ELSEVIER

Biochimica et Biophysica Acta 1370 (1998) 218–234

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

The aminosterol antibiotic squalamine permeabilizes large unilamellar phospholipid vesicles

Barry S. Selinsky [a,*], Zhao Zhou [a], Kristin G. Fojtik [a], Stephen R. Jones [a], Norman R. Dollahon [b], Ann E. Shinnar [c]

[a] *Department of Chemistry, Villanova University, Villanova, PA 19085-1699, USA*
[b] *Department of Biology, Villanova University, Villanova, PA 19085-1699, USA*
[c] *Department of Chemistry, Barnard College, New York, NY 10027, USA*

Received 21 October 1997; accepted 28 October 1997

Abstract

The ability of the shark antimicrobial aminosterol squalamine to induce the leakage of polar fluorescent dyes from large unilamellar phospholipid vesicles (LUVs) has been measured. Micromolar squalamine causes leakage of carboxyfluorescein (CF) from vesicles prepared from the anionic phospholipids phosphatidylglycerol (PG), phosphatidylserine (PS), and cardiolipin. Binding analyses based on the leakage data show that squalamine has its highest affinity to phosphatidylglycerol membranes, followed by phosphatidylserine and cardiolipin membranes. Squalamine will also induce the leakage of CF from phosphatidylcholine (PC) LUVs at low phospholipid concentrations. At high phospholipid concentrations, the leakage of CF from PC LUVs deviates from a simple dose–response relationship, and it appears that some of the squalamine can no longer cause leakage. Fluorescent dye leakage generated by squalamine is graded, suggesting the formation of a discrete membrane pore rather than a generalized disruption of vesicular membranes. By using fluorescently labeled dextrans of different molecular weight, material with molecular weight ≤ 4000 g/mol is released from vesicles by squalamine, but material with molecular weight $\geq 10{,}000$ is retained. Negative stain electron microscopy of squalamine-treated LUVs shows that squalamine decreases the average vesicular size in a concentration-dependent manner. Squalamine decreases the size of vesicles containing anionic phospholipid at a lower squalamine/lipid molar ratio than pure PC LUVs. In a centrifugation assay, squalamine solubilizes phospholipid, but only at significantly higher squalamine/phospholipid ratios than required for either dye leakage or vesicle size reduction. Squalamine solubilizes PC at lower squalamine/phospholipid ratios than PG. We suggest that squalamine complexes with phospholipid to form a discrete structure within the bilayers of LUVs, resulting in the transient leakage of small encapsulated molecules. At higher squalamine/phospholipid ratios, these structures release from the bilayers and aggregate to form either new vesicles or squalamine/phospholipid mixed micelles.

Keywords: Aminosterol; Antimicrobial; Membrane-lytic agent

Abbreviations: LUVs, large unilamellar vesicles; SUVs, small unilamellar vesicles; CF, carboxyfluorescein; ANTS, 8-aminonaphthalene-1,3,6 trisulfonic acid; DPX, *p*-xylene-bis-pyridinium bromide; PC, phosphatidylcholine; PG, phosphatidylglycerol; CL, cardiolipin; PS, phosphatidylserine; FD-4, FD-70, fluorescein-labeled dextrans with molecular weight 4000 and 70,000 g/mol, respectively; TMR-10, tetramethylrhodamine-labeled dextran with molecular weight 10,000 g/mol; MIC, minimum inhibitory concentration

* Corresponding author. Fax: +1-610-519-7167; E-mail: selinsky@rs6chem.vill.edu

PII S0005-2736(97)00265-4

1. Introduction

The increasing incidence of antibiotic-resistant bacteria and public awareness of chronic infection has intensified efforts to discover novel antimicrobial agents. A variety of higher organisms have been found to synthesize and secrete antimicrobial substances which supplement the cellular and humoral immune systems. Many of the antimicrobial agents have been identified as peptides: defensins from mammals, magainins from frogs, tachyplesins from the horseshoe crab, and cecropins from insects [1,2]. These peptides all have fewer than 50 amino acids, of which a high proportion are the basic amino acids lysine and arginine. In the presence of a membrane containing acidic lipids, or in a membrane mimetic environment, many of the peptides have the ability to form amphipathic α-helices. The number of basic amino acids and the propensity of the peptide to form an amphipathic helix influences the antimicrobial activity and selectivity of these agents [3].

The novel antimicrobial agent *squalamine* (3β-*N*-1-{*N*-[3-(4-aminobutyl)]-1,3-diaminopropane}-7α,24ξ-dihydroxy-5-cholestane 24(*R*)-sulfate) was recently isolated from the dogfish shark [4]. The structure of this water-soluble cationic steroid is shown in Fig. 1. Squalamine exhibits antimicrobial activity against a broad spectrum of microorganisms including *Escherichia*, *Staphylococcus*, *Streptococcus*, *Candida*, and *Paramecium*. The synthesis of squalamine from 5-cholenic acid has been completed in 17 steps [5], and the synthetic squalamine has identical activity to the natural aminosterol from shark.

Due to its broad antimicrobial specificity, squalamine is a promising therapeutic agent in the treatment of microbial infections resistant to conventional antibiotics. Squalamine contains a polyamine moiety which will be protonated at neutral pH. The positive charges could bind to negatively charged phospholipids on the surface of bacterial cells. Antimicrobial peptides such as magainins have been shown to preferentially bind to anionic phospholipids [6–8], and are believed to lyse bacterial cells by forming a transient pore through their plasma membrane [9,10].

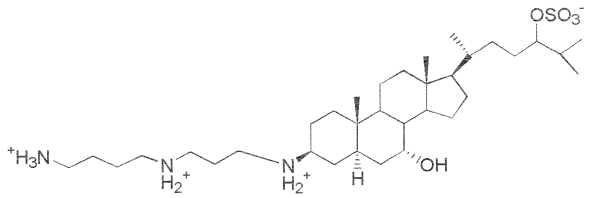

Fig. 1. The structure of the antimicrobial aminosterol squalamine.

Many studies directed towards understanding the mechanism of peptide antibiotics have been facilitated by the relative ease of preparing synthetic peptides in high purity and yield. The mechanism by which squalamine kills bacterial cells has not been studied until now, in part because only limited quantities of isolated squalamine have been available. Our laboratory has begun a systematic physico–chemical approach to understanding squalamine's mechanism of action.

In this report, squalamine has been added to large unilamellar vesicles (LUVs), prepared from one of several different phospholipids and loaded with the self-quenching fluorescent dye carboxyfluorescein (CF). Squalamine is shown to promote the release of CF from LUVs comprised of anionic phospholipids. Squalamine also promotes leakage of CF from vesicles comprised of the zwitterionic lipid phosphatidylcholine, but only at a much higher squalamine/lipid molar ratio, and only at low vesicle concentrations. By using vesicles with encapsulated 8-aminonapthalene-1,3,6 trisulfonic acid (ANTS) and *p*-xylene-bis-pyridinium bromide (DPX), dye leakage was determined to follow a graded mechanism, as opposed to an all-or-none mechanism. The size of the squalamine-induced membrane defect was measured using fluorescent-labeled dextrans. Also, the surfactant-like nature of squalamine was tested using a variety of biochemical and microscopic experiments. From these results, a model describing the interaction of squalamine with phospholipid bilayers is proposed.

2. Materials and methods

2.1. Chemicals

Egg yolk phosphatidylcholine (PC), phosphatidylglycerol derived from egg yolk (PG), beef heart

cardiolipin (CL), and brain phosphatidylserine (PS) were purchased from Avanti Polar Lipids (Alabaster, AL). Carboxyfluorescein (CF) was purchased from Fluka (Ronkonkoma, NY), and purified by Sephadex LH-20 column chromatography [11]. 8-Aminonapthalene-1,3,6 trisulfonic acid (ANTS), *p*-xylene-bis-pyridinium bromide (DPX), and tetramethylrhodamine-labeled dextran (MW = 10 kD) were purchased from Molecular Probes (Eugene, OR). Orange OT and 1-phenyl-*N*-napthalamine were purchased from Aldrich (Milwaukee, WI). The fluorescein-labeled dextrans FD-4 (MW = 4 kD) and FD-70 (MW = 70 kD), as well as Sephacryl S-400-HR, were purchased from Sigma (St. Louis, MO). All other chemicals were reagent grade and used without further purification.

Squalamine was isolated from the liver of the shark *Squalus acanthias* as previously described [4], and purified as the trifluoroacetate salt. As determined by elemental analysis, the salt contains two trifluoroacetate groups, resulting in an effective molecular weight for squalamine of 923.5 g/mol. Aqueous stock squalamine solutions used for leakage measurements were prepared at a concentration of 1 mg/ml (1.08 mM). Stock solutions were stored at 4°C.

2.2. Vesicle preparation

Large unilamellar vesicles (LUVs) with encapsulated CF were prepared by extrusion [12], using a hand-held apparatus (Avestin, Ottawa, Canada). Approximately 12 mg of lipid in chloroform solution was dried under nitrogen gas, and then under vacuum for at least 2 h. The dried lipid was resuspended into buffer containing CF (20 mM Pipes, 10 mM NaCl, 1 mM EDTA, 100 mM CF, pH 7.0) and vigorously vortexed. The lipid suspension was freeze–thawed 10 cycles by alternately placing the sample in a dry ice–acetone bath and a water bath thermostatically regulated at 30°C. The lipid suspension was then extruded through one or two 100 nm polycarbonate filters for 29 passes. Extravesicular CF was separated from the loaded vesicles by passage through a 1×15 cm BioGel P6DG size exclusion chromatography column, eluting with assay buffer (20 mM Pipes, 150 mM NaCl, 1 mM EDTA, pH 7.0; assay buffer is osmotically balanced to the CF buffer encapsulated within the vesicles [6]). The vesicles were harvested and stored at room temperature (20–22°C) for leakage studies, which were performed on the same day as vesicle preparation. The phospholipid concentration of the vesicular solutions were measured by total phosphate assay [13].

To measure the mechanism of leakage (see later), LUVs were prepared encapsulated with both the fluorophore 8-aminonapthalene-1,3,6 trisulfonic acid (ANTS) and the quenching agent *p*-xylene-bis-pyridinium bromide (DPX). Lipid was extruded in the same manner as for CF loaded vesicles, except that ANTS/DPX buffer (10 mM Pipes, 10 mM NaCl, 1 mM EDTA, 50 mM ANTS, 50 mM DPX, pH 7.0) was used. Unencapsulated dyes were separated from vesicles using a 1×18 cm Sephadex G-75 size exclusion column. In our hands, size exclusion chromatography using P6DG best separates unencapsulated CF from LUVs, while Sephadex G-75 best separates free calcein or ANTS/DPX from LUVs. Harvested vesicles were stored at 4°C until use. No loss of encapsulated fluorophore is observed within a week of vesicle preparation.

2.3. Leakage measurements

The leakage of CF from LUVs was measured using an Aminco Bowman spectrofluorimeter maintained at 30°C, with $\lambda_{ex} = 490$ nm and $\lambda_{em} = 520$ nm. An aliquot of LUVs (typically 10–100 μl) was added to assay buffer in a quartz fluorescence cuvette and allowed to equilibrate to 30°C for 5 min. A small aliquot (2–20 μl) of squalamine from an aqueous stock solution of 1 mg/ml was added to the cuvette, and the solution vigorously stirred. The total solution volume in the cuvette was 3.0 ml. Fluorescence readings were recorded every 15 s for 2 min. After 2 min, 30 μl of a 10% Triton X-100 solution was added to lyse all vesicles, and the fluorescence recorded. Some experiments were performed with a Photon Technologies, Quantamaster spectrofluorimeter using the same experimental conditions. With this instrument, sample fluorescence is recorded continuously.

Carboxyfluorescein leakage from LUVs induced by squalamine addition was recorded as the percent

leakage at 1 min, using the relationship

$$\% \text{ Leakage at 1 min} = \left[\frac{(F_{t=1\,\text{min}} - F_0)}{(F_\infty - F_0)} \right] * 100\% \quad (1)$$

where F_0 is the background fluorescence intensity before squalamine addition, F_∞ is the fluorescence intensity after adding Triton X-100 to a final concentration of 0.1%, and $F_{t=1\ \text{min}}$ is the fluorescence intensity at 1 min after squalamine addition. Leakage is expressed as the percentage of encapsulated dye released.

Some of the acquired leakage data was used to indirectly calculate a binding isotherm, employing a method previously described in detail [6,14,15]. First, the fractional leakage is measured as a function of squalamine concentration at several fixed concentrations of lipid. Next, a set of lines parallel to the abscissa is drawn through the dose–response curves, giving for each line a set of pairs of [squalamine$_{\text{init}}$], [lipid] values that produce an equivalent fractional leakage. [Squalamine$_{\text{init}}$] and [lipid] are the total molar concentrations of squalamine and lipid, respectively, and are functionally related to the concentrations of free and bound squalamine by the conservation of mass equation

$$[S_0] = [S_f] + r[L] \quad (2)$$

where $[S_0]$ is the total squalamine concentration, $[S_f]$ is the concentration of free squalamine, and r is the molar concentration of bound squalamine per lipid. Since all pairs of $[S_0]$, $[L]$ values that produce a given fractional leakage must also produce the same value of r, plots of $[S_0]$ vs. $[L]$ at constant fractional leakages are linear, with slopes of r and intercepts of $[S_f]$. The set of r, $[S_f]$ values obtained from these plots constitutes the binding isotherm, where the slope of a line drawn through the experimental points yields the isotherm in units of reciprocal concentration. For this study, only data acquired using low phospholipid concentrations ($\leq 5\ \mu$M) can be used to calculate binding isotherms; at high lipid concentrations, the concentration of free squalamine is too low to provide a valid measurement.

2.4. Mode of dye leakage induced by squalamine

To distinguish between 'all-or-none' leakage vs. 'graded' leakage, the amount of self-quenching exhibited by LUVs after squalamine treatment was measured. The details of the method have been described in detail elsewhere [9,16]. In brief, vesicles were prepared either from PG, PS, or PC encapsulating a fluorophore (50 mM ANTS) and a quenching agent (50 mM DPX). First, DPX is incrementally added to untreated vesicles disrupted with Triton X-100, and a normalized fluorescence $F_{\text{DPX}}/F_{\text{max}} = Q_{\text{out}}$ was measured as a function of the DPX concentration. Next, squalamine was added to a new population of vesicles, and the fluorescence increase monitored until a stable fluorescence value was reached. The vesicle solution was then titrated with quenching agent (90 mM DPX, 10 mM Pipes, 1 mM EDTA, pH 7.0), and the total fluorescence (F_{total}) was measured as a function of the DPX concentration. After the final addition of DPX, Triton X-100 was added to determine F_{max}. The quantity Q_{total} was defined to be equal to $F_{\text{total}}/F_{\text{max}}$. The result is the acquisition of Q_{total} and Q_{out} for each concentration of DPX, which can be used to calculate the internal quenching (Q_{ins}) and the fraction of ANTS released by squalamine (f_{out}) through the equation

$$Q_{\text{total}} = Q_{\text{out}} \cdot f_{\text{out}} + Q_{\text{ins}} \cdot (1 - f_{\text{out}}). \quad (3)$$

From this data, curves relating the internal quenching of the vesicles relative to the external fluorescence are generated. If the internal quenching Q_{ins} is independent of the extent of dye release, the leakage mechanism is 'all-or-none'. If Q_{ins} varies with dye release, the leakage mechanism is 'graded'.

2.5. Sizing the squalamine-generated membrane pore through the leakage of fluorescent dextrans

Membrane pores induced in bilayers by the addition of squalamine were sized using the methodology developed by Ladokhin et al. [17]. Vesicles containing the fluorescein labeled dextrans FD-4 (2 mg/ml) and FD-70 (4 mg/ml), and tetramethylrhodamine-labeled dextran (3 mg/ml, MW = 10,000; abbreviated TMR-10) were prepared by extrusion. Vesicles

were separated from extravesicular fluorescent dye by column chromatography, using a 38×1 cm Sephacryl S-400-HR chromatography column eluting with 10 mM HEPES, 50 mM KCl, 1 mM EDTA, 3 mM sodium azide, pH 7.0, at room temperature (20–22°C), with a flow rate of 1.2 ml per minute. Column fractions (0.75 ml) were collected. The harvested LUVs were treated with either squalamine (0.5 ml of 1.0 mg/ml squalamine in distilled water) or an equal volume of distilled water as a control, and incubated for 10 min. The vesicle/squalamine mixture was placed on a second Sephacryl S-400-HR column, and vesicles separated from dye which leaked from the vesicles due to the addition of squalamine. Column fractions were analyzed for fluorescence and phospholipid. The vesicles were harvested, lyophilized, resuspended in 1.0 ml of 0.1% Triton X-100, chromatographed using a Sephacryl S-400-HR column, and analyzed by fluorescence spectroscopy.

2.6. Electron microscopy

Large unilamellar vesicles were prepared as described previously, using PC or a mixture of PC/PG (70%/30% w/w). Vesicle solution (50 μl) was added into a test tube, into which was added either 10 μl of distilled water or 10 μl of squalamine solution. The tubes were incubated at room temperature for 2 min, and then sampled for electron microscopy.

Grids were prepared by suspending formvar in 0.25% ethylene dichloride for about 2 min, and then putting the formvar on a glass slide. The plastic formvar was floated on distilled water for 10 min. Copper grids (500 mesh, Electron Microscopy Scientific, Washington, DC) were placed on the formvar film. Finally, the copper grids were dried overnight by vacuum evaporation for carbon coating. The coated grids were used immediately.

Negative staining of the liposome samples was performed by placing a small drop of vesicular sample on the freshly prepared, dried surface for 8 min and drawing off the excess liquid from the side with filter paper. A drop of 2% (w/v) ammonium molybdate (prefiltered through a 100 nm membrane) at pH 8.0 was placed on the grids and allowed to stain for 30 s. Excess stain was removed, the grids were air-dried and viewed with a Hitachi H-600 electron microscope operated at 75 kV. The normal magnification was corrected by calibration of the microscope using a carbon grating replica (2160 crossed lines/mm; E.F. Fullam, Schenectady, NY). All samples were examined under the electron microscope within 4 h of preparation. Vesicle diameters were measured from electron micrographs taken at a magnification of 20,000–40,000.

2.7. Critical micelle concentration measurements

The critical micelle concentration (cmc) of squalamine was measured by two different dye partitioning assays. In one assay, solutions of squalamine in distilled water were prepared ranging in concentration between 3 μM and 2.3 mM. To 3 ml of each solution was added 10 μl of 1-phenyl-*N*-naphthylamine (10 mM in 95% ethanol), and the solutions incubated at 30°C for either 30 min or 24 h to achieve equilibrium. The fluorescence of 1-phenyl-*N*-naphthylamine was measured at 30°C in an Aminco–Bowman spectrofluorimeter, using $\lambda_{ex} = 336$ nm and $\lambda_{em} = 462$ nm [18]. For the second assay, 1 ml of squalamine solution was added to 5 mg of Orange OT. The samples were incubated with agitation for 18 h at 30°C, and then the absorbance of the solubilized dye was measured at 492 nm using a Spectronic 21 spectrophotometer [19].

2.8. Squalamine-induced solubilization of phospholipid multilamellar vesicles

Multilamellar vesicles (MLVs) were prepared from both PG and PC by vigorous vortexing of dried lipid into buffer (10 mM HEPES, 150 mM NaCl, 1 mM EDTA, pH 7.0). Approximately 550 nmoles of phospholipid were transferred into polycarbonate centrifuge tubes, and diluted with 1.8 ml of the same buffer. Squalamine solutions of varying concentration are added to give a final volume of 3.0 ml, and vigorously vortexed. After 10 min, the tubes are centrifuged using a Beckman L8 Ultracentrifuge in a 70.1 Ti rotor at 50,000 rpm ($171{,}000 \times g$) for 90 min. After centrifugation, the supernatant was sampled for phospholipid using a total phosphate assay [13].

3. Results

3.1. Lysis of carboxyfluorescein-loaded large unilamellar vesicles

The ability of squalamine to induce the leakage of the fluorescent dye carboxyfluorescein from LUVs has been evaluated by measuring the time course of leakage of CF from PG LUVs (Fig. 2). The leakage kinetics appear to be biphasic, with a rapid initial leakage of CF followed by a much slower phase. Similar kinetics have been observed using vesicles comprised of the anionic lipids PS and CL (data not shown).

The leakage of dye from the vesicles was defined as the percentage of the total encapsulated CF released from the LUVs 1 min after the addition of squalamine. The total fluorescence trapped in the vesicles was determined by lysing the vesicles with Triton X-100. The leakage at 1 min was measured for each concentration of lipid and squalamine tested. The leakage was then plotted vs. the squalamine concentration for several different PG concentrations, and the data shown in Fig. 3. The data shown in Fig. 3 are typical results describing the leakage of CF from anionic LUVs by squalamine; similar data was acquired using the anionic phospholipids PS and CL.

3.2. Lipid specificity of squalamine

The relative affinity of squalamine for anionic lipids was determined by leakage measurements us-

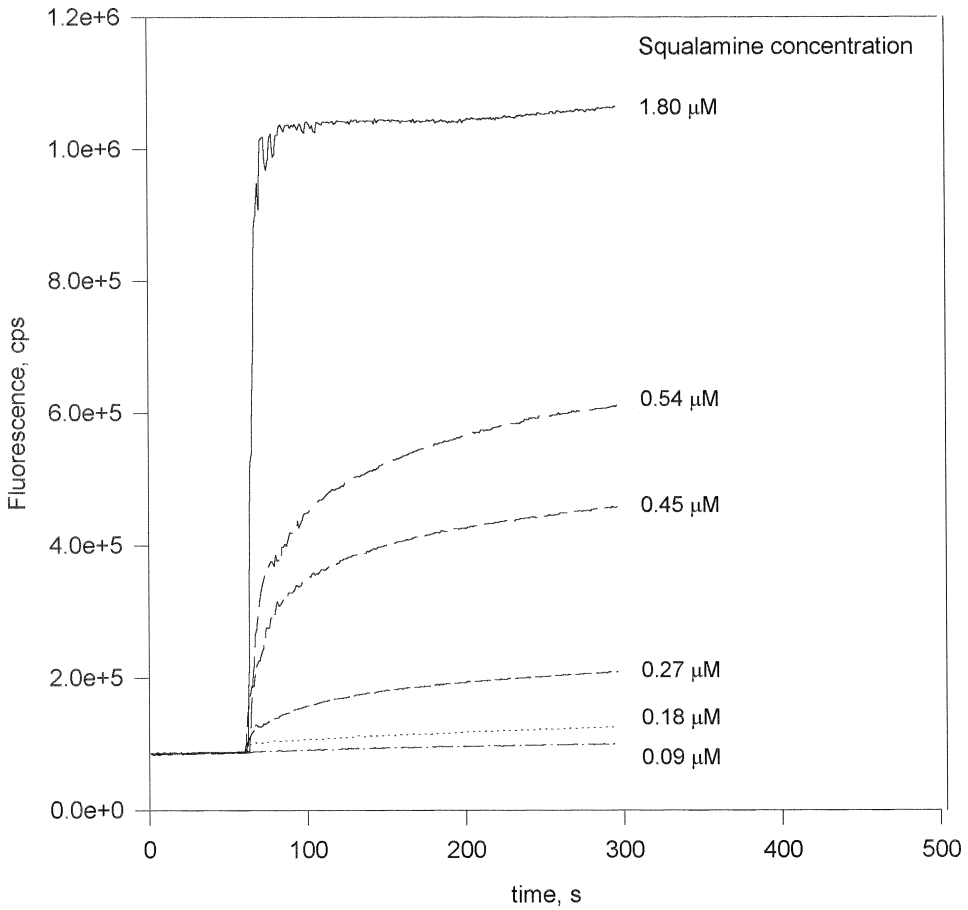

Fig. 2. The kinetics of carboxyfluorescein leakage from PG LUVs. Squalamine was added to PG LUVs (phospholipid concentration = 4.9 μM) to the final concentration indicated on the Figure, and the increase in CF fluorescence recorded. The fluorescence units are recorded in counts per second.

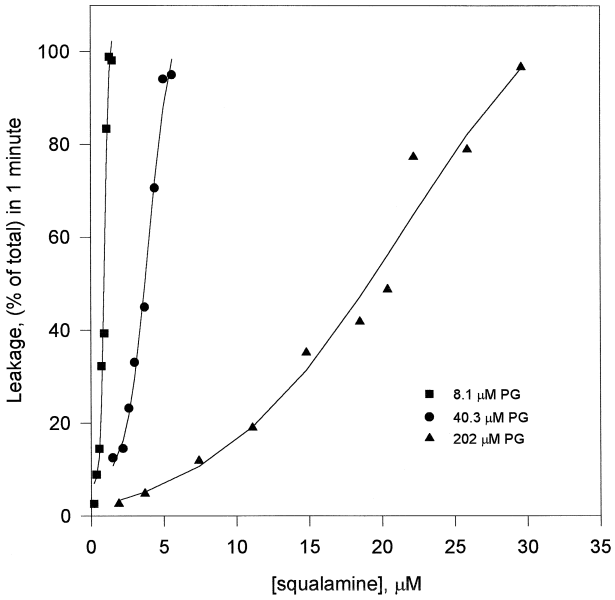

Fig. 3. A plot of leakage rate vs. squalamine concentration for three different concentrations of PG LUVs. The leakage rate was defined as the percentage of the total encapsulated CF released 1 min after the addition of squalamine.

ing LUVs prepared from the anionic lipids PG, PS, and CL. Carboxyfluorescein leakage induced by squalamine was determined as described above, and this leakage was plotted against the logarithm of the squalamine/phospholipid molar ratio for the different phospholipid concentrations tested (Fig. 4). At high lipid concentrations, the curves in Fig. 4 superimpose, demonstrating that under these conditions the measured leakage depends only upon the squalamine/lipid ratio. This suggests that at high phospholipid concentrations, the squalamine binds tightly to the anionic lipid headgroups, resulting in a very small concentration of unbound squalamine in bulk solution. At low lipid concentrations, the curves describing the relationship between the squalamine/lipid ratio and the leakage separate, indicating the presence of a steady-state concentration of free squalamine in solution.

By using the data presented in Fig. 4, the relative binding affinity of squalamine for the different phospholipid headgroups can be assessed in two ways. First, from the data acquired at high phospholipid concentrations, the molar ratio of squalamine to phospholipid required to induce 50% contents leakage in 1 min can be estimated. This ratio will be reported as its base 10 logarithm and referred to as an ED_{50} value (for effective dose). The more negative the ED_{50}, the

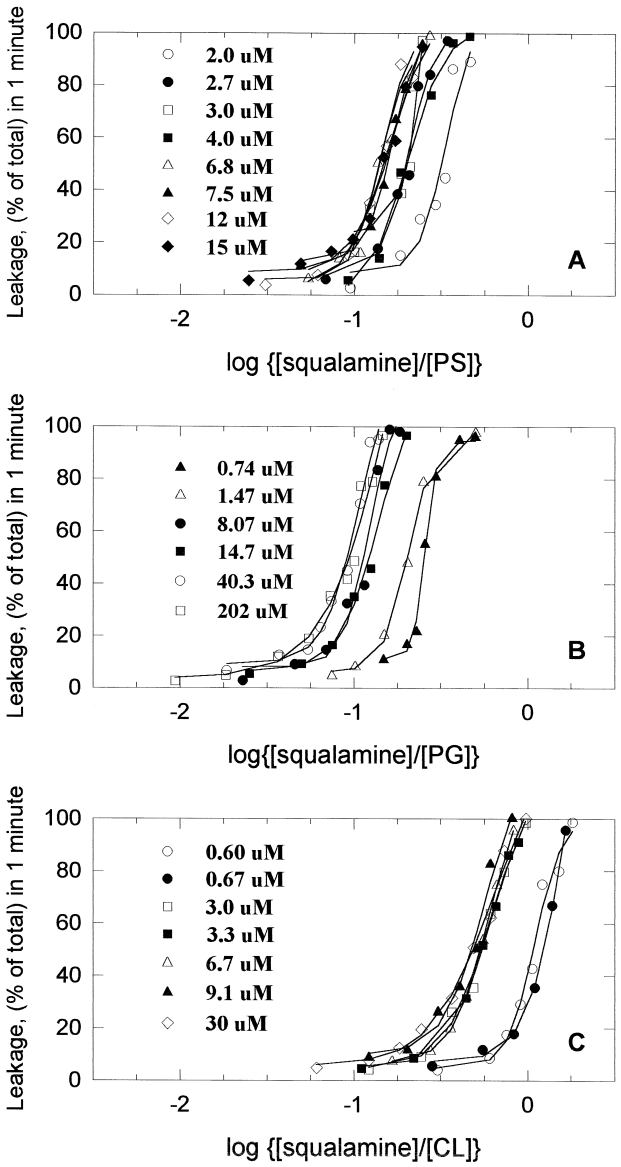

Fig. 4. Dose–response curves describing the rate of leakage of CF from anionic lipid LUVs as a function of the logarithm of the squalamine/lipid molar ratio. At low lipid concentrations, the dose–response curves separate; at higher concentrations, the leakage rate is solely dependent upon the squalamine/lipid molar ratio. (A) Phosphatidylserine LUVs; (B) Phosphatidylglycerol LUVs; (C) Cardiolipin LUVs. The solution phospholipid concentrations for each curve are listed for each study. The errors in the leakage rate and the [squalamine]/[phospholipid] molar ratios are estimated to be $\pm 5\%$.

Table 1
Binding parameters describing the interaction of squalamine with LUVs prepared from a single phospholipid species

Phospholipid	$K_{b\ apparent}$ (M^{-1})	ED_{50}
PG	1.6×10^{6}	−0.95
PS	9.4×10^{5}	−0.85
CL	3.6×10^{5}	−0.30
PC	6.5×10^{4}	ND

The $K_{b\ apparent}$ is calculated from leakage data acquired at lipid phosphate concentrations ≤ 5 mM, as described in the text.
The error in the $K_{b\ apparent}$ calculated in this manner is estimated to be $\pm 20\%$ [7].
The ED_{50} is the log {[squalamine]/[phospholipid]} representing the midpoint of the sigmoidal curves shown in Fig. 4 for saturating lipid concentrations, where the curves superimpose.
No value for ED_{50} could be determined for PC vesicles due to the lack of superimposable dose–response curves (see Fig. 5).

greater the affinity of squalamine for that particular lipid. Second, leakage data can be used to calculate a binding isotherm describing the association of squalamine with phospholipid, as described in Section 2.3 [6,14,15]. The binding isotherms are calculated from leakage curves generated at phospholipid concentrations of less than 5 μM, where the curves do not superimpose and where the concentration of free squalamine is significant relative to the concentration of lipid-bound squalamine.

Both the ED_{50} values and the apparent binding constants ($K_{b\ apparent}$) describing the binding of squalamine to phospholipids are reported in Table 1. Squalamine binds approximately equally well to PS and PG vesicles, with apparent binding constants of approximately 10^{6} M^{-1}. Squalamine binds somewhat less well to CL vesicles ($K_{b\ apparent} = 3.6\times10^{5}$ M^{-1}). However, as CL contains two phosphate groups per phospholipid, the affinity of squalamine per lipid phosphate is not much less for CL than for PG or PS. For this reason, we have used PG as a typical anionic phospholipid in later experiments probing the lytic mechanism for squalamine.

3.3. The binding of squalamine to zwitterionic lipid vesicles

The ability of squalamine to induce CF leakage from LUVs composed of PC, a zwitterionic phospholipid, was also measured. As shown in Fig. 5, squalamine does cause the leakage of CF from PC LUVs at low lipid concentrations. This leakage can be used to calculate an apparent binding constant of 6.5×10^{4} M^{-1} (Table 1). However, at higher lipid concentrations, and unlike the behavior observed between squalamine and anionic phospholipids, the leakage behavior changes. Specifically, the relationship between the leakage and the logarithm of the [squalamine]/[phospholipid] ratio is no longer sigmoidal, and is better described as linear. Also, some of the carboxyfluorescein becomes inaccessible to squalamine leakage; the maximum leakage decreases with increasing lipid concentration (see inset to Fig. 5). Since the leakage behavior changes with PC concentration, no ED_{50} value could be estimated for this lipid at high lipid concentrations.

3.4. Mode of carboxyfluorescein leakage induced by squalamine

Carboxyfluorescein may be released from LUVs through one of two different mechanisms. Either all of the dye is released from a population of vesicles,

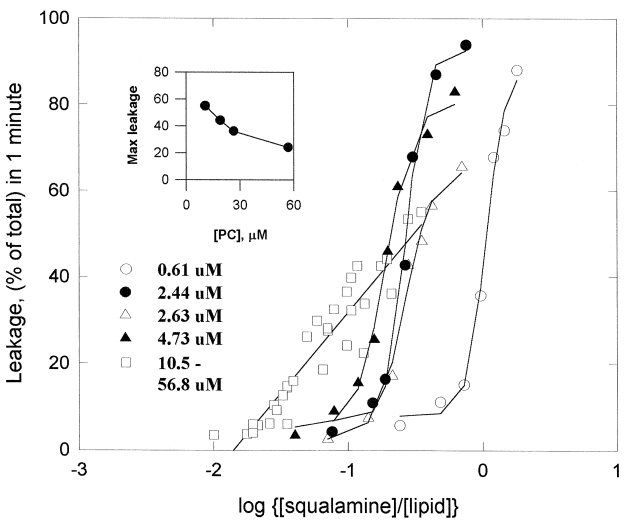

Fig. 5. Dose–response curves describing the leakage rate of CF from PC LUVs as a function of the logarithm of the squalamine/lipid molar ratio. At high lipid concentrations, the dose–response relationship differs from that measured at low lipid concentrations. The errors in the leakage rate and the [squalamine]/[phospholipid] molar ratios are estimated to be $\pm 5\%$. Inset: The maximum observed leakage rate for PC vesicles decreases with the PC concentration in the fluorescence cuvette.

leaving a second population of intact vesicles containing all of the CF initially encapsulated (‘all-or-none’ leakage), or some portion of CF is released from all of the vesicles (‘graded’ leakage). Traditionally, all-or-none leakage is distinguished from graded leakage by adding a pore-forming agent to vesicles containing CF, separating released CF from the vesicles using a size exclusion column, and measuring the fluorescence quenching in the vesicles. Comparison of the percent quenching before and after adding the permeabilizing agent determines the mode of leakage. If the internal quenching does not change, then the leakage is ‘all-or-none’. If the internal quenching varies as a function of added agent, then the leakage is ‘graded’.

Recently, a method was described which allows for the direct measurement of the leakage mechanism [9,16]. To distinguish between the two mechanisms, LUVs were loaded with the fluorescent dye ANTS and the quenching agent DPX. The amount of fluorescence quenching for the dye ANTS after the addition of squalamine to the vesicles can be used to determine whether the leakage is all-or-none or graded (see rationale described in Section 2.4). The direct method has several advantages over separation with a size exclusion column. In the direct method, a series of internal quenching measurements can be made on the same vesicle sample, while in the column method, only a single quenching measurement can be made on each sample. The direct method also requires less lytic agent, important since only small quantities of squalamine were available.

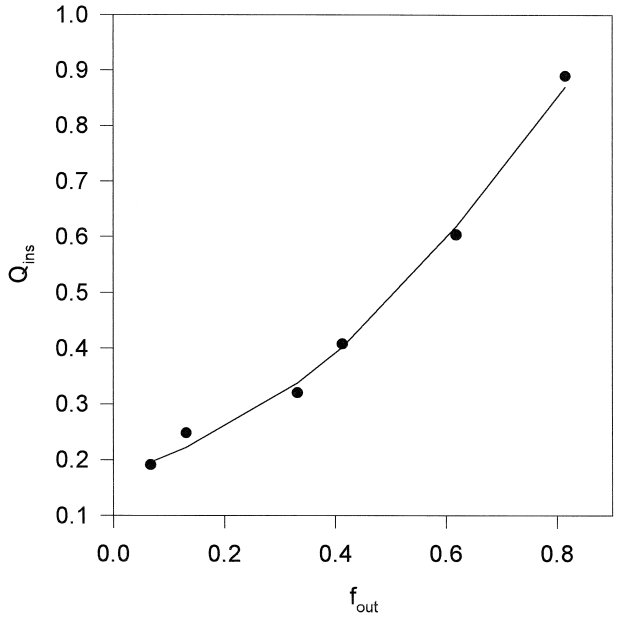

Fig. 6. The results of the all-or-none experiment using squalamine and PG LUVs. The increase in Q_{in} as a function of f_{out} indicates a graded leakage mechanism.

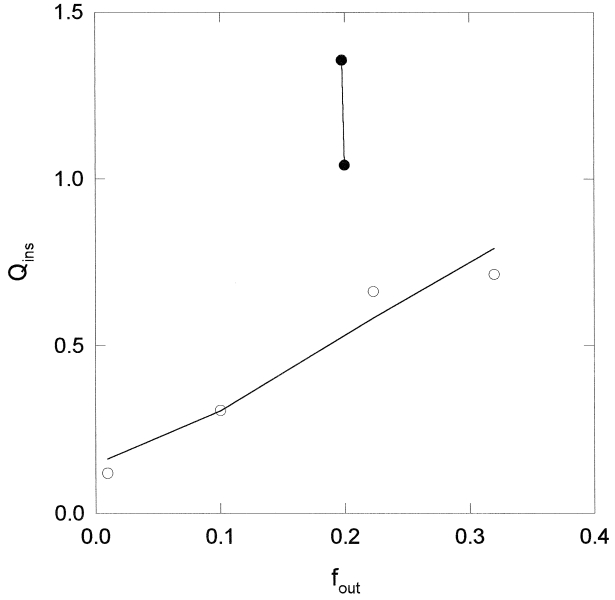

Fig. 7. The results of the all-or-none experiment using squalamine and PC LUVs. The results at low PC concentrations indicate a graded leakage mechanism. Above approximately 10 μM lipid, the experimental data deviates from expected, in parallel to the leakage results shown in Fig. 5. Open circles: 0.6–6 μM PC; Closed circles: 15–40 μM PC.

As shown in Fig. 6, the leakage of the fluorescent dye ANTS induced by squalamine from anionic lipid vesicles is proportional to the *fraction of ANTS* released by squalamine. Hence, squalamine appears to operate via a graded mechanism. This suggests that a transient pore is formed by squalamine when interacting with phospholipid vesicles. The same experiment was repeated using squalamine and the zwitterionic lipid PC (Fig. 7). At low lipid concentrations, the experimental results indicate that the leakage of ANTS from PC vesicles also proceeds by a graded mechanism. At high PC concentrations, the experimentally determined data pairs Q_{ins} and f_{out} no longer fall on the same smooth curve, but lie on approximately the

same value of f_{out} (see Fig. 7). This anomalous result mirrors the leakage data obtained with high PC concentrations.

3.5. The size of the membrane pore

If squalamine forms a discrete membrane pore of defined size, then smaller molecules should pass through the pore more easily than large molecules. We have examined the leakage of the small fluorescent dyes CF, calcein, and ANTS. All are approximately equal in molecular weight, and all leak from vesicles at the same rate at a given squalamine/lipid ratio (data not shown). To estimate the size of the pore generated by squalamine, vesicles were prepared encapsulating three different labeled dextrans: fluorescein-labeled dextran with a molecular weight of 70,000 g/mol (FD-70), tetramethylrhodamine-labeled dextran with a molecular weight of 10,000 g/mol (TMR-10), and fluorescein-labeled dextran with a molecular weight of 4,000 (FD-4). These dyes are separable from each other, and from LUVs, using Sephacryl S-400-HR size exclusion chromatography (Fig. 8). Curiously, the 10 K dextran labeled with tetramethylrhodamine elutes from the column after the fluorescein-labeled 4 K dextran; we attribute this discrepancy to interactions between the tetramethylrhodamine and the column media. In a control study, a tetrakisporphrin-labeled 10 K dextran elutes between the 70 K and 4 K fluorescein-labeled dextrans, as expected (data not shown). Since the spurious elution behavior results in better separation of the dextrans, the tetramethylrhodamine-labeled material was used.

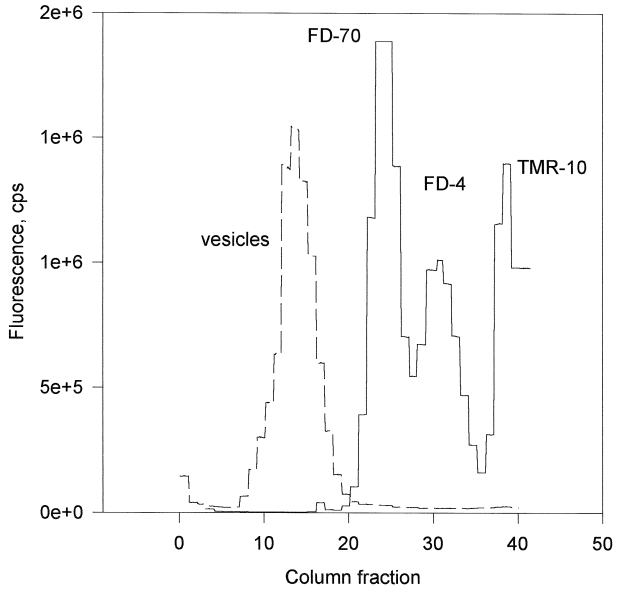

Fig. 8. The separation of fluorescently labeled dextrans and LUVs with encapsulated fluorophores by Sephacryl S-400-HR column chromatography. The column elution buffer was 10 mM HEPES, 50 mM KCl, 1 mM EDTA, 3 mM sodium azide, pH 7.0, at room temperature (20–22°C), with a flow rate of 1.2 ml per minute. The ordinant axis represents column fraction number (0.75 ml fractions), while the abscissa is relative fluorescence (λ_{ex} = 491 nm; λ_{em} = 516 nm) in counts per second.

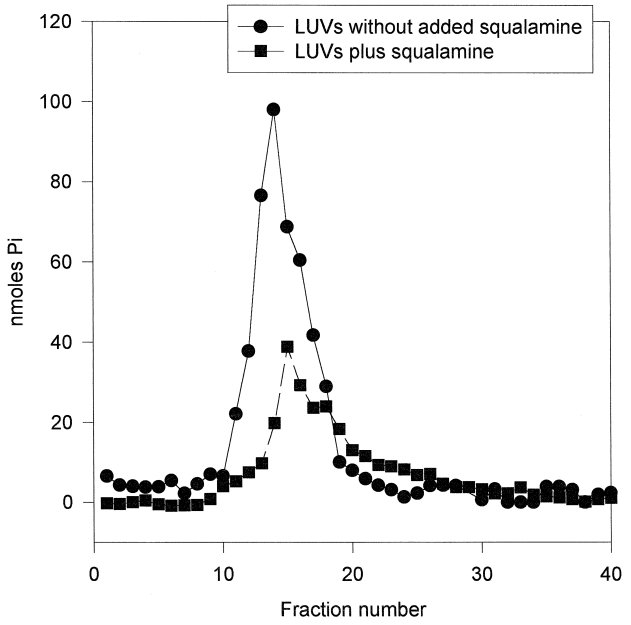

Fig. 9. Total phosphate analysis of fractions from the Sephacryl S-400-HR column. Elution conditions were identical to those given in Fig. 8. Closed circles: PG LUVs applied to the column in the absence of squalamine; closed squares: squalamine-treated PG LUVs (at a squalamine/phospholipid ratio of 0.058/1).

PG vesicles containing three fluorescent dextrans were subjected to size exclusion chromatography (Fig. 9). Analysis of phosphate content shows that the LUVs elute as a single peak. When PG vesicles are treated with squalamine, however, the elution profile changes. The elution time (v_e) remains the same for the largest LUVs, but the phosphate concentration is decreased in the peak. Phosphate is also detected in later fractions. The change in v_e suggests size heterogeneity due to the formation of smaller vesicles.

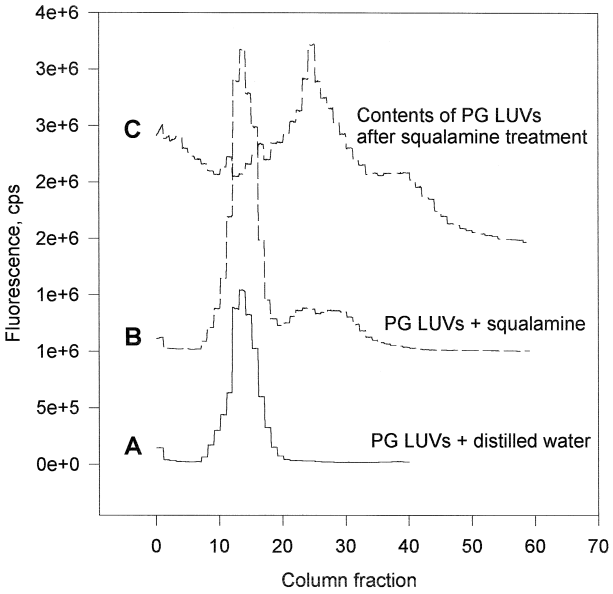

Fig. 10. Fluorescence analysis of fractions collected from the Sephacryl S-400-HR column. Elution conditions were identical to those given in Fig. 8. (A) PG LUVs treated with distilled water. (B) Squalamine-treated PG LUVs, where the molar ratio of squalamine/PG is 0.058/1. (C) The contents of the LUVs found in fractions 13–19 from the bottom trace, released from the vesicles as described in Section 2. The three traces were manually offset from one another; the background fluorescence in all cases is less than 10,000 cps.

Fig. 10 describes the effect of squalamine on the release of fluorescent dextrans from PG LUVs. When PG LUVs containing three fluorescent dextrans are treated with distilled water, no leakage of the fluorescent dextrans is observed (Fig. 10A). Squalamine, when added to vesicles at a squalamine/phospholipid ratio of 0.058/1, induces the release of FD-4 from PG LUVs (Fig. 10B). Some of the FD-70 appears to be released as well, but the amount is difficult to quantitate, since some smaller vesicles generated by squalamine elute in the same column fractions (see Fig. 9). The increased fluorescence in these fractions may be due to fluorescent dextrans within the smaller vesicles.

To confirm the leakage of FD-4 from the vesicles and the retention of the larger dextrans, after treatment with squalamine and separation on the Sephacryl S-400-HR column, the larger vesicles (column fractions 13–19; see Fig. 9) were harvested, concentrated by lyophilization, suspended in buffer containing 0.1% Triton X-100, and rechromatographed. After squalamine treatment, only the FD-70 and TMR-10 remain within the vesicles; all of the FD-4 has leaked out (Fig. 10C). This indicates that the size of the pore created by squalamine is large enough to allow a

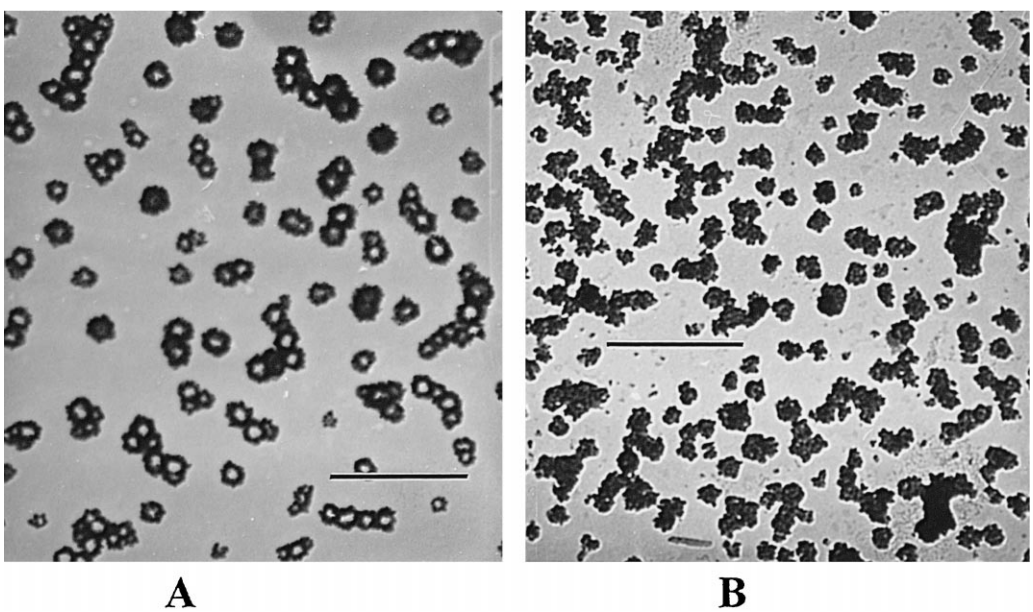

Fig. 11. Negative-stain electron micrographs of PG/PC (3/7 w/w) LUVs. (A) PG/PC vesicles prepared in the absence of squalamine. (B) PG/PC vesicles prepared after treatment with squalamine at a squalamine/phospholipid molar ratio of 0.047/1. Both micrographs are magnified 20,000 times, and the bar on the micrograph corresponds to 1 μM.

molecule with a molecular weight of 4000 g/mol to leak out, but not sufficiently large to allow a molecule with molecular weight of 10,000 g/mol to leak. Since the hydrodynamic radius of FD-4 is reported to be 1.85 nm [9] and that of a 10 K molecular weight dextran is 2.2 nm [20], this suggests that the size of the membrane defect caused by squalamine is between 1.8–2.2 nm.

3.6. Electron microscopy

Since squalamine induces the leakage of fluorescent dyes encapsulated within LUVs, we were curious to see if gross morphological changes in the vesicles accompanied the leakage event. Vesicles were prepared using PC or a mixture of PC/PG (70/30 w/w), and the effect of squalamine on these vesicles observed by negative stain electron microscopy. Pure PG vesicles were also prepared but not analyzed due to poor staining. In the absence of squalamine, unilamellar vesicles were observed in all cases with diameters of approximately 95 nm (Fig. 11A), consistent with LUVs previously prepared by extrusion [12]. Upon the addition of squalamine to the vesicles at squalamine/phospholipid ratios relevant to contents leakage, some changes in the appearance of the vesicles are observed. In particular, darkly stained patches appear on the surface of many of the vesicles in the presence of squalamine (Fig. 11B). The patches appear on both PC and PC/PG vesicles.

The sizes of the vesicles were measured before and after squalamine treatment. As seen in Fig. 12, the vesicular diameters decreased as a function of the squalamine to phospholipid molar ratio. This result corroborates our interpretation of the size exclusion chromatography study, i.e., that squalamine results in the formation of smaller vesicles. Vesicles prepared from PC were less sensitive to squalamine than vesicles prepared from either PG or PG/PC mixtures.

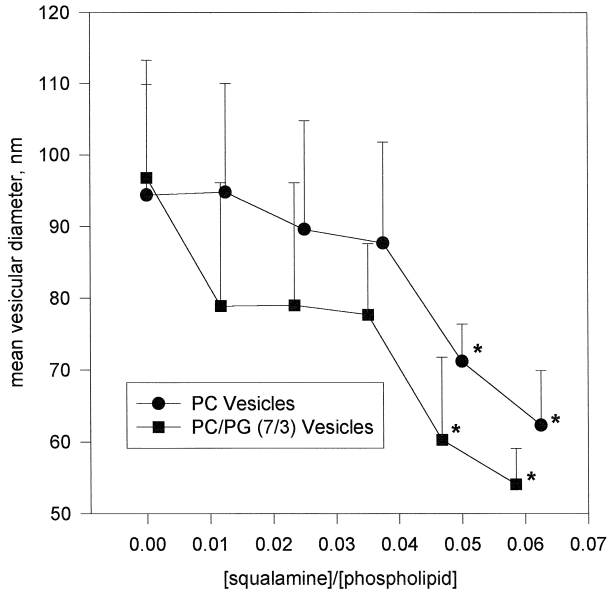

Fig. 12. The mean vesicular diameter decreases in the presence of squalamine. Squalamine was added to either PC LUVs or PC/PG (7/3 w/w) LUVs in the ratios shown in the Figure. Negative stain electron micrographs were taken and analyzed for vesicle size. Between 50–100 vesicles per micrograph were analyzed, and the points represent the mean diameter with the error bars representing one standard deviation. Points marked with an asterisk are different from the zero squalamine controls at the 99% confidence level.

3.7. Critical micelle concentration measurements

Squalamine has some structural similarity to a bile acid; this prompted us to hypothesize that squalamine might work through a detergent-like disruption of a lamellar membrane. An attempt was made to study the cmc of squalamine by measuring the partitioning of spectroscopic probes that are known to incorporate into the hydrophobic environment of detergent micelles. Two different probes were used: 1-phenyl-*N*-naphthylamine, which is fluorescent in a hydrophobic environment [18], and Orange-OT, which is insoluble in aqueous solutions but has a high extinction coefficient at 492 nm when solubilized in a hydrophobic environment [19]. Neither of the two dyes partitioned into a hydrophobic environment in squalamine solutions ranging in concentration between 3 μM and 2.3 mM. Both dyes did partition into a hydrophobic environment in the presence of the bile acid deoxycholate, allowing us to determine a cmc of 7.1 mM (data not shown), comparable to literature values [21]. Since this validates the method, we therefore conclude that squalamine must not form sufficiently large aggregates to define a distinct hydrophobic environment in the concentration range tested.

We were also concerned that squalamine micelles might form in a concentrated stock solution, and that upon dilution into a solution containing lipid vesicles

these micelles would rapidly generate holes in membranes before the micelles could dissociate into monomeric squalamine. The interaction of squalamine micelles with membranes, followed by dissolution into aminosterol monomers, could explain the biphasic nature of the leakage behavior. To insure that squalamine micelles were not causing leakage, squalamine was added from 1.0 mg/ml (1.08 mM), 0.1 mg/ml, and 0.01 mg/ml stock solutions into cuvettes containing PG vesicles with encapsulated CF. The leakage rates measured after adding squalamine to the same final concentration agreed within 5%, regardless of the concentration of the squalamine stock solution. As one final test, squalamine was added to buffer in the fluorescence cuvette to the same final concentration as above (3 μM), incubated at 30°C for 5 min, and then PG vesicles containing CF were added to the cuvette. The leakage rate measured using this experimental protocol was identical to that measured by adding squalamine to a cuvette already containing PG vesicles. These various studies suggest that CF leakage from vesicles does not result from the interaction of squalamine micelles with vesicle membranes.

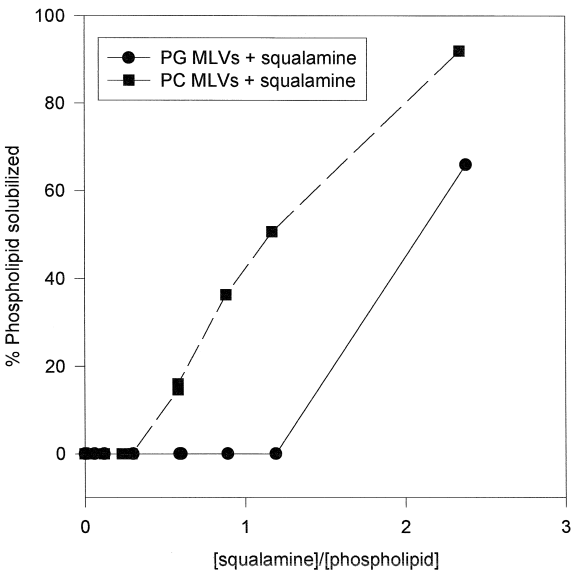

Fig. 13. Phospholipid solubilized by squalamine from PC or PG MLVs. Phospholipid in buffer was treated with squalamine in the ratios shown in the Figure, and then centrifuged. Intact MLVs sedimented to the bottom of the centrifuge tube, while solubilized phospholipid remained in the supernatant and was measured by total phosphate assay. Circles: PC MLVs; Squares: PG MLVs.

3.8. Phospholipid solubilization

As a final indication of possible micelle formation, the ability of squalamine to solubilize phospholipid from multilamellar vesicles (MLVs) was measured. Phospholipid (0.18 mM) is mixed with squalamine (0–0.44 mM) in a polycarbonate centrifuge tube, incubated for 10 min, and centrifuged. The supernatant is then assayed for phospholipid. As shown in Fig. 13, squalamine can solubilize both PC and PG MLVs at very high squalamine/phospholipid ratios. Here, PC is solubilized at lower squalamine concentrations than PG, in contrast to the leakage and electron microscopy results where LUVs containing PG are more sensitive to squalamine than PC vesicles.

4. Discussion

4.1. The lipid selectivity of squalamine

In this report, squalamine is shown to effect the leakage of small fluorescent molecules from LUVs prepared from both anionic and zwitterionic LUVs. Squalamine exhibits a distinct preference for anionic lipid. As seen in Table 1, squalamine causes the leakage of CF equally well from PG and PS LUVs, but less efficiently from CL LUVs. The binding constants reported in Table 1, however, were determined relative to total phospholipid concentration. If instead the binding constants were calculated relative to total lipid phosphate, the binding of squalamine to CL LUVs would approach the levels observed for PG and PS. The minimal selection between anionic phospholipids and the clearly weaker binding to PC LUVs suggests that an electrostatic interaction between the squalamine polyamine and lipid phosphate is a primary determinant of membrane binding.

The kinetics of CF leakage indicate a rapid initial phase, followed by a rapid decrease in dye leakage until no additional material leaks from the vesicles (Fig. 2). These biphasic leakage kinetics might be explained by an ‘all-or-none’ mechanism. Upon addition of squalamine, some of the vesicles totally empty their contents, while a second population is unaffected and retain all of their encapsulated dye. Fur-

ther experimental analysis of the leakage mechanism (Figs. 6 and 7) indicates that the leakage of fluorescent dyes induced by squalamine is graded in nature. The leakage mechanism was determined using only PG and PC vesicles, with the assumption that squalamine will interact with LUVs prepared from any anionic lipid in like manner. The graded leakage mechanism observed for both anionic and zwitterionic phospholipid must be accounted for in any mechanism explaining the action of squalamine on membranes.

The kinetic behavior of squalamine-induced CF leakage is reminiscent of that observed for the antimicrobial peptide magainin-2-amide. Upon addition to LUVs, magainin collects on the surface of lipid vesicles, aggregates, and inserts into the bilayer as a multimeric pore. The peptides comprising the transient pore relax and redistribute on both leaflets of the vesicle bilayer. After relaxation, the peptide concentrations on both sides of the bilayer are insufficient to generate new pores, and the dye leakage ceases [22]. While it is unlikely that squalamine interacts with LUVs in exactly the same manner as magainin, a general mechanism describing squalamine-induced dye leakage may consist of three similar steps: (1) membrane binding and aggregation, (2) pore formation, and (3) pore relaxation and dilution.

4.2. Squalamine decreases the size of LUVs

One of the unexpected observations with squalamine is that at squalamine/phospholipid ratios sufficient to cause CF leakage, squalamine creates size heterogeneity in vesicles. The average vesicular size decreases from about 95 nm in the absence of squalamine to less than 70 nm when the squalamine/phospholipid ratio exceeds 1/20 (Fig. 12). The decrease in vesicular size is observed both by electron microscopy and size exclusion chromatography (Fig. 9).

4.3. Is squalamine a detergent?

One interpretation for the mechanism of vesicular and cell lysis caused by squalamine is a detergent-like disruption of the phospholipid bilayer. Detergents have been proposed to disrupt phospholipid liposomes via a three-stage mechanism [23]. In stage I, detergent monomers are incorporated into phospholipid bilayers until the liposome is saturated, at which point mixed phospholipid–detergent micelles form and stage II begins. By stage III, the lamellar to micellar transition is complete, with no remaining liposomes and all of the phospholipids are found in mixed micelles. The three-stage mechanism has been used to understand the action of many detergents, including Triton X-100, octyl glucoside, and sodium cholate [24–29].

In some ways, the ability of squalamine to form holes in membranes resembles the interaction of simple detergents with lipid membranes. Entrapped CF is released from anionic lipid vesicles when added squalamine exceeds a critical squalamine/phospholipid molar ratio. The same behavior is observed in the interaction between egg yolk lecithin and either the bile salt surfactant sodium cholate [26] or the nonionic surfactant Triton X-100 [29,30]. Also, when squalamine is added to LUVs, the vesicular size decreases as a function of the squalamine/phospholipid molar ratio (Fig. 12). In the interaction between PC and sodium cholate, the formation of small vesicles is used to explain the appearance of an isotropic ^{31}P NMR resonance [26]. At high squalamine/phospholipid molar ratios, squalamine can solubilize both PG and PC from MLVs, behavior typical of a surfactant.

In other ways, squalamine does not exhibit detergent-like behavior. Squalamine is a hydrophobic steroid with polar substituents at positions 3, 7, and 24 on the steroid backbone. As a result, the separation of hydrophobic and hydrophillic regions in squalamine is difficult to define. Since no critical micelle concentration can be measured by methods used here, squalamine seems incapable of forming micelles in the absence of phospholipid. Squalamine does solubilize phospholipid, but only at very high squalamine/phospholipid ratios, much higher than required for dye leakage or reduction of average vesicular size.

The lipid selectivity of squalamine is also striking. The ability of squalamine to induce the leakage of dye encapsulated within PC LUVs is much different from that for LUVs prepared from anionic lipid. At higher PC concentrations, a population of CF resists leakage; this amount increases as a function of phos-

pholipid concentration. There is lipid selectivity in the squalamine-induced formation of small vesicles (where PG vesicles demonstrate size heterogeneity at lower squalamine/phospholipid ratios than for PC vesicles), and in squalamine-induced lipid solubilization (where PC is solubilized at lower squalamine concentrations than PG). To the best of our knowledge, only one study has previously examined the interaction of different lipids with a detergent (Triton X-100) [31]. While some solubility differences were noted, none were related to lipid charge.

4.4. Membrane disruption and antimicrobial activity

Squalamine is a potent antimicrobial agent, with minimum inhibitory concentrations (MICs) of less than 10 μM against most microorganisms [4]. In the experiments reported here, squalamine is studied at similar concentrations, but the LUVs suspensions are much more dilute than the cellular suspensions used in determining MICs. As a result, the lytic behavior observed with squalamine and LUVs may not relate to the antimicrobial activity of squalamine. Since squalamine does preferentially interact with anionic lipid, the selectivity of squalamine for prokaryotic over eukaryotic cells and the low hemolytic activity of squalamine indicates that squalamine selects for cellular surfaces based upon electrostatic interactions. Future studies in this laboratory will examine the interactions of squalamine to bacterial membranes, to see if squalamine can lyse bacterial membranes, and if so, to determine the concentration dependence of the lytic activity.

4.5. A model for squalamine-induced bilayer disruption

We hypothesize that squalamine first binds to the surface of phospholipid vesicles, where binding affinity is related to vesicle charge and the surface organization of the lipid. Binding is initially electrostatic in nature, and absolutely requires the presence of phospholipid. Squalamine is soluble in distilled water to concentrations of 20 mg/ml (21 mM), but is insoluble in 10 mM phosphate buffer. The solubility behavior suggests a strong, specific interaction between squalamine and phosphate, presumedly at the protonated polyamine nitrogens. In studies of other sterols the polar moieties have been shown to reside at the bilayer surface. For example, cholesterol 3-sulfate orients in zwitterionic membranes with the sulfate group at the bilayer surface. Cholesterol sulfate is a bilayer stabilizing agent, but in the presence of calcium ions destabilizes phosphatidylethanolamine bilayers [32]. Also, studies using a spin-labeled sterol, with a nitroxide added to the side chain in the approximate position of the squalamine sulfate group, suggest that the nitroxide is located at the surface of the membrane in close proximity with the choline *N*-methyl groups [33]. These studies suggest that negatively charged substituents attached to sterols prefer to sit at the surface of a bilayer, but might change their orientation and destabilize bilayers under appropriate conditions. Future squalamine studies in our laboratory will examine in more detail the effects of pH and phospholipid structure upon membrane binding and stability.

After binding, squalamine aggregates on the vesicle surface. The molecular nature of the aggregate is unknown at present, but is suggested in negative stain electron micrographs of squalamine-treated vesicles. Darkly staining patches are apparent, which might result from binding of the negatively charged molybdate stain to squalamine amine groups not tied to phospholipid phosphate, or from invaginations where the negative stain could accumulate. The aggregates create a defect in the membrane, allowing the release of vesicular contents with molecular weight of less than 10,000 g/mol. The antimicrobial peptide defensin generates a pore of similar size in its interaction with phospholipid membranes [17].

While the structure of the squalamine aggregate is unknown, we would suggest that the aggregate contains both squalamine and phospholipid. The antimicrobial peptide magainin has been demonstrated to include bound phospholipid in its pore structure [34]. The formation of small vesicles, and the ultimate solubilization of phospholipid by squalamine supports a association between aminosterol and phospholipid. Also, the aggregate is not stable in the membrane, and will relax to a different structure. However, the relaxation mechanism is somewhat different when squalamine is complexed to anionic lipid than when complexed to zwitterionic lipid. Squalamine, complexed with either anionic or zwitterionic lipid, will extract itself from LUVs in the form of small

vesicles. Vesicles containing squalamine and anionic lipid exist as such until sufficient squalamine is added to completely saturate all vesicles, at which time solubilization into squalamine/phospholipid mixed micelles occurs. Vesicles containing squalamine and zwitterionic lipid can act as a squalamine 'sponge', interacting with free squalamine and other squalamine/PC complexes, protecting PC vesicles with less bound squalamine and allowing them to retain more encapsulated dye.

In a recent report, an analog of squalamine was shown to act as an anion ionophore in PG membranes [35]. A model for an anion pore was proposed, where the positively charged amine groups in the analog line the channel and the more polar sterol interfaces with the lipid acyl chains. There is no evidence in our experiments which supports a model of this type. In particular, the size of the pore generated in the squalamine-induced lysis of membranes must be large enough to allow the passage of fluorescent dyes. For this squalamine analog, the membrane permeability was limited to small ions. In fact, the observation of ion transport using the squalamine analog depended upon the continued encapsulation of a pH-sensitive fluorescent dye within phospholipid vesicles. Although this analog possesses some of the structural elements of squalamine, the orientation of these elements and the structure of the sterol ring are much different. Finally, we have tested squalamine in both anionic and zwitterionic LUVs, and have found no evidence for ionophoric activity using the methodologies described in the unpublished report. [1] The antimicrobial mechanism for squalamine may be different from the antimicrobial mechanism for the squalamine analog.

Acknowledgements

The authors would like to thank Dr. William A. Kinney of Magainin Pharmaceuticals, for his kind gift of squalamine, and also Dr. John I. Williams of Magainin Pharmaceuticals for his critical reading of the manuscript.

[1] R. Smith, A. Frangiosi, L. Pedersen, and B.S. Selinsky, unpublished studies.

References

[1] H.G. Boman, Cell 65 (1991) 205–207.
[2] M. Zasloff, Curr. Opin. Immunol. 4 (1992) 3–7.
[3] W.L. Maloy, U.P. Kari, Biopolymers (Peptide Sci.) 37 (1995) 105–112.
[4] K.S. Moore, S. Wehrli, H. Roder, M. Rogers, J.N. Forrest Jr., D. McCrimmon, M. Zasloff, Proc. Natl. Acad. Sci. USA 90 (1993) 1354–1358.
[5] R.M. Moriarty, S.M. Tuladhar, L. Gao, S. Wehrli, Tetrahedron Lett. 35 (1994) 8103–8106.
[6] E. Grant Jr., T.J. Beeler, K.M.P. Taylor, K. Gable, M.A. Roseman, Biochemistry 31 (1992) 9912–9918.
[7] K. Matsuzaki, M. Harada, S. Funakoshi, N. Fujii, K. Miyajima, Biochim. Biophys. Acta 1063 (1991) 162–170.
[8] A. Vaz Gomes, A. de Waal, J.A. Berden, H.V. Westerhoff, Biochemistry 32 (1993) 5365–5372.
[9] W.C. Wimley, M.E. Selsted, S.H. White, Protein Sci. 3 (1994) 1362–1373.
[10] K. Matsuzaki, O. Murase, H. Tokuda, S. Funakoshi, N. Fujii, K. Miyajima, Biochemistry 33 (1994) 3342–3349.
[11] P.I. Lelkes, in: G. Gregoriadis (Ed.), Liposome Technology, Vol. III: Targeted Drug Delivery and Biological Interaction, CRC Press, Boca Raton, FL, 1984, pp. 225–246.
[12] R.C. MacDonald, R.I. MacDonald, B.Ph.M. Menco, K. Takeshita, N.K. Subbarao, L.-R. Ju, Biochim. Biophys. Acta 1061 (1991) 297–303.
[13] G.R. Bartlett, J. Biol. Chem. 234 (1959) 466–468.
[14] K. Matsuzaki, M. Harada, T. Handa, S. Funakoshi, N. Fujii, H. Yajima, K. Miyajima, Biochim. Biophys. Acta 981 (1989) 130–134.
[15] G. Schwartz, R.-T. Zong, T. Popescu, Biochim. Biophys. Acta 1110 (1986) 97–104.
[16] A.S. Ladokhin, W.C. Wimley, S.H. White, Biophys. J. 69 (1995) 1964–1971.
[17] A.S. Ladokhin, M.E. Selsted, S.H. White, Biophys. J. 72 (1997) 1762–1766.
[18] Y. Moroi, Micelles: Theoretical and Applied Aspects, Plenum, New York, NY, 1992, pp. 211–213.
[19] H. Schott, J. Phys. Chem. 70 (1966) 2966–2973.
[20] M.P. Bohrer, W.M. Deen, C.R. Robertson, J.L. Troy, B.M. Brenner, J. Gen. Physiol. 74 (1979) 583–593.
[21] A. Helenius, D.R. McCaslin, E. Fries, C. Tanford, Meth. Enzymol. 56 (1979) 734–749.
[22] K. Matsuzaki, O. Murase, N. Fujii, K. Miyajima, Biochemistry 34 (1995) 6521–6526.
[23] D. Lichtenberg, R.J. Robson, E.A. Dennis, Biochim. Biophys. Acta 737 (1983) 285–304.
[24] M.L. Jackson, C.F. Schmidt, D. Lichtenberg, B.J. Litman, A.D. Albert, Biochemistry 21 (1982) 4576–4582.
[25] S. Almog, T. Kushnir, S. Nir, D. Lichtenberg, Biochemistry 25 (1986) 2597–2605.
[26] R. Schubert, K. Beyer, H. Wolburg, K.-H. Schmidt, Biochemistry 25 (1986) 5263–5269.
[27] F.M. Goni, M.-A. Urbaneja, J.L.R. Arrondo, A. Alonso, A.A. Durrani, Eur. J. Biochem. 160 (1986) 659–665.

[28] M.-T. Paternostre, M. Roux, J.-L. Rigaud, Biochemistry 27 (1988) 2668–2677.
[29] Y. Liu, S.L. Regen, J. Am. Chem. Soc. 115 (1993) 708–713.
[30] J. Ruiz, F.M. Goni, A. Alonso, Biochim. Biophys. Acta 937 (1988) 127–134.
[31] M.A. Urbaneja, J.L. Nieva, F.M. Goni, A. Alonso, Biochim. Biophys. Acta 904 (1987) 337–345.
[32] J.J. Cheetham, R.J.B. Chen, R.M. Epand, Biochim. Biophys. Acta 1024 (1990) 367–372.
[33] S. Banerjee, G.K. Trivedi, S. Srivastave, R.S. Phadke, Steroids 59 (1994) 377–382.
[34] K. Matsuzaki, O. Murase, N. Fujii, K. Miyajima, Biochemistry 35 (1996) 11361–11368.
[35] D. Deng, T. Dewa, S.L. Regen, J. Am. Chem. Soc. 118 (1996) 8975–8976.