447-475, Academic Press, New York.
Srivastava, S., Phadke, R. S., & Govil, G. (1984) *Indian J. Chem. 23*, 1148-1153.
States, D. J., Haberkorn, R. A., & Ruben, D. J. (1982) *J. Magn. Reson. 48*, 286-292.
Stockton, G. W., Polnaszek, C. F., Tulloch, A. P., Hasan, F., & Smith, I. C. P. (1976) *Biochemistry 15*, 954-966.
Tallon, M. A., Shenbagamurthi, P., Marcus, S., Becker, J. M., & Naider, F. (1987) *Biochemistry 26*, 7767-7774.
Thayer, A. M., & Kohler, S. J. (1981) *Biochemistry 20*, 6831-6834.
Thorner, J. (1980) in *Molecular Genetics of Development: An Introduction to Recent Research on Experimental Systems* (Leighton, T. J., & Loomis, W. A., Jr., Eds.) pp 119-117, Academic Press, New York.
Wakamatsu, K., Okada, A., Suzuki, M., Higashijima, T., Masui, Y., Sakakibara, S., & Miyazawa, T. (1986) *Eur. J. Biochem. 154*, 607-615.
Wakamatsu, K., Okada, A., Miyazawa, T., Masui, Y., Sakakibara, S., & Higashijima, T. (1987) *Eur. J. Biochem. 163*, 331-338.
Wüthrich, K. (1984) *Biomed. Res., Suppl. 5*, 151-160.
Wüthrich, K., Billeter, M., & Braun, W. (1984) *J. Mol. Biol. 180*, 715-740.
Zuiderweg, E. R. P., Kaptein, R., & Wüthrich, K. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 5837-5841.

Quantitative Fluorescence Measurement of Chloride Transport Mechanisms in Phospholipid Vesicles†

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ABSTRACT: A quantitative fluorescence assay has been developed to measure Cl flux across liposomal membranes for use in chloride transporter reconstitution studies. A Cl-sensitive fluorophore [6-methoxy-*N*-(3-sulfopropyl)quinolinium; SPQ] was entrapped into phospholipid/cholesterol liposomes formed by bath sonication, high-pressure extrusion, and detergent dialysis. Liposomes containing entrapped SPQ were separated from external SPQ by passage down a Sephadex G25 column. There was <10% leakage of SPQ from liposomes in 8 h at 4 °C and in 2 h at 23 °C. Cl influx (J_{Cl} in millimolar per second or nanomoles per second per centimeter squared) was determined from the time course of SPQ fluorescence, measured by cuvette or stopped-flow fluorometry, in response to inward Cl gradients. In 90% phosphatidylcholine/10% cholesterol liposomes at 23 °C, J_{Cl} in response to a 50 mM inward Cl gradient was 0.06 ± 0.01 mM·s^{-1} (SD, n = 3) in the absence and 0.27 ± 0.02 mM·s^{-1} in the presence of a K/valinomycin voltage clamp (0 mV), showing that the basal Cl "leak" is conductive; J_{Cl} increased (1.7 ± 0.1)-fold in the presence of a 60-mV inside-positive diffusion potential. Accuracy of chloride influx rates determined by the SPQ method was confirmed by measurement of ^{36}Cl uptake. In liposomes voltage-clamped to 0 mV, J_{Cl} was linear with external [Cl] (0-100 mM), independent of pH gradients, and strongly dependent on temperature (activation energy 18 ± 1 kcal/mol, 12-42 °C) as predicted for channel-independent Cl diffusion. To test this method for measurement of rapid Cl transport rates, liposomes were reconstituted with the Cl/OH exchanger tributyltin. Tributyltin incorporation gave rapid, pH gradient driven Cl influx (J_{Cl} = 13 mM·s^{-1}, no pH gradient; 24 mM·s^{-1}, 1.5-unit pH gradient). These results establish a rapid and accurate method for measurement of Cl influx in liposomes suitable for reconstitution studies.

Purification and physical characterization of chloride transport proteins require a quantitative functional assay for chloride permeability across membranes reconstituted with candidate chloride transporters. The transport assay should ideally (a) have good time resolution, (b) require small quantities of transport protein, (c) be sufficiently rapid for the screening of multiple samples, and (d) be applicable for measurement of neutral and conductive chloride transport. For example, the half-time for chloride equilibration for a single continuously open, 50-pS chloride channel in a 0.2-μm-diameter liposome in 50 mM Cl would be ~10 ms in the absence of a potential gradient (Fong et al, 1988). Standard ^{36}Cl uptake methods (Karniski & Aronson, 1985) or chloride-sensitive electrode methods (Dubinski & Monti, 1986) are inadequate to measure this very rapid chloride transport, particularly if a limited amount of chloride transport protein is available.

We report here a simple fluorescence assay for chloride transport in liposomes based on the use of the entrapped chloride-sensitive fluorophore 6-methoxy-*N*-(3-sulfopropyl)-quinolinium (SPQ). SPQ has peak excitation and emission wavelengths of 350 and 445 nm, respectively. SPQ fluorescence is quenched by chloride by a collisional mechanism with a response time for changes in chloride concentration of under 1 ms (Illsley & Verkman, 1987). SPQ fluorescence is not altered by pH, bicarbonate, sulfate, nitrate, phosphate, or cations. The method gives a continuous record of chloride

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influx across liposomal membranes and is suitable for measurement of chloride transients of <5 ms. Measurement of chloride transport in liposomes containing SPQ was validated quantitatively by parallel measurements of fluorescence and ^{36}Cl uptake in liposomes. Studies of the dependence of Cl transport on membrane potential, temperature, and pH gradients showed that Cl moves across liposome membranes by a simple conductive mechanism. Effects of liposome cholesterol content and method of preparation were examined to minimize the basal Cl leak for reconstitution studies. The SPQ method was applied to study the Cl transport mechanism in liposomes reconstituted with the Cl/OH (H/Cl) ionophore tributyltin.

Materials and Methods

Materials. SPQ was synthesized as described by Krapf et al. (1988b) and recrystallized 3 times from 1:1 methanol/water. $H^{36}Cl$ was obtained from New England Nuclear (Bedford, MA). Tributyltin chloride was purchased from Aldrich Chemical Co. (Milwaukee, WI). Egg phosphatidylcholine (PC), cholesterol, and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Sephadex G-25M exclusion columns were purchased from Pharmacia (Piscataway, NJ). For ^{36}Cl uptake measurements, a column (Kontes D-420161; San Leandro, CA) containing Dowex-1-chloride, mesh 50–100 (Sigma), was used.

Liposome Preparation. Liposomes were prepared by three procedures that have been used for protein reconstitution studies. The first is a modification of a *bath sonication* procedure (Carruthers & Melchior, 1983; Kasahara & Hinkle, 1977). Phospholipids and cholesterol at specified mole ratios were mixed in chloroform and dried under an N_2 stream. The mixture was dissolved in 2 mL of diethyl ether, dried, and resuspended in 2 mL of a chloride-free "loading buffer" containing 10 mM SPQ. The lipid suspension was sonicated for 10–15 min in a bath sonicator (Laboratory Supply Co., Hicksville, NY) at 23 °C under flowing N_2. External SPQ was removed by passage of liposomes down a G-25M exclusion column; the first 10 drops of cloudy eluate were pooled for subsequent experiments. Electron micrographs of sonicated liposomes, obtained by the negative staining technique, showed a mean liposome diameter of 0.2 μm, similar to that reported previously (Kasahara & Hinkle, 1977).

For preparation of liposomes by *extrusion* (Hope et al., 1985; Mayer et al., 1985, 1986), dried lipid was suspended in the loading buffer at a concentration of 10 mg/mL. Lipids were subject to 5 freeze–thaw cycles and 10 passages through a 200-nm Nucleopore filter (Pleasanton, CA) at 200–400 psi using a commercial extruder (Lipex Biomembranes, Inc., Vancouver, British Columbia, Canada). External SPQ was removed by exclusion chromatography as above. The diameter of liposomes extruded through a 200-nm filter was reported to be 151 nm as determined by freeze–fraction electron microscopy (Mayer et al., 1986). For preparation of liposomes by *detergent dialysis*, lipid was suspended in loading buffer containing 1% NP-40 detergent (Nonident P-40, Sigma) at a concentration of 5 mg/mL. Detergent was removed by stirring the suspension with 0.35 mg/mL Bio-Beads SM-2, prepared as described by Holloway (1973), for 3 h at 4 °C. Liposomes were decanted and subjected to exclusion chromatography. The majority of liposomes prepared by this method had diameters of 100–300 nm (Weigele & Barchi, 1982).

Fluorescence Measurements. Cuvette fluorometry measurements were performed on an SLM 48000 fluorometer (Urbana, IL) interfaced to an IBM PC/AT computer. Fluorescence was excited by a single monochromator at 350 nm (8-nm band-pass) in series with a Schott UG-1 black glass filter (Duryea, PA), and measured through a Schott GG420 cuton filter. Experiments were performed by using acrylic cuvettes (Sarstadt, FGR) placed in a thermostated cuvette holder. Solutions were stirred continuously with a Teflon-coated magnetic stirring bar. The mixing time was under 1 s. All solutions were filtered through 0.22-μm HAWP Millipore filters (Bedford, MA) to remove dust particles.

Chloride influx experiments were initiated by addition of 200 μL of a liposome suspension (0.5 mM phospholipid) to 2 mL of an isoosmotic, isotonic buffer containing chloride to give specified ion and/or pH gradients. Absolute rates of chloride influx (J_{Cl}) in units of millimolar per second were obtained by the fluorescence time course using a two-point calibration method as described previously (Chen et al, 1988). The fluorescence corresponding to zero chloride (F_i) was that measured immediately after suspension of liposomes. The fluorescence corresponding to the external chloride concentration (F_s) was obtained at the end of every experiment by addition of ~20 μM tributyltin, which caused rapid equilibration of internal and external chloride. Tributyltin did not itself alter the fluorescence signal. Complete chloride equilibration after tributyltin addition was evidenced by the insensitivity of the fluorescence signal to addition of 0.01% Triton X-100, which caused liposome lysis and thus ensured complete equilibration of internal and external chloride activity.

J_{Cl} was calculated from the measured initial slope of the fluorescence decrease [$dF(0)/dt$], F_i, and F_s, and the known final chloride concentration $[Cl]_s$ from the equation:

$$J_{Cl} = [(F_i - F_s)(K + 1/[Cl]_s)]^{-1}[dF(0)/dt] \quad (1)$$

where K is the Stern–Volmer constant for SPQ quenching by chloride (60 M^{-1} at 150 mM internal gluconate; Chen et al., 1988). Equation 1 is valid when the internal chloride concentration is zero initially. To determine J_{Cl} in units of nanomoles per second per centimeter squared, J_{Cl} in units of millimolar per second was divided by the liposome surface to volume ratio (3×10^5 cm^{-1}).

Stopped-Flow Experiments. Rapid kinetic measurements were performed at 23 °C on a Hi-Tech SF51 stopped-flow apparatus (Wiltshire, England) interfaced to a Digital Equipment Corp. MINC/23 computer (Maynard, MA). The instrument dead time was <2 ms. Samples were illuminated by using a stabilized 100-W tungsten–halogen light source in series with a 360 ± 10 nm six-cavity interference filter (Brattleboro, VT) and a Schott KG-3 infrared blocking filter. Fluorescence was detected through two Schott GG420 nm cuton filters. The maximum rate of data acquisition was 10 points/ms.

Stopped-flow experiments were performed by mixing 0.075 mL of a liposome suspension (100 μM PC/10 μM cholesterol) containing entrapped SPQ with an equal volume of buffer containing chloride. The time course of SPQ fluorescence was used to calculate rates of chloride influx as described above. The total quenchable signal ($F_i - F_s$) was determined from the total change in fluorescence following mixture of liposomes with 150 mM KSCN, which quenches >99% of SPQ fluorescence (Illsley & Verkman, 1987).

Radioactive Chloride Uptake Measurements. ^{36}Cl uptake was measured at 23 °C by a modification of the ion-exchange method of Gasko et al. (1976). Polypropylene columns plugged with porous polyethylene disks were packed with the strongly basic anion-exchange resin Dowex-1-chloride and washed with distilled water, 1 M NaOH, 1 M formic acid, and distilled water until the pH was between 4 and 5. Columns

were washed with 10 mL of 150 mM potassium gluconate/10 mM HEPES, pH 7.5, immediately before use.

Uptake was initiated by mixing 200 μL of liposomes (200 μM PC/20 μM cholesterol) containing 100 mM NMG gluconate, 50 mM potassium gluconate, 10 mM HEPES, and 10 mM SPQ, pH 7.5 (with or without 20 μg of valinomycin), with 200 μL of 100 mM NMG gluconate, 25 mM KCl, 25 mM $K^{36}Cl$, and 10 mM HEPES, pH 7.5. For uptake measurements with reaction times under 60 s, the mixture was held by capillary action in a pipet tip fitted into the top of the column. For time points >60 s, the reaction took placed in a separate vessel. After incubation, the reaction mixture was sucked through the column by application of a vaccum; the column was washed immediately with 10 mL of 150 mM potassium gluconate/10 mM HEPES, pH 7.5. Eluate samples were mixed with a scintillant (Aquasol, New England Nuclear) and counted in a scintillation counter (Model 1500; Packard Instrument Co., Downers Grove, IL). ^{36}Cl uptake was normalized for recovery of PC through the column (typically >90%) by phosphate assay (Gomori, 1942). The zero-time ^{36}Cl uptake, representing nonspecific ^{36}Cl binding to liposomes, was subtracted from all values. Chloride uptake was expressed in millimolar from the product of the external chloride concentration (50 mM) and the fraction of ^{36}Cl uptake (in cpm) at specified times relative to that at 1 h.

Results

For previous studies in isolated membrane vesicles and intact cells, SPQ has been loaded by prolonged incubation at low temperatures (24 h at 4 °C) or by more rapid incubation at higher temperature (30 min at 37 °C) (Chen et al, 1988; Krapf et al., 1988a). Under these conditions, there was incomplete equilibration between external and internal SPQ; however, there was sufficient internal SPQ to make the fluorescence measurements of chloride transport. For liposome experiments, entrapment of SPQ during the liposome formation procedure was most efficient.

SPQ (10 mM) was added to the buffer used to suspend the dried phospholipid/cholesterol mixtures for entrapment into liposomes. External SPQ was removed effectively by a single passage down an exclusion column (see Materials and Methods) in under 1 min. Compared with washing methods requiring multiple centrifugations, the column method was more rapid and less traumatic to liposome integrity. Once entrapped in liposomes prepared by any of the three procedures, SPQ is very impermeant. On the basis of measurements of the total quenchable signal ($F_i - F_s$, see eq 1) as a function of time following the column separation procedure, SPQ leakage was <10% in 8 h at 4 °C or in 2 h at 23 °C (three samples tested). The leak rate was not affected by ionic composition. The lower leak rate of SPQ in liposomes than in membrane vesicles or in intact proximal tubule cells (Chen et al., 1988; Krapf et al., 1988a) may be due to a lower surface-to-volume ratio in liposomes and to lack of proteins which may provide a nonspecific route for SPQ exit. In recent experiments using cultured epithelial cells and fibroblasts (Verkman et al., 1989), SPQ leaked out <10% in 60 min at 37 °C, suggesting that the surface-to-volume ratio is the major determinant of leakage rate.

To confirm the accuracy of chloride influx measurement in liposomes by SPQ fluorescence, parallel fluorescence and ^{36}Cl uptake measurements were performed (Figure 1). In response to a 50 mM inward chloride gradient, there was a slow time course of decreasing SPQ fluorescence due to chloride influx. The initial rate of chloride influx was enhanced (4.4 ± 0.3)-fold (SD, n = 3) with addition of valinomycin,

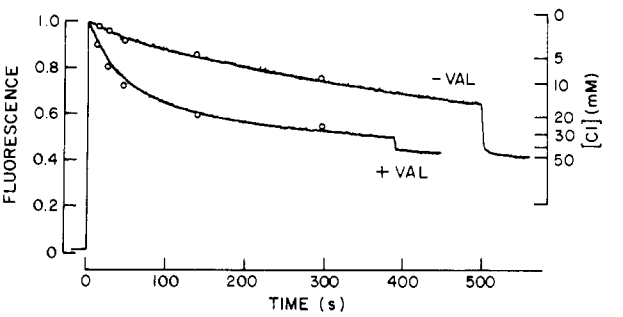

FIGURE 1: Time course of chloride influx into liposomes measured by SPQ fluorescence and ^{36}Cl uptake. Liposomes prepared by bath sonication (final concentrations 50 μM PC and 5 μM cholesterol) containing 100 mM NMG gluconate, 50 mM potassium gluconate, 10 mM HEPES/Tris, and 10 mM SPQ with and without 5 μg of valinomycin were suspended in an isoosmotic, isotonic buffer at 23 °C in which 50 mM gluconate was replaced by Cl. The time course of SPQ fluorescence (solid curves) was monitored and averaged over 1-s time intervals as described under Materials and Methods. Near the completion of the fluorescence experiments, tributyltin was added to equilibrate internal and external Cl. The scale on the right-hand ordinate was calculated from the positions of 0 mM Cl (fluorescence intensity at zero time) and 50 mM Cl (fluorescence intensity after tributyltin addition), and the calibration curve for SPQ quenching by Cl (Chen et al, 1988). Parallel ^{36}Cl uptake measurements were performed (open circles) as described under Materials and Methods. ^{36}Cl uptake results were plotted on the same scale as SPQ fluorescence data to compare uptake rates directly.

suggesting that much of the chloride influx occurs by a conductive route. Upon addition of the chloride ionophore tributyltin, there was rapid equilibration of external and internal chloride. The right-hand axis shows the calculated internal chloride concentrations corresponding to measured fluorescence values. ^{36}Cl uptake values for data obtained in the presence and absence of valinomycin are plotted on this scale. The ^{36}Cl uptake data are in close agreement with chloride uptake determined from SPQ fluorescence measurements.

The physical characteristics of the chloride pathway in liposomes were examined from the dependence of J_{Cl} on valinomycin concentration, membrane potential, chloride concentration, pH gradients, and temperature. In these studies, liposomes consisting of 90% PC/10% cholesterol were prepared by the bath sonication procedure. When the experimental conditions given in the legend to Figure 1 were used, J_{Cl} measured at valinomycin concentrations of 0, 1, 2.5, and 5 μg/mL was (in $mM \cdot s^{-1}$) 0.06 ± 0.01, 0.19 ± 0.01, 0.27 ± 0.02, and 0.27 ± 0.03 (SD, n = 2). These results indicate that the major route for chloride influx into liposomes occurs by a conductive mechanism and establish the concentration range of valinomycin required to produce an adequate voltage clamp for elimination of diffusion potentials. To show directly that J_{Cl} is voltage-driven, J_{Cl} was measured in response to a 60-mV inside positive membrane potential. For these experiments, liposomes containing 250 mM NMG gluconate, 25 mM potassium gluconate, 10 mM HEPES/Tris, 10 mM SPQ, and valinomycin were suspended in an isotonic buffer containing 25 mM Cl and 25 mM K (0 mV) or 250 mM K (+60 mV). J_{Cl} increased from 0.15 ± 0.01 $mM \cdot s^{-1}$ (SD, n = 3) to 0.25 ± 0.02 $mM \cdot s^{-1}$ for a 60-mV internal positive diffusion potential. This (1.7 ± 0.1)-fold increase in J_{Cl} is in agreement with the 1.6-fold increase in J_{Cl} predicted by the Goldman equation for chloride transport by a single ion conductance mechanism.

The dependence of J_{Cl} on the magnitude of the inward chloride gradient is shown in Figure 2, top. Experiments were performed using a K/valinomycin voltage clamp at 0 mV. There was no change in SPQ fluorescence in the absence of

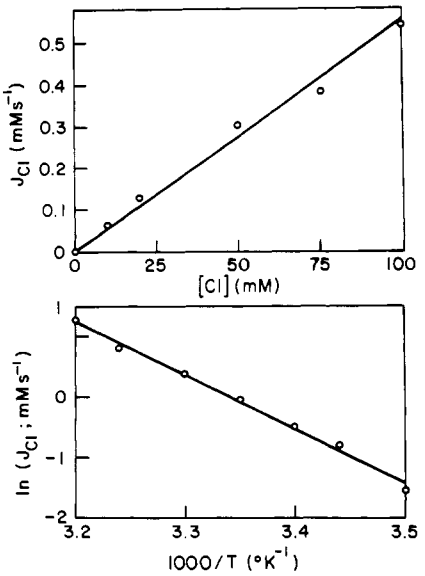

FIGURE 2: Characteristics of Cl transport across liposomal membranes. (Top) Experiments were performed as in Figure 1 in the presence of valinomycin for several inward Cl gradients. J_{Cl} values were calculated from the fluorescence data by using eq 1. Each point is the average of triplicate determinations. (Bottom) Arrhenius plot for Cl transport. J_{Cl} was measured in voltage-clamped liposomes for a 50 mM inward Cl gradient at varying temperatures. Measurements were performed in triplicate. The fitted activation energy was 18 ± 1 kcal/mol.

external chloride. J_{Cl} increased linearly with increasing external chloride concentration, as expected for a simple diffusional leak pathway. In response to a 1.5 pH unit inward H gradient (external pH 6.0), J_{Cl} did not change significantly (0.27 ± 0.02 mM/s, − gradient, vs 0.29 ± 0.03 mM/s, + gradient), showing little contribution of H/Cl cotransport to the basal Cl leak in liposomes. Figure 2, bottom, shows the temperature dependence of J_{Cl} measured in the presence of a K/valinomycin voltage clamp. The data fit well to a single activation energy of 18 ± 1 kcal/mol, similar to the activation energies measured for transport of water and nonelectrolytes across pure lipid bilayers (Finkelstein, 1976; Fettiplace & Haydon, 1980).

To demonstrate the application of the SPQ fluorescence method for measurement of chloride flux in liposomes containing a rapid chloride transporter, a low concentration (5 nM) of tributyltin was added to liposomes. Chloride influx was measured by stopped-flow fluorescence (Figure 3). In response to addition of 150 mM KSCN in the presence of valinomycin, there was rapid quenching of SPQ over a 5-s time course. Because >99% of the SPQ fluorescence is quenched under these conditions, the amplitude of this signal represents the total signal from SPQ entrapped in the liposomes. Chloride influx was very slow in the absence of tributyltin and valinomycin (J_{Cl} = 0.07 mM/s). In the presence of tributyltin, J_{Cl} was 13 mM/s, which increased to 24 mM/s in the presence of a 1.5 pH unit inward H gradient. These results demonstrate that rapid chloride influx in liposomes is measurable by stopped-flow SPQ fluorescence and that tributyltin functions, at least in part, as a Cl/OH (or H/Cl) transporter.

Experiments were performed to examine whether J_{Cl} in the absence of ionophore could be decreased by changing the method of liposome preparation or the liposome composition. A minimal Cl leak in liposomes is desirable for protein reconstitution studies so that the incremental Cl flux due to a functional transport protein is well above the base line. As described under Materials and Methods, liposomes were prepared by bath sonication, extrusion, and detergent dialysis.

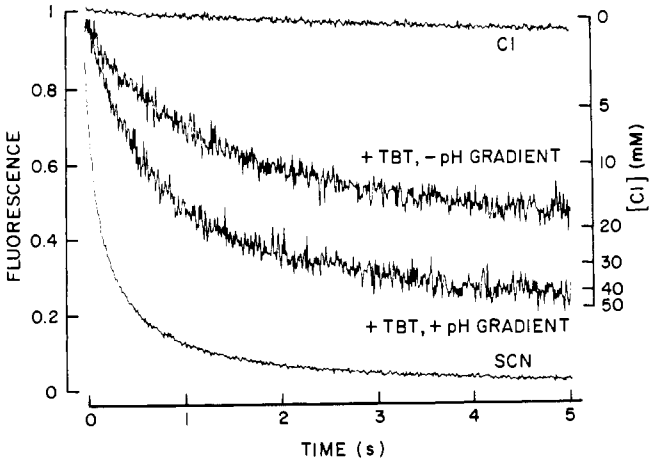

FIGURE 3: Chloride influx into liposomes reconstituted with tributyltin. Liposomes (100 μM PC/10 μM cholesterol) prepared by bath sonication and containing 100 mM NMG gluconate, 50 mM potassium gluconate, 10 mM HEPES/Tris, and 10 mM SPQ, pH 7.5, were subjected to a 50 mM inward Cl gradient in a stopped-flow apparatus as described under Materials and Methods. The time course of SPQ fluorescence is shown. The curve labeled "Cl" was obtained in the absence of valinomycin and tributyltin. "+TBT" indicates inclusion of 5 nM tributyltin in the liposome suspension. "+pH gradient" indicates that the outside pH was 6.0, giving a 1.5 pH unit inward H gradient. The "SCN" curve was obtained by mixing liposomes containing 10 μg of valinomycin (but no tributyltin) with KSCN to give a final SCN concentration of 150 mM. Curves obtained in the absence of tributyltin represent the average of 10 experiments; to show the signal-to-noise ratio of these stopped-flow studies, curves obtained in the presence of tributyltin represent a single measurement. The concentration scale on the right-hand ordinate was calculated by using a Stern–Volmer constant for SPQ quenching by Cl of 60 M^{-1}. Calculated J_{Cl} values are given in the text.

J_{Cl} measured in voltage-clamped liposomes consisting of 90% PC/10% cholesterol in response to a 50 mM inward Cl gradient was 0.26 ± 0.02 mM/s (n = 2, bath sonication), 0.17 ± 0.02 mM/s (n = 3, extrusion), and 0.22 ± 0.01 mM/s (n = 2, detergent dialysis). These results indicate similar passive Cl permeabilities in liposomes prepared by very dissimilar procedures; Cl permeability was lowest in the liposomes prepared by extrusion.

The effects of cholesterol, which decreases liposome fluidity, and phosphatidylserine (PS), which gives liposomes a negative surface charge, on J_{Cl} were measured. Liposomes were prepared by extrusion to minimize the influence of liposome composition on liposome size. In PC liposomes containing 0, 15, 30, 40, and 45 mol % cholesterol and subject to a 50 mM inward Cl gradient under voltage-clamp conditions, J_{Cl} was 0.23, 0.19, 0.14, 0.09, and 0.07 mM/s, respectively (n = 2). In liposomes consisting of 30% PS/70% PC, J_{Cl} was 0.22 mM/s (n = 2).

Discussion

The results presented here establish the application of SPQ fluorescence for quantitative measurement of chloride flux into liposomes. A single stopped-flow measurement of fluorescence provides a continuous record of intraliposomal chloride concentration. Because SPQ responds to changes in chloride concentration in <1 ms, very rapid rates of chloride influx are measurable. Parallel assessment of chloride influx into liposomes by fluorescence and by ^{36}Cl uptake showed that SPQ fluorescence reports chloride influx rates accurately. In previous reports in biological membrane vesicles (Chen et al, 1988) and in red cells (Illsley & Verkman, 1987), it was shown that SPQ did not itself alter rates of passive or protein-mediated chloride transport.

Chloride transport mechanisms can be examined from the dependence of initial chloride flux on gradients of pH (e.g., Cl/OH exchange) or ion concentration (e.g., Na/Cl cotransport), or on induced membrane potentials established by K gradients in the presence of valinomycin (e.g., Cl conductance). Multi-ion-coupled chloride transport mechanisms can be studied by use of multiple simultaneous gradients. In basolateral membrane vesicles from renal cortex, the presence of a $NaHCO_3$/Cl countertransport system was established by demonstration of chloride influx driven by simultaneous outward gradients of Na and HCO_3 (Chen & Verkman, 1988). The high sensitivity and nonlinear response characteristics of SPQ fluorescence to changes in chloride concentration (50% fluorescence quenching at 8 mM Cl) make it possible to measure initial rates of chloride influx, well before ion or pH gradients have dissipated. Under some circumstances, such as with very rapid chloride influx measured by using high Cl concentrations, it may be desirable to use a chloride-sensitive fluorescent indicator with lower sensitivity to chloride (e.g., 50% fluorescence quenching at 10–50 mM Cl). A number of suitable indicators have been synthesized and characterized recently (Krapf et al., 1988b). Many of these indicators have a lower octanol/water partition coefficient than SPQ, assuring a very low leakage rate after entrapment in liposomes.

The effects of valinomycin and induced membrane potential on chloride influx in liposomes indicate that the major "leak" pathway for chloride is conductive in nature; the low Cl permeability of liposomes in the absence of valinomycin (Figure 1) probably represents Cl influx by parallel K and Cl, and H/OH and Cl, conductances. The lack of stimulation of Cl influx by an inward H gradient suggests that H/Cl cotransport is minimal in these experiments. In 90% PC/10% cholesterol liposomes prepared by bath sonication, the chloride permeability coefficient (P_{Cl}) was 1.3×10^{-8} cm/s under voltage-clamp conditions. This value is ~40-fold greater than P_{Cl} or P_{Na} measured in planar bilayers (Cass et al., 1970).[1] The reason for this difference is not known. The small curvature of a 100–200-nm liposome compared to a planar bilayer or small differences bilayer liposome composition could account for this 40-fold difference. There may be other strategies not tested here to decrease further the passive Cl conductance of liposomes.

The low rate of neutral chloride leak makes the detection of an additional neutral chloride pathway inserted by the reconstitution procedure easy. If a voltage clamp is not used in the reconstitution assay, it is important to demonstrate that the incremental chloride flux due to reconstitution is neutral and not coupled electrically to gradients of a counterion. This can be accomplished by showing that the incremental chloride flux is inhibited specifically, or by showing independence of membrane potential (by use of a potentiometric indicator) on chloride gradients. For reconstitution of a conductive chloride transporter (e.g., Cl channel), an adequate voltage clamp is required to eliminate the development of diffusion potentials which would impede chloride transport. Under these conditions, it is desirable to minimize the conductive leak in liposomes so that the incremental chloride conductance due to reconstituted proteins is measurable. Addition of cholesterol to the phospholipid and use of the extrusion method of liposome preparation result in a partial reduction of passive Cl permeability. For transporter reconstitution studies, the lipid environment is an important determinant of both liposome chloride leak and transport activity, making it necessary to screen a series of lipids or lipid combinations to optimize functional studies.

The results presented for liposomes reconstituted with tributyltin show that the incremental chloride permeability conferred by a reconstituted chloride transport system is measurable directly by SPQ fluorescence. The half-times for chloride transport in the presence of tributyltin in Figure 3 were ~1 s. With increasing concentrations of tributyltin, half-times of under 10 ms were measurable. Therefore, the fluorescence method described here is suitable for measurement of rapid chloride transport rates on liposomes conferred by chloride channels or carriers.

Registry No. SPQ, 119971-42-5; Cl^-, 16887-00-6; cholesterol, 57-88-5.

[1] The maximal conductance of planar lipid bilayers in 0.1 M NaCl is 10^{-7} S·cm^{-2} (Cass et al, 1970). If this is ascribed entirely to either Na or Cl, then the Na or Cl flux in the absence of a potential becomes 10^{-4} μEq·cm^2·h^{-1} (Linderholm, 1951; Schultz & Zalusky, 1963), corresponding to a permeability coefficient of 3×10^{-10} cm·s^{-1}.

References

Carruthers, A., & Melchior, D. L. (1983) *Biochemistry 22*, 5795–5807.

Cass, A., Finkelstein, A., & Krespi, V. (1970) *J. Gen. Physiol. 56*, 109–124.

Chen, P.-Y., & Verkman, A. S. (1988) *Biochemistry 27*, 655–660.

Chen, P.-Y., Illsley, N. P., & Verkman, A. S. (1988) *Am. J. Physiol. 254*, F114–F120.

Dubinsky, W. P., & Monti, L. B. (1986) *Am. J. Physiol. 251*, C713–C720.

Fettiplace, R., & Haydon, D. A. (1980) *Physiol. Rev. 60*, 510–550.

Finkelstein, A. (1976) *J. Gen. Physiol. 68*, 127–135.

Fong, P., Illsley, N. P., Widdicombe, J., & Verkman, A. S. (1988) *J. Membr. Biol. 104*, 233–239.

Gasko, O. P., Knowles, A. F., Schertzer, H. G., Suolinni, E. M., & Racker, E. (1976) *Anal. Biochem. 72*, 57–65.

Gomori, G. (1942) *J. Lab. Clin. Med. 27*, 955–960.

Holloway, P. W. (1973) *Anal. Biochem. 53*, 304–308.

Hope, M. J., Bally, M. B., Webb, G., & Cullis, P. R. (1985) *Biochem. Biophys. Acta 812*, 55–65.

Illsley, N. P., & Verkman, A. S. (1987) *Biochemistry 26*, 1215–1219.

Karniski, L. P., & Aronson, P. S. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6362–6385.

Kasahara, M., & Hinkle, P. C. (1977) *Biochemistry 16*, 7384–7390.

Krapf, R., Berry, C. A., & Verkman, A. S. (1988a) *Biophys. J. 53*, 955–962.

Krapf, R., Illsley, N. P., Tseng, H. C., & Verkman, A. S. (1988b) *Anal. Biochem. 169*, 142–150.

Linderholm, H. (1952) *Acta Physiol. Scand. 27*, 1–100.

Mayer, L. D., Hope, M. J., Cullis, P. R., & Janoff, A. S. (1985) *Biochim. Biophys. Acta 817*, 193–196.

Mayer, L. D., Hope, M. J., & Cullis, P. R. (1986) *Biochim. Biophys. Acta 858*, 161–168.

Schultz, S. G., Zalusky, R., & Gass, A. E. (1964) *J. Gen. Physiol. 48*, 375–388.

Verkman, A. S., Sellers, M., Ketchum, R., Widdicombe, J. H., & Chao, A. C. (1989) *Kidney Int. 35*, 492.

Weigele, J. B., & Barchi, R. L. (1983) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3651–3655.