) 'microvesicles' of diameter 150 nm, sedimentable at 16000 *g*; and (ii) 'nanovesicles' of diameter 60 nm, sedimentable at 100000 *g* [35,36]. To determine the approximate size of the vesicles generated by SLO induction, serial steps of centrifugation were performed. The medium from SLO-treated cells was first centrifuged at 1500 *g* for 10 min to remove any detached cells and released nuclei. The resulting supernatant was then centrifuged at 16000 *g* for 30 min. After this second centrifugation, more than 90% of released AP was recovered in the supernatant and could be fully sedimented by centrifugation at 100000 *g* for 30 min. This result suggests that the vesicles generated from SLO-treated ROS cells belong to the category of 'nanovesicles'.

GPI-anchored proteins are enriched in the SLO-released vesicles

To determine whether the SLO-released vesicles contain other GPI-anchored proteins in addition to AP, we used an experimental approach that combines sulpho-NHS-biotin cell-surface labelling, Triton X-114 phase separation and PI-PLC

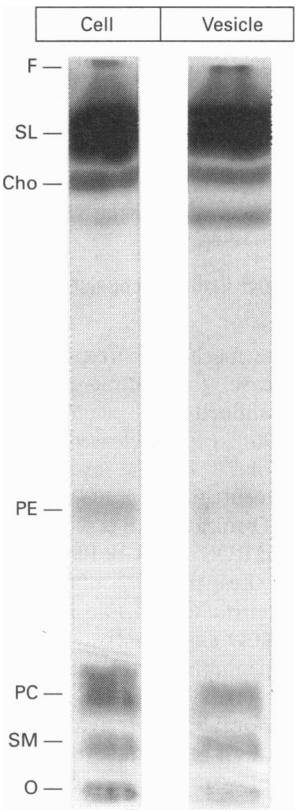

Figure 5 Lipid composition of SLO-released vesicles

Lipids from two wells of intact ROS cells (Cell) and SLO-released vesicles from 14 wells of ROS cells (Vesicle) were separated by t.l.c., and stained with Coomassie Blue as described in the Materials and methods section. T.l.c. origin (O), solvent front (F), and the mobilities of pure standard lipids, simple lipids (SL; e.g. triacylglycerols), cholesterol (Cho), phosphatidylethanolamine (PE), phosphatidylcholine (PC) and sphingomyelin (SM) are indicated at the left of the Figure. Essentially the same results were obtained in one other independent experiment.

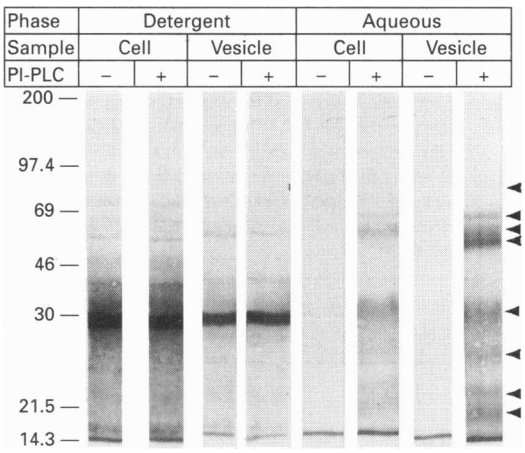

Figure 6 SLO-released vesicles are enriched with GPI-anchored proteins

ROS cells (24 wells) were surface-labelled with sulpho-NHS-biotin. Four wells of labelled cells were used for total analysis, and the other 20 wells were treated with SLO (2 HU/ml) and $CaCl_2$ (1 mM) to induce vesicle release. Detergent extracts from both control cells (Cell) and SLO-released vesicles (Vesicle) were treated in the absence (lane –) or presence (lane +) of PI-PLC (2 units/ml). After phase separation with Triton X-114, proteins in both the aqueous and detergent phases were precipitated and analysed as described in the Materials and methods section. Note the differences in the amounts of proteins applied to the gel (one-tenth versus half of the total recovered) and the time of AP-catalysed colour development (10 versus 90 min) between the detergent- and aqueous-phase proteins. GPI-anchored proteins specifically released by PI-PLC are indicated at the right with arrowheads. Essentially the same results were obtained in two other independent experiments. Molecular-mass markers are indicated at the left, in kDa: myosin heavy chain, 200; phosphorylase *b*, 97.4; BSA, 69; ovalbumin, 46; carbonic anhydrase, 30; soybean trypsin inhibitor, 21.5; lysozyme, 14.3.

treatment [31,32]. PI-PLC was used to release specifically GPI-anchored proteins into the aqueous phase after temperature-induced phase separation with Triton X-114. This aqueous phase was further depleted of any contaminating intact GPI-anchored proteins with phenyl-Sepharose treatment. As shown in Figure 6, at least eight proteins, of molecular masses approx. 80, 65, 62, 60, 32, 26, 22 and 20 kDa, were specifically released into the aqueous phase by PI-PLC treatment of detergent extract of SLO-released vesicles. Thus the vesicles contain at least eight GPI-anchored proteins. It remains to be determined which one is AP, although 65–70 kDa is the expected molecular mass of mammalian AP. Intact ROS cells contained the same profile of GPI-anchored proteins (Figure 6). To explore the possibility that GPI-anchored proteins are enriched in these vesicles compared with intact cells, total detergent-phase proteins (mainly integral membrane proteins, in addition to GPI-anchored proteins) from the phase separation were also analysed. As shown in Figure 6, the profile of biotin-labelled proteins (exclusively integral plasma-membrane proteins, including GPI-anchored proteins) in the vesicles was similar to that of the intact cells, further supporting the suggestion that the vesicles are derived from the plasma membrane. Two proteins running close together in the 30 kDa region were the most prominently labelled (Figure 6). The profile of labelled proteins in ROS cells is dramatically different from that of either the apical or the basolateral surface of Madin–Darby canine kidney (MDCK) cells [31]. The two surfaces of MDCK cells also exhibit distinct profiles of biotin-labelled proteins [31]. It should be pointed out that the GPI-anchored proteins are relatively minor protein constituents of both the plasma membrane and the SLO-released vesicles (see the legend to Figure 6). Consequently, the decrease in staining of detergent-phase proteins after PI-PLC digestion was not obvious in Figure 6. However, it is striking that the intact cells contained much more total labelled proteins, but less GPI-anchored proteins, than the vesicles (Figure 6). These data indicate that biotinylated GPI-anchored proteins are substantially enriched in the detergent extract of the SLO-released vesicles compared with that obtained from the cells. Although we believe that this result is due to a preferential redistribution of GPI-anchored proteins from the plasma membrane into the vesicle, it could also be due to an increase in their ability to be extracted from the vesicles by Triton X-114. Previous studies with GPI-anchored AP in several cell types indicate that it is extracted inefficiently by cold Triton [21,23].

The effect of SLO is dependent on both cholesterol in the plasma membrane and temperature-induced aggregation

In order to understand further the mechanism for SLO induction of plasma-membrane vesicles enriched with GPI-anchored proteins, the factors affecting SLO interaction with the plasma membrane were investigated. As the prototype of cholesterol-binding thiol-activated bacterial cytolysins, SLO has been extensively studied for the mechanism of cholesterol binding and pore formation in the plasma membrane [24,25]. It is well established that SLO first binds with cholesterol in the membrane and then aggregates at 37 °C to form semi-circular and circular pores [24,25]. We first examined whether cholesterol binding is

Table 2 Effects of cholesterol and low temperature on SLO induction of AP release from ROS cells

ROS cells were pretreated with either BSS or SLO (2 HU/ml) and/or 0.52 mM cholesterol for 20 min at 4 °C. The treated cells were washed with BSS to remove unbound SLO. Cells were then incubated with 0.5 ml of fresh BSS supplemented with 1 mM $CaCl_2$ at 37 °C for 60 min. The amount of AP released into the media is expressed as a percentage of the maximal response. Values are means ± S.D. (two independent experiments). The cells were also examined for permeability with 0.25% Trypan Blue: Yes, positive staining; No, negative staining.

Incubation temperature	SLO	Cholesterol	AP release (% of max.)	Trypan Blue staining of nuclei
37 °C	−	−	11.5 ± 4.5	No
37 °C	+	−	100	Yes
37 °C	−	+	11.5 ± 2.5	No
37 °C	+[a]	+	15 ± 4	No
37 °C	+[b]	+	82 ± 2	Yes
4 °C	+	−	9.8 ± 2.1	No

[a] SLO and cholesterol were mixed together, preincubated for 5 min at 4 °C and then the mixture was added to cell wells.

[b] SLO was first added to cell wells and incubated for 5 min at 4 °C before cholesterol was added.

essential for SLO to induce AP release. As shown in Table 2, preincubation of SLO with exogenous cholesterol (0.52 mM) before addition to cells abolished SLO-mediated AP release. This result indicates that contaminants in the SLO preparation were unlikely to be responsible for AP release. If exogenous cholesterol was added to cells 5 min later than SLO, the SLO effect was only slightly inhibited. This result is consistent with the suggestion that SLO binds cholesterol rapidly and that such binding is almost irreversible [24,25]. We then determined whether temperature-induced SLO aggregation is also required for the effect. Incubation of cells with SLO at 4 °C for as long as 60 min failed to induce any release of AP above the background level (Table 2). Both exogenous cholesterol and low temperature also blocked SLO-induced permeabilization of ROS cells (Table 2). Taken together, it is suggested that the effect of SLO is dependent on both cholesterol in the plasma membrane and temperature-induced aggregation and cell permeabilization.

To explore the possibility that the effect of SLO could simply be due to its ability to permeabilize the cells and allow influx of Ca^{2+} into the cells, we examined the effects of a Ca^{2+} ionophore and several other cholesterol-binding agents. A23187 is an extensively used ionophore relatively specific for Ca^{2+}. This ionophore at 30 μM induced a significant release of AP (about 47% of the SLO response) into the medium only in the presence of 1 mM $CaCl_2$, although it failed to induce permeabilization to Trypan Blue (Table 3). Nystatin, an antifungal cholesterol-binding agent, at a concentration of as high as 0.2 mg/ml failed both to permeabilize cells and to induce significant release of AP (Table 3). This observation supports the above suggestion, that cholesterol binding alone is not sufficient for induction of AP release and that cell permeabilization is essential. However, two other cholesterol-binding agents, β-escin and filipin, were able to permeabilize cells, but failed to induce AP release (Table 3), suggesting that both cholesterol binding and cell permeabilization are not sufficient for induction of AP release. Purified GPI-PLD also failed to elicit AP release from ROS cells treated with nystatin, filipin or β-escin. Control experiments showed that nystatin, filipin or β-escin exhibited no inhibitory effects toward AP activity itself (results not shown). Therefore it is suggested that SLO must possess some unique properties which afford its ability to induce vesiculation, or that β-escin and filipin, or contaminants in them, inhibit vesiculation.

We also examined two additional cholesterol-binding agents, saponin and digitonin, for their effects on AP release. They share similar structures, consisting of an aglucone moiety and a sugar. The aglucone moiety in digitonin is a steroid, whereas that in saponin may be a steroid or a triterpene. Both agents permeabilized ROS cells and induced similarly significant AP release by themselves (Table 3). These agents were more detrimental to cells than SLO, in that they caused cells to detach and be fragmented. Ca^{2+} potentiated saponin-mediated AP release by 3.8-fold, but had no effect on digitonin. Bovine serum at 2% (v/v) stimulated digitonin- and saponin-mediated AP release by 1.7- and 4.2-fold respectively (Table 3), whereas GPI-PLD purified from bovine serum had no effect (results not shown). The discrepancy between the digitonin and saponin effects could well be explained by the presence of the unique triterpene–sugar structure in saponin. To determine whether the mechanism for AP release by digitonin and saponin is anchor cleavage/proteolysis or vesiculation,

Table 3 Comparison between SLO and other cholesterol-binding agents and a Ca^{2+} ionophore for their abilities to release AP from ROS cells

ROS cells were pretreated with the indicated agents at the specified concentrations. Cells were then incubated at 37 °C for 60 min in the presence of carrier control, 1 mM $CaCl_2$, or 2% (v/v) bovine serum (BS). The amount of AP released into the media is expressed as a percentage of the maximal response elicited by SLO. Values represent means ± S.D. (no of independent experiments). After collection of media, the cells were also examined for permeability with 0.25% Trypan Blue: Yes, positive staining; No, negative staining.

Agents (concentration)	AP release (% of maximal SLO response)			Trypan Blue staining of nuclei
	No addition	+ $CaCl_2$	+ BS	
Control	4 ± 1 (3)	11 ± 3 (4)	10 ± 2 (3)	No
Cholesterol-binding agents				
SLO (2 HU/ml)	38 ± 8 (5)	100 (5)	84 ± 12 (4)	Yes
Saponin (0.15 mg/ml)	48 ± 7 (3)	184 ± 22 (3)	201 ± 20 (2)	Yes
Digitonin (0.1 mg/ml)	43 ± 12 (3)	43 ± 17 (3)	72 ± 13 (2)	Yes
Nystatin (0.2 mg/ml)	10 ± 2 (2)	19 ± 3 (2)	17 ± 4 (2)	No
Filipin (0.2 mg/ml)	5 ± 1 (3)	8 ± 1 (3)	9 ± 2 (2)	Yes
β-Escin (50 μg/ml)	6 ± 1 (3)	7 ± 2 (3)	8 ± 2 (2)	Yes
Calcium ionophore				
A23187 (30 μM)	10 ± 1 (3)	47 ± 9 (3)	16 ± 5 (2)	No

Triton X-114 phase separation and ultracentrifugation analysis were performed. Most of the AP released by digitonin and saponin was recovered in the detergent phase and in the pellet fraction after Triton X-114 phase separation and 100000 *g* spin, respectively (Table 1). This result is quite similar to that for SLO. Furthermore, lipids were identified in the pellet, and a similar lipid composition to that in SLO-released material was demonstrated (results not shown). Taken together, these results are consistent with vesiculation as the mechanism for AP release induced by digitonin and saponin.

DISCUSSION

Recent studies have suggested that GPI-anchored proteins may be concentrated in small plasma-membrane invaginations called caveolae [18–20,37]. Caveolae have been postulated to be 'message centres' for the cell, on the basis of the observation that many signalling molecules, e.g. trimeric GTPases, small GTPases, non-receptor protein tyrosine kinases, and Ca^{2+} channels and pumps, may be enriched in this organelle [38–42]. Caveolae are believed to have a distinct lipid composition, with relatively high concentrations of cholesterol and sphingolipids [18,21–23]. This could explain the observation that the clustering of GPI-anchored proteins can be prevented or disrupted by depletion of membrane cholesterol through blocking cholesterol biosynthesis or by sequestration of membrane cholesterol with cholesterol-binding agents [18,22,23]. An interesting possibility is that this clustering is responsible for the inability of purified GPI-PLD to cleave cell-surface GPI-anchored proteins [15]. The present study has been undertaken to test this hypothesis.

The present results demonstrate that treatment of ROS cells with SLO, a well-characterized cholesterol-binding agent, results in a time- and concentration-dependent release of GPI-anchored AP into the extracellular medium. Unexpectedly, this release is not mediated by GPI-PLD cleavage, since purified GPI-PLD does not further augment this SLO effect and, furthermore, the GPI anchor of the released AP is intact, as determined by its ability to bind detergents. Instead, vesiculation was shown to be the most likely mechanism for SLO induction of AP release, based on the observations that: (i) the released AP has an intact GPI anchor; (ii) it can be sedimented at 100000 *g*; and (iii) the 100000 *g* pellet contained lipids. The SLO effect is dependent on both cholesterol binding and temperature-induced aggregation of SLO molecules. Two other cholesterol-binding agents, saponin and digitonin, can also induce release of a substantial amount of AP, possibly through a similar vesiculation process. It should be pointed out that not all cholesterol-binding agents have this effect. Nystatin, filipin and β-escin are unable to elicit AP release, although both nystatin and filipin have previously been shown to disrupt clustering of GPI-anchored proteins [18], and both filipin and β-escin can elicit cell permeabilization. Thus the mechanism by which SLO, saponin and digitonin induce vesiculation remains to be determined. During preparation of this paper, a report appeared in which saponin, but not nystatin, was found to be capable of stimulating human serum GPI-PLD to cleave Thy-1 from a mouse T-lymphoma cell line Y191 [43]. By contrast, in the present study, saponin and all other cholesterol-binding agents tested were shown to be unable to activate bovine serum GPI-PLD-mediated release of AP from ROS cells. The reason for this striking discrepancy between the two studies is currently unclear.

Our results thus do not provide any support for the hypothesis [43] that clustering of GPI-anchored proteins is responsible for their resistance to cleavage by GPI-PLD. Furthermore, the notion that most of the GPI-anchored proteins exist in clusters on the cell surface has itself been challenged by two independent lines of research. First, experiments employing fluorescence recovery after photobleaching (FRAP) and fluorescence resonance energy transfer (FRET) measurements have demonstrated that only the newly synthesized GPI-anchored molecules are relatively immobile and clustered, and such clustering decays within 1 h of arrival on the cell surface [44]. Second, experiments using direct coupling of fluorophore to anti-(GPI-anchored-protein) monoclonal antibodies have further revealed that the previously observed clustering of GPI-anchored proteins may have been induced by the secondary polyclonal antibodies used for localization [45]. Instead, GPI-anchored proteins normally are found to distribute diffusely on the cell surface and thus may not be constitutively clustered in caveolae. They enter into these structures independently only after cross-linking with polyclonal antibodies [45]. Potentially, this clustering event may occur *in vivo* after activation of endogenous 'cross-linkers'. Taken together, these results suggest that it is unlikely that clustering of GPI-anchored proteins is involved in GPI-PLD resistance. In fact, clustering of GPI-anchored proteins into caveolae under currently undefined conditions may provide a favourable micro-environment for GPI-PLD action.

The vesicles released by SLO treatment of ROS cells exhibit a distinct lipid composition from that of intact cells. For example, both cholesterol and sphingomyelin are relatively enriched, whereas phosphatidylethanolamine is particularly poor in these vesicles. It has long been observed that treatment of erythrocytes with A23187 and Ca^{2+} results in production of two types of vesicles: microvesicles and nanovesicles, of diameter 150 nm and 60 nm respectively [35,36]. The present results are consistent with the suggestion that the SLO-released vesicles belong to the category of nanovesicles, on the basis of both sedimentation and lipid-composition analysis. The nanovesicles from erythrocytes also require 100000 *g* to be sedimented, and contain enriched sphingomyelin and less phosphatidylethanolamine compared with microvesicles and cells [35]. This lipid composition might be necessary for the formation of nanovesicles, since the extreme curvature of the nanovesicle membrane would lead to an excess of outer leaflet area over inner leaflet, and thus to an increase in the amounts of those lipids normally present in the outer leaflet (e.g. cholesterol and sphingomyelin) relative to those in the inner leaflet (e.g. phosphatidylethanolamine) [35].

The present study demonstrates that ROS cells express at least eight GPI-anchored proteins. These GPI-anchored proteins are all present, and may even be enriched, in the SLO-released vesicles compared with intact cells. The enrichment of GPI-anchored proteins and cholesterol/sphingolipids in the vesicles induced by SLO would be reminiscent of recent observations on caveolae [18–21,37,39]. Furthermore, caveolae of diameter 60 nm could be regarded as a type of nanovesicle. However, the relationship between these two entities remains to be explored. In addition to erythrocytes, many other cell types, including platelets [46] and tumour cells [47–49], are also capable of vesiculating under certain conditions. In those cases where GPI-anchored proteins were examined, they are all enriched in the released vesicles [35,46,47,50]. Considering that these vesicles have been shown, in many cases, to contain relatively high concentrations of cholesterol and sphingomyelin, and that GPI-anchored proteins appear to associate preferentially with these lipid species, the enrichment of GPI-anchored proteins in these vesicles would be expected.

Vesiculation probably occurs *in vivo*, since membrane vesicles have been found in the body fluids, such as serum [48–50]. It has been postulated that vesiculation during erythrocyte senescence and consequent loss of decay-accelerating factor and CD59 may

contribute to the clearing of aged erythrocytes by autologous complement-mediated cell lysis [50]. Furthermore, blood from cancer patients is known to contain an increased amount of membrane vesicles [47–49]. Considering that these vesicles are potentially enriched with GPI-anchored proteins of diverse physiological functions, loss of these proteins could contribute to many pathological processes.

This is the first study to demonstrate clearly that SLO is capable of inducing cells to vesiculate, and that this process is further augmented by serum. These findings are of potential pathological significance in human streptococcal diseases. SLO is produced by β-haemolytic group A streptococci, which represent the major human pathogens of the genus *Streptococcus* [24]. High concentrations of SLO have been detected in patients with streptococcal infections and rheumatic fever [24]. Therefore, it is possible that the ability of SLO to induce cells to vesiculate may contribute to its role in human streptococcal pathogenesis. Further studies on the mechanism for SLO induction of vesiculation will increase understanding not only of basic principles of membrane fusion and budding, but of the pathological mechanism of SLO in human streptococcal diseases as well.

We thank Dr. Jau-Yi Li for purification of GPI-PLD from bovine serum. This work was supported, in part, by NIH Grants GM-40083 and GM-35873, a Grant-in-Aid from the American Heart Association, and a gift from Samuel W. Rover.

REFERENCES

1 Low, M. G. (1989) Biochim. Biophys. Acta **998**, 427–454
2 Cross, G. A. M. (1990) Annu. Rev. Cell Biol. **6**, 1–39
3 Field, M. C. and Menon, A. K. (1993) in Lipid Modifications of Proteins (Schlesinger, M. J., ed.), pp. 83–134, CRC Press, Boca Raton
4 Englund, P. T. (1993) Annu. Rev. Biochem. **62**, 121–138
5 Low, M. G. (1992) in Lipid Modification of Proteins: a Practical Approach (Hooper, N. M. and Turner, A. J., eds), pp. 117–154, Oxford University Press, Oxford
6 Davitz, M. A., Hereld, D., Shak, S., Krakow, J., Englund, P. T. and Nussenzweig, V. (1987) Science **238**, 81–84
7 Low, M. G. and Prasad, A. R. S. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 980–984
8 Xie, M., Sesko, A. M. and Low, M. G. (1993) Am. J. Physiol. **265**, C1156–C1168
9 Xie, M. and Low, M. G. (1994) Biochem. J. **297**, 547–554
10 Metz, C. N., Thomas, P. and Davitz, M. A. (1992) Am. J. Pathol. **140**, 1275–1281
11 Stadelmann, B., Zurbriggen, A. and Brodbeck, U. (1993) Cell Tissue Res. **274**, 547–552
12 Metz, C. N., Zhang, Y., Guo, Y., Tsang, T. C., Kochan, J. P., Altszuler, N. and Davitz, M. A. (1991) J. Biol. Chem. **266**, 17733–17736
13 Davitz, M. A., Hom, J. and Schenkman, S. (1989) J. Biol. Chem. **264**, 13760–13764
14 Huang, K.-S., Li, S., Fung, W.-J. C., Hulmes, J. D., Reik, L., Pan, Y.-C. E. and Low, M. G. (1990) J. Biol. Chem. **265**, 17738–17745
15 Low, M. G. and Huang, K.-S. (1991) Biochem. J. **279**, 483–493
16 Metz, C. N., Brunner, G., Choi-Muira, N. H., Nguyen, H., Gabrilove, J., Caras, I. W., Altszuler, N., Rifkin, D. B., Wilson, E. L. and Davitz, M. A. (1994) EMBO J. **13**, 1741–1751
17 Brunner, G., Metz, C. N., Nguyen, H., Gabrilove, J., Patel, S. R., Davitz, M. A., Rifkin, D. B. and Wilson, E. L. (1994) Blood **83**, 2115–2125
18 Rothberg, K. G., Ying, Y.-S., Kamen, B. A. and Anderson, R. B. W. (1990) J. Cell Biol. **111**, 2931–2938
19 Ying, Y.-S., Anderson, R. G. W. and Rothberg, K. G. (1992) Cold Spring Harbor Symp. Quant. Biol. **57**, 593–604
20 Anderson, R. G. W., Kamen, B. A., Rothberg, K. G. and Lacey, S. W. (1992) Science **255**, 410–411
21 Brown, D. A. and Rose, J. K. (1992) Cell **68**, 533–544
22 Chang, W.-J., Rothberg, K. G., Kamen, B. A. and Anderson, R. G. W. (1992) J. Cell Biol. **118**, 63–69
23 Cerneus, D. P., Ueffing, E., Posthuma, G., Strous, G. J. and Van der Ende, A. (1993) J. Biol. Chem. **268**, 3150–3155
24 Bhakdi, S. and Tranum-Jensen, J. (1987) Rev. Physiol. Biochem. Pharmacol. **107**, 147–223
25 Bhakdi, S., Weller, U., Walev, I., Martin, E., Jonas, D. and Palmer, M. (1993) Med. Microbiol. Immunol. **182**, 167–175
26 Huang, K.-S., Li, S. and Low, M. G. (1991) Methods Enzymol. **197**, 567–575
27 Bordier, C. (1981) J. Biol. Chem. **256**, 1604–1607
28 Bligh, E. G. and Dyer, W. J. (1959) Can. J. Biochem. Physiol. **37**, 911–917
29 Billah, M. M., Lapetina, E. G. and Cuatrecasas, P. (1981) J. Biol. Chem. **256**, 5399–5403
30 Xie, M., Jacobs, L. S. and Dubyak, G. R. (1991) J. Clin. Invest. **88**, 45–54
31 Lisanti, M. P., Sargiacomo, M., Graeve, L., Saltiel, A. R. and Rodriguez-Boulan, E. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 9557–9561
32 Lisanti, M. P., Caras, I. W., Davitz, M. A. and Rodriguez-Boulan, E. (1989) J. Cell Biol. **109**, 2145–2156
33 Bollag, D. M. and Edelstein, S. J. (1991) Protein Methods, Wiley–Liss, New York
34 Wong, Y. W. and Low, M. G. (1992) Clin. Chem. **38**, 2517–2525
35 Allan, D., Thomas, P. and Limbrick, A. R. (1980) Biochem. J. **188**, 881–887
36 Allan, D. and Thomas, P. (1981) Biochem. J. **198**, 433–440
37 Lisanti, M. P., Tang, Z. and Sargiacomo, M. (1993). J. Cell Biol. **123**, 595–604
38 Stefanová, I., Horejsí, V., Ansotegui, I. J., Knapp, W. and Stockinger, H. (1991) Science **254**, 1016–1019
39 Sargiacomo, M., Sudol, M., Tang, Z. and Lisanti, M. P. (1993) J. Cell Biol. **122**, 789–807
40 Fujimoto, T. (1993) J. Cell Biol. **120**, 1147–1157
41 Fujimoto, T., Nakade, S., Miyawaki, A., Mikoshiba, K. and Ogawa, K. (1992) J. Cell Biol. **119**, 1507–1513
42 Anderson, R. G. W. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 10909–10913
43 Bergman, A.-S. and Carlsson, S. R. (1994) Biochem. J. **298**, 661–668
44 Hannan, L. A., Lisanti, M. P., Rodriguez-Boulan, E. and Edidin, M. (1993) J. Cell Biol. **120**, 353–358
45 Mayor, S., Rothberg, K. G. and Maxfield, F. R. (1994) Science **264**, 1948–1951
46 Kobayashi, T., Okamato, H., Yamada, J.-I., Setaka, M. and Kwan, T. (1984) Biochim. Biophys. Acta **778**, 210–218
47 Masella, R., Cantafora, A., Guidoni, L., Luciani, A. M., Mariutti, G., Rosi, A. and Viti, V. (1989) FEBS Lett. **246**, 25–29
48 Carr, J. M., Dvorak, A. M. and Dvorak, H. F. (1984) Cancer Res. **45**, 5944–5951
49 Taylor, D. D. and Black, P. H. (1986) in Developmental Biology (Steinberg, M., ed.), pp. 33–57, Plenum, New York
50 Bütikofer, P., Kuypers, F. A., Xu, C. M., Chiu, D. T. Y. and Lubin, B. (1989) Blood **74**, 1481–1485

Received 6 June 1994/23 August 1994; accepted 7 September 1994