Fluorescence measurement of chloride transport in monolayer cultured cells

Mechanisms of chloride transport in fibroblasts

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ABSTRACT The methodology has been developed to measure Cl activity and transport in cultured cells grown on a monolayer using the entrapped Cl-sensitive fluorophore 6-methoxy-*N*-[3-sulfopropyl] quinolinium (SPQ). The method was applied to a renal epithelial cell line, LLC-PKI, and a nonepithelial cell line, Swiss 3T3 fibroblasts. SPQ was nontoxic to cells when present for >48 h in the culture media. To load with SPQ (5 mM), cells were made transiently permeable by exposure to hypotonic buffer (150 mOsm, 4 min). Intracellular fluorescence was monitored continuously by epifluorescence microscopy using low illumination intensity at 360 ± 5 nm excitation wavelength and photomultiplier detection at >410 nm. Over 60 min at 37°C, there was no photobleaching and <10% leakage of SPQ out of cells; intracellular SPQ fluorescence was uniform. SPQ fluorescence was calibrated against intracellular [Cl] using high K solutions containing the ionophores nigericin and tributyltin. The Stern-Volmer constant (K_q) for quenching of intracellular SPQ by Cl was 13 M^{-1} for fibroblasts and LLC-PKI cells. In the absence of Cl, SPQ lifetime was 26 ns in aqueous solution and 3.7 ± 0.6 ns in cells, showing that the lower K_q in cells than in free solution (K_q = 118 M^{-1}) was due to SPQ quenching by intracellular anions. To examine Cl transport mechanisms, the time course of intracellular [Cl] was measured in response to rapid Cl addition and removal in the presence of ion or pH gradients. In fibroblasts, three distinct Cl transporting systems were identified: a stilbene-inhibitable Cl/HCO_3 exchanger, a furosemide-sensitive Na/K/2Cl cotransporter, and a Ca-regulated Cl conductance. These results establish a direct optical method to measure intracellular [Cl] continuously in cultured cells.

INTRODUCTION

Chloride movement across cell plasma membranes plays a central role in cell volume and pH regulation, and in many epithelia, in the vectorial transepithelial transport of Cl. Altered Cl transport in the apical membrane of several types of epithelial cells is a central defect in cystic fibrosis (Widdicombe et al., 1985; Frizzell et al., 1986). Double-barreled Cl-sensitive microelectrodes and ^{36}Cl tracer methods have been used to study Cl transport in intact cultured cells, however there are technical difficulties inherent in each method. Microelectrodes are difficult to use in small cultured cells (Oberleithner et al., 1986) and have imperfect Cl selectivity and response times (Chao and Armstrong, 1987). ^{36}Cl tracer methods cannot be used to monitor cell Cl continuously, and have poor time resolution because of the low specific activity of ^{36}Cl. For study of the conductance properties of single Cl channels, the patch-clamp technique is the method of choice.

We report here the use of an intracellular Cl-sensitive fluorophore, SPQ, to obtain accurate continuous records of intracellular Cl activity in monolayer cultured cells. SPQ is quenched by Cl by a collisional mechanism without change in the shape of its excitation and emission spectra (Illsley and Verkman, 1987). SPQ fluorescence responds to changes in Cl activity in <1 ms, and in aqueous solution, is not sensitive to HCO_3, SO_4, pH, NO_3, and physiological cations. SPQ has been used to quantitate Cl transport in a variety of biological membrane vesicles (Chen et al., 1988; Chen and Verkman, 1988; Pearce and Verkman, 1989), in liposomes reconstituted with Cl transporters (Verkman et al., 1989*b*) and in the intact kidney proximal tubule (Krapf et al., 1988*a*). SPQ has not been used previously to study Cl transport in cultured cells.

Methodology has been developed to load and trap SPQ in cultured cells, to measure SPQ fluorescence without photodynamic artifacts, to calibrate SPQ vs. intracellular [Cl], and to define Cl transport mechanisms across cell plasma membranes. Continuous records of intracellular [Cl] were obtained in cells loaded with SPQ without photobleaching or toxicity, and with minimal (<10%) SPQ leakage from cells in 60 min at 37°C. This methodology was applied to measure the intracellular Cl activity and the kinetics of Cl/HCO_3 exchange, Na/K/2Cl cotransport, and Cl conductance in fibroblasts.

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METHODS

Materials

SPQ was synthesized as described previously (Krapf et al., 1988*b*) and triply recrystallized from 1:1 methanol/H_2O. SPQ gave a single spot on reverse phase thin layer chromatography (1:35 chloroform/methanol). 4,4'-Diisothiocyanatodihydrostilbene-2,2'-disulfonic acid (H_2DIDS) and 2,7-bis-carboxyethyl-5(and 6)-carboxy-fluorescein tetracetoxymethyl ester (BCECF) were purchased from Molecular Probes Inc., Junction City, OR. All other reagents were purchased from Sigma Chemical Co., St. Louis, MO. The ionophores nigericin (K/H exchanger) and tributyltin (Cl/OH exchanger) were added from ethanolic stock solutions. The compositions of solutions used in these studies are given in Table 1.

Cell culture

LLC-PK1 cells (ATCC No. CCL-101, passages 221–240) and Swiss 3T3 fibroblasts (ATCC No. CCL-92, passages 121–128) were obtained from the University of California San Francisco Cell Culture Facility. Cells were grown to confluence on 18-mm-diameter round glass coverslips in Dulbecco's modified Eagle's medium (DME-H21) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air. Experiments were performed on LLC-PK1 cells 2–5 d after seeding and on Swiss 3T3 fibroblasts 7–14 d after seeding. There was no effect of addition of 5 mM SPQ to the cell culture medium for 48 h on the appearance or growth of fibroblasts and LLC-PK1 cells.

Cell loading procedure

A number of loading procedures were tested to maximize uniform loading of SPQ into cells in a brief period of time without cell membrane damage. Passive diffusion, hypotonic shock, detergent, and scratch-loading techniques were tested (McNeil, 1989) using different SPQ concentrations (5–15 mM), loading times (4–30 min), and buffer osmolalities (150–300 mOsm). Good loading without cell trauma was obtained consistently in all cells tested (LLC-PK1 and MDCK renal cells, Swiss 3T3 fibroblasts, tracheal epithelial cells in primary culture) by incubation in a 1:1 mixture of solution 1 and distilled H_2O containing 5 mM SPQ for 4 min at 23°C (see Results). Cells were then washed for 1 min with solution 1 before transfer to the perfusion chamber.

Cell perfusion chamber

A perfusion chamber was designed to measure cell fluorescence in response to rapid solution changes in an inverted epifluorescence microscope using short working distance immersion objectives (Fig. 1). The coverslip containing loaded cells (facing upward) was placed on a thin latex gasket on the bottom half of the chamber. The top half of the chamber was then screwed down onto the coverslip to make a watertight seal. Solutions bathing the cells were infused through stainless steel tubing through the top half of the chamber. The chamber volume was 180 μl. The microscope objective (glycerol immersion) was separated from the cell layer by a single coverslip (thickness, 0.12 mm). The top half of the chamber was bounded by a 10-mm-diameter glass coverslip for brightfield, differential interference contrast or phase-contrast microscopy measurements.

The perfusion system was optimized to eliminate bubbles, which introduce noise in the fluorescence data tracings, and to select rapidly among up to eight perfusion solutions maintained at constant temperature. Solutions were gravity fed into the perfusion chamber from glass bottles placed in a constant temperature water bath 80 cm above the microscope stage. Solutions were selected using four-port D4 valves (Hamilton Co., Reno, NV). Perfusion solutions containing HCO_3 were gassed with 95% O_2/5% CO_2 continuously during the experiment. Constant temperature (37°C) was maintained by circulating fluid from the temperature bath through wide-bore rubber tubing in which ran PE-190 polyethylene tubing from the individual perfusion solutions. Stability of the temperature control system was checked by a microthermistor placed in the fluid exit path just distal to the perfusion chamber. The fluid leaving the perfusion chamber passed through an in-line Gilmont No. 2 flow meter (Greatneck, NY) and drained into a reservoir 17 cm below the level of the microscope stage giving a perfusion rate of ~15 ml/min.

Fluorescence microscopy measurements

SPQ fluorescence was measured using a Nikon inverted epifluorescence microscope (Diaphot, Japan). All glass lenses in the excitation path were replaced by fused silica lenses of identical specifications (Melles Griot, Irvine, CA). To eliminate photobleaching and to maximize stability of the light source, fluorescence was excited at 360 ± 5 nm by a 100-W tungsten-halogen lamp powered by a stabilized DC supply in series with an O.D. 2 neutral density filter, a UGl black glass filter (Schott Glass Co., Duryea, PA), and a 360 ± 5 nm six-cavity interference filter (Omega Optical Co., Brattleboro, VT). Excitation light was reflected by a 400-nm fused silica dichroic mirror having low autofluorescence (Omega Optical Co.) and illuminated the cells through a 40×

TABLE 1 Composition of solutions

Component	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Na	130	122	122	25	25	25	25	110	110	110	110	15	15	15	15	101	101	0	0	0	0
K^+	5	5	5	120	120	120	120	5	5	5	5	120	120	120	120	5	5	5	5	120	120
Ca^{2+}	2	7	2	8	2	8	2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	7	2	2	2	8	2
Mg^{2+}	2	2	2	2	2	2	2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	2	2	2	2	2	2
Cl^-	114	0	110	0	153	0	128	0	0	30	30	0	0	30	30	0	114	0	114	0	114
Gluconate$^-$	29	120	0	165	0	140	9	76	111	46	81	130	95	100	65	115	0	9	0	130	14
HCO_3^-	0	25	25	0	0	25	25	40	5	40	5	5	40	5	40	0	0	0	0	0	0
Mannitol	0	100	100	50	50	100	100	50	50	50	50	50	50	50	50	50	50	252	50	100	100
Hepes/Tris	5	0	0	5	5	0	0	0	0	0	0	0	0	0	0	5	5	5	5	5	5
pH	7.40	7.44	7.44	7.40	7.40	7.44	7.44	7.74	6.63	7.74	6.63	6.63	7.74	6.63	7.74	7.40	7.40	7.40	7.40	7.40	7.40

All solutions contain 5 mM D-glucose. Solution 19 contains 101 mM choline$^+$.

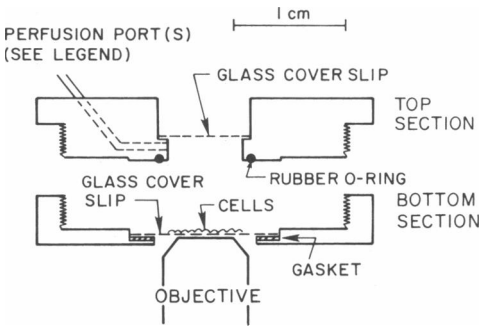

FIGURE 1 Cross-section of cell perfusion chamber. The perfusion chamber was constructed of aluminum. Dimensions are to scale. The perfusion ports were stainless steel 16-gauge tubing. There were two such ports, which entered the chamber tangentially in the horizontal plane; the entrance points of the two ports were 30° apart. See text for other details.

glycerol immersion quartz objective (Leitz-Wezlar, FRG; N.A. 0.65, working distance 0.35 mm). Emitted light was filtered by a 410-nm low-autofluorescence cut-on filter and detected by a model R928S photomultiplier (Hamamatsu Corp., Middlesex, NJ) powered by a high voltage supply/amplifier (Ealing Corp., South Natick, MA). Typically a 200-namp photomultiplier current was detected at 625 V. The amplified signal was digitized by an analog-to-digital converter (Interactive Microware, College Point, PA) at a rate of 30 points/s and averaged over 1-s intervals by a PC/AT computer (IBM Corp., Danbury, CT). Concentric circular iris diaphragms were used to illuminate and measure ~50% of the field of vision (10–15 cells).

To check for uniformity of cell staining, cells were imaged by a SIT camera (DAGE-MTI Inc., Michigan City, IN) using a 100× oil immersion objective (Nikon, fluortar, N.A. 1.30, working distance 0.17 mm). Imaging hardware was purchased from Data Translation, Marlboro, MA. The camera was modified to operate at fixed gain for quantitative imaging. Software for image analysis and ratio imaging was written and compiled in Microsoft Fortran 4.0.

Lifetime microscopy measurements

Nanosecond SPQ lifetimes were measured in single cells using an LS-1 pulsed lifetime instrument (Photon Technology International, Princeton, NJ) interfaced to the epifluorescence microscope. The light source was a 25 KHz N_2 flashlamp operating at 5 kV. Light was filtered by a 360 ± 5 nm interference filter. Emitted light was filtered by a 410-nm cut-on filter and detected by a gated photomultiplier that was activated for 100-ps intervals at varying times after the rise in the flashlamp pulse. Approximately 2 min of data acquisition was required to measure the SPQ lifetime in a single illuminated cell. Background fluorescence decay (15–20% of total signal), measured before cell SPQ loading, was subtracted from the SPQ fluorescence decay. Lifetimes were calculated from flashlamp and fluorescence decay data by standard fitting procedures.

^{36}Cl uptake measurements

^{36}Cl tracer experiments were carried out to determine intracellular Cl concentration and to validate the ionophore calibration procedure. To measure Cl concentration, fibroblasts grown on plastic six-well plates were incubated with 1.5 ml of solutions 4 and 5 containing 2 μCi ^{36}Cl, and 2 μCi of the nonmetabolized cell volume indicator ^{3}H-*O*-methyl glucose. After equilibration of ^{36}Cl and ^{3}H-*O*-methyl glucose across the cell (45 min), cells were washed three times with 2 ml of buffer 4 at 2°C. Cells were dissolved in 2 ml of 0.1 N NaOH, and radioactivity was counted in a scintillation counter. Cell Cl concentration was calculated from the solution Cl concentration, the ratio of solution ^{36}Cl cpm to ^{3}H cpm, and the ratio of cell ^{36}Cl to ^{3}H cpm. Nonspecific binding (<10%) obtained by incubation of ^{36}Cl and ^{3}H-*O*-methyl glucose with cells for <5 s was subtracted from equilibrium cpm. To examine the kinetics of ^{36}Cl equilibration, cells were labeled with 2 μCi/1.5 ml of ^{3}H-*O*-methyl glucose for 60 min in buffer 4. ^{36}Cl influx was initiated by addition of specified mixtures of buffers 4 and 5 containing 2 μCi/1.5 ml ^{3}H-*O*-methyl glucose and ^{36}Cl with or without the ionophores nigericin (5 μM) and tributyltin (10 μM).

RESULTS

Cell loading with SPQ

Several procedures were tested to load SPQ into cells including passive diffusion, scratch-loading, hypotonic loading, and detergent treatment. The loaded cells were washed with isotonic buffer (solution 1) and placed in the cell chamber for continuous perfusion with specified buffers at 37°C. Table 2 shows a comparison of the loading efficiencies of each method, expressed as an SPQ signal-to-background ratio. The hypotonic loading procedure using a 150 mOsm buffer gave the best results. Loading by passive diffusion, the scratch method, and detergents was ineffective. Images of Swiss 3T3 fibroblasts and LLC-PKl cells loaded with SPQ by the hypotonic loading method are shown in Fig. 2. The cell

TABLE 2 Comparison of several methods for loading fibroblasts with SPQ

Loading method	SPQ signal-to-background ratio	SPQ leakage Rate
		%/h
Hypotonic shock with 5 mM SPQ for 4 min		
150 mOsm	1.6–1.8	6–8
225 mOsm	1.3–1.5	9–15
Isotonic loading with		
5 mM SPQ for 4 min	1.07–1.11	—
20 mM SPQ for 30 min	1.1–1.3	4–8
Scratch loading with 5 mM SPQ	1.0–1.1	—
Detergent loading 5 mM SPQ	1.09–1.12	9–16

SPQ was loaded into Swiss 3T3 fibroblasts by hypotonic shock using two different buffer osmolalities, isotonic loading (passive diffusion) under two sets of conditions, scratch loading (multiple needle scratches in the presence of SPQ), and detergent loading (0.01–0.1% saponin, 10 min). A range is shown for experiments performed two to four times.

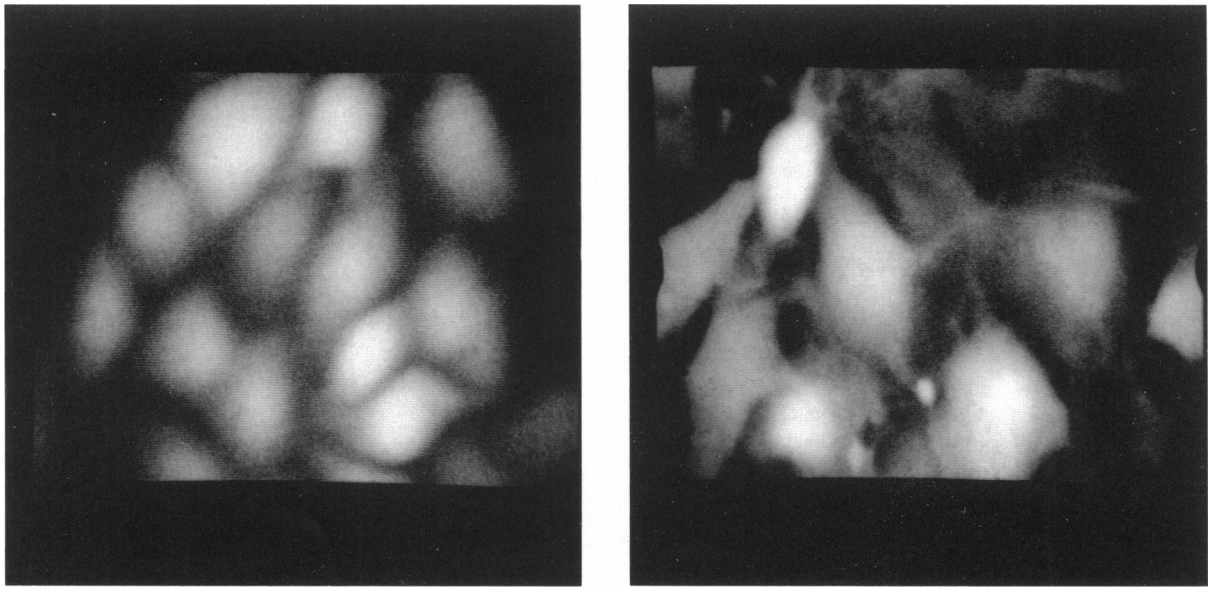

FIGURE 2 Photographs of fibroblasts (*left*) and LLC-PK1 cells (*right*) labeled with SPQ. Cells were perfused with solution 2 at 37°C after SPQ loading and imaged as described in Methods. Background signal measured in the absence of cells was subtracted digitally. Final magnification is 1,250.

fluorescence was distinctly demarcated and appeared to be uniform within individual cells. There were no bright spots due to SPQ binding to cell components or precipitation in the intercellular space.

The intracellular distribution of an ion indicator is an important concern in the interpretation of fluorescence data. An ideal indicator is confined to the cytoplasmic compartment and does not bind to intracellular membranes or proteins. To assess quantitatively the distribution of SPQ in loaded cells, a ratio imaging technique was used to compare the distribution of SPQ with that of the extensively used pH indicator, BCECF, which was assumed to reside in the cytoplasmic compartment. A ratio image was required to normalize the SPQ intensities for differences in cell thickness. Cells loaded with SPQ and BCECF were imaged separately for SPQ (excitation 360 nm) and BCECF (excitation 480 nm) fluorescence. The calculated ratio image was very uniform without distinct variations across the cell. Standard deviations were 4% (LLC-PK1) and 6% (3T3 fibroblasts) for calculated ratio values.

SPQ leakage

The rate of SPQ leakage from loaded cells was quantitated from the time course of loss of cell fluorescence when cells were perfused with solutions not containing SPQ. These and subsequent experiments were performed using photomultiplier detection to integrate the signal intensities from 10–15 cells. The background signal due to cell and instrument autofluorescence was obtained at the end of each experiment by perfusion with 150 mM KSCN in the presence of 5 μM valinomycin which has been shown to quench >99% of SPQ fluorescence (Krapf et al., 1988*a*). Signal intensities measured after addition of KSCN did not differ from those measured in cells not loaded with SPQ.

Fig. 3 (*top*) shows the time course of SPQ fluorescence

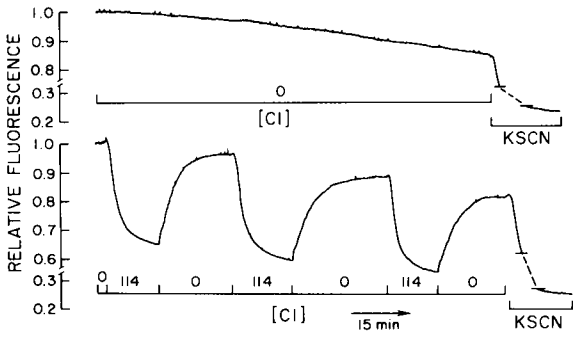

FIGURE 3 Leakage of SPQ from cells. (*Top*) Swiss 3T3 fibroblasts were perfused continuously at 37°C with solution 2. Fluorescence from 10–15 cells was monitored as described in Methods. KSCN in the presence of valinomycin was added at the end of the experiment to quench intracellular SPQ fluorescence. (*Bottom*) Fibroblasts were loaded with SPQ and perfused continuously at 37°C. Perfusion solutions were switched between solutions 2 and 3. In the presence of Cl, intracellular Cl activity was measured to be 50 mM.

in 3T3 fibroblasts perfused with buffer not containing SPQ (solution 2) at 37°C. There was a decrease in cell fluorescence at a rate of 7 ± 1%/h (SE, n = 4). This rate was not affected by illuminating cells for 30 s out of every 10 min, indicating that the fluorescence decrease was due to SPQ leakage but not to fluorescence photobleaching. Addition of KSCN at the end of the experiment quenched the remaining SPQ fluorescence rapidly. Similar experiments in LLC-PKl cells gave a leakage rate of 8 ± 2%/h (n = 3) at 37°C. To show that the rate of SPQ leakage was not influenced by addition of Cl to and removal of Cl from the bathing solution, the time course of SPQ fluorescence was measured in response to a series of Cl addition/removal cycles (Fig. 3, *bottom*). Fibroblast SPQ fluorescence decreased and increased reversibly in response to Cl addition to and removal from the bathing solution. The rate of SPQ leakage was 6%/h, similar to the leakage rate measured in the absence of Cl at 37°C.

Calibration of intracellular SPQ fluorescence

Quantitative analysis of SPQ fluorescence signals from cells requires knowledge of the relationship between SPQ fluorescence and intracellular Cl activity. Because SPQ does not have an isosbestic wavelength, a two-point calibration scheme was necessary in every experiment in which SPQ fluorescence was measured in the absence of Cl, and in the presence of infinite Cl (KSCN + valinomycin, see above). Intermediate Cl activities can then be calculated from the two calibration points and the relationship between relative SPQ fluorescence and cell Cl activity (Chen et al., 1988).

SPQ fluorescence was calibrated against intracellular Cl activity by a double ionophore technique. Intracellular Cl was set equal to external Cl using a high K buffer, the K/H exchange ionophore nigericin, and the Cl/OH exchange ionophore tributyltin (Krapf et al., 1988*a*). The high K buffer in the presence of nigericin clamps intracellular pH at the external pH, and strongly depolarizes cell membrane potential. Tributyltin addition in pH-clamped cells results in equal intracellular and external Cl activities.

Fig. 4 (*top*) shows a calibration of LLC-PKl cells at 37°C. Cell fluorescence changed reversibly in response to a series of external Cl concentrations. The progressively decreasing fluorescence signal results from SPQ leakage. In the presence of ionophores at 37°C, the rate of SPQ leakage was 10–20%/h (n = 4). The faster leakage rate in the presence of ionophores has been noted in other ionophore calibration procedures, such as the pH calibration of BCECF in the presence of nigericin, and is thought to be due to cell toxicity. Tributyltin is the only known compound with Cl ionophoric activity.

Two types of control studies were performed to show

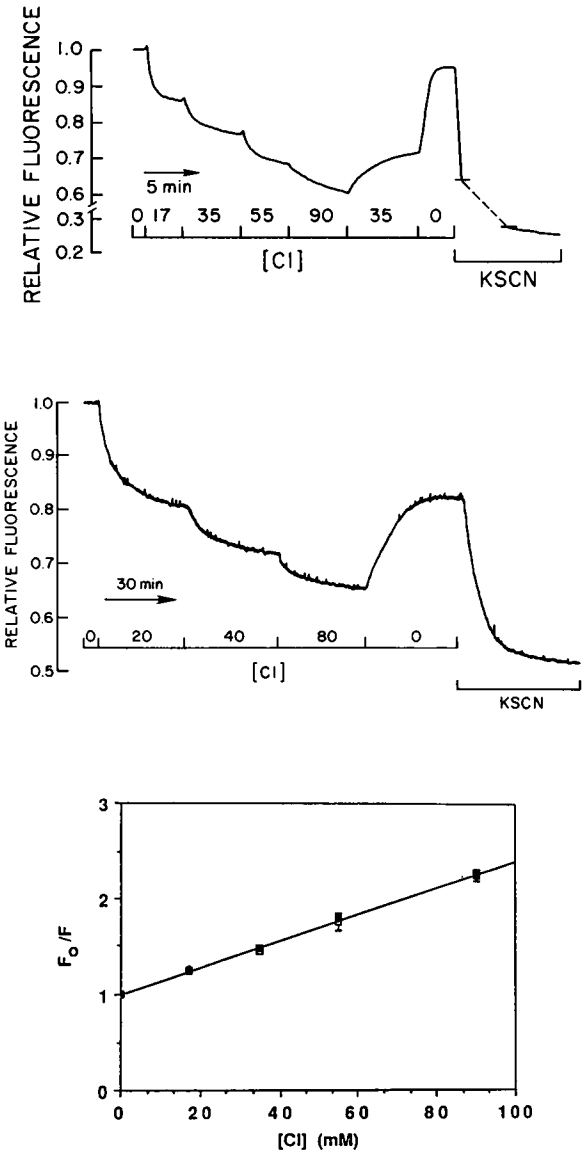

FIGURE 4 Intracellular calibration of SPQ. (*Top*) LLC-PKl cells were perfused continuously at 37°C with solutions containing the ionophores nigericin (5 μM) and tributyltin (10 μM). Solutions were made to contain different Cl concentrations by mixture of solutions 4 and 5. (*Middle*) Swiss 3T3 fibroblasts were perfused with different Cl concentrations by mixture of solutions 6 and 7 in the absence of ionophores (see text). (*Bottom*) Results from two (LLC-PKl cells) and three (fibroblasts) separate calibrations performed in the presence of ionophores are summarized. The calibration in fibroblasts was carried out in the presence of HCO_3 using mixtures of solutions 6 and 7. The ordinate (F_o/F) is the total fluorescence measured in the absence of Cl (fluorescence at zero Cl minus that after SCN quenching) divided by the fluorescence in the presence of Cl (fluorescence at a given Cl minus that after SCN quenching). Error bars are 1 SE for fibroblasts (*open squares*) and LLC-PKl cells (*solid squares*). Data were fitted to the Stern-Volmer quenching equation, $F_o/F = 1 + K_{Cl}[Cl]$, where K_{Cl} is the Stern-Volmer constant calculated to be 12.9 ± 0.2 M^{-1}. [Cl] and K_{Cl} were expressed in concentration units. To obtain activities, [Cl] is multiplied by 0.75 and K_{Cl} is divided by 0.75.

that complete Cl equilibration was obtained in the ionophore calibration. As presented below, ^{36}Cl equilibration was >90% complete in 2 min under the conditions of the calibration experiments. In a second study, SPQ fluorescence was calibrated against intracellular Cl in the absence of ionophores (Fig. 4, *middle*). The calibration was performed in fibroblasts in a high-K, low-Na buffer which was similar to intracellular ion concentrations. Because there is no primary active Cl transport, intracellular and extracellular Cl activities should be nearly equal after a sufficiently long equilibration time.[1] Solutions containing different Cl concentrations were perfused for 30 min each, resulting in >90% Cl equilibration across the membrane. Note that in the absence of ionophores, the SPQ leakage rate in these experiments was 8%/h. Results of the ionophore and nonionophore calibration methods did not differ significantly.

Fig. 4 (*bottom*) shows that calibration curves of relative SPQ fluorescence vs. cell Cl activity are similar for LLC-PK1 cells and 3T3 fibroblasts. The calibration data were fitted to the Stern-Volmer relation for use in the calculation of transmembrane Cl transport rates. It is remarkable that the SPQ sensitivity to Cl (Stern-Volmer constant 13 M^{-1}) was approximately ninefold lower than that reported in aqueous solution (118 M^{-1}). The Stern-Volmer constant obtained by the nonionophore calibration experiment in fibroblasts was 16 M^{-1}.

Single cell nanosecond lifetimes of SPQ

SPQ lifetimes were measured to determine the mechanism of the decreased Cl sensitivity of SPQ in cells compared with that in aqueous solution. If SPQ were quenched by non-Cl components in the intracellular environment, then the SPQ lifetime in the absence of Cl would be less than that in aqueous solution, and the Stern-Volmer constant for SPQ quenching by Cl would decrease.[2] If intracellular SPQ sensitivity were decreased because of decreased intracellular Cl diffusion or restricted access of Cl to SPQ, the SPQ lifetimes in cells and aqueous solution would be similar in the absence of Cl.

Fig. 5 shows the nanosecond decay of SPQ fluorescence in a single fibroblast and in aqueous solution. In aqueous solution, the SPQ lifetime was 26 ns, similar to that reported previously (Illsley and Verkman, 1987). In three separate experiments, the SPQ lifetime in the absence of Cl (3.7 ± 0.6 ns) was approximately sevenfold less than that in aqueous solution. In 0.2 μm diameter phosphatidylcholine liposomes loaded with 10 mM SPQ in the absence of Cl, the SPQ lifetime was 23 ± 1 ns, showing that the decreased SPQ lifetime in cells is not an effect of compartmentation. The difference in SPQ lifetimes in free solution and cells accounts quantitatively for the relatively low SPQ sensitivity in cells; SPQ fluorescence

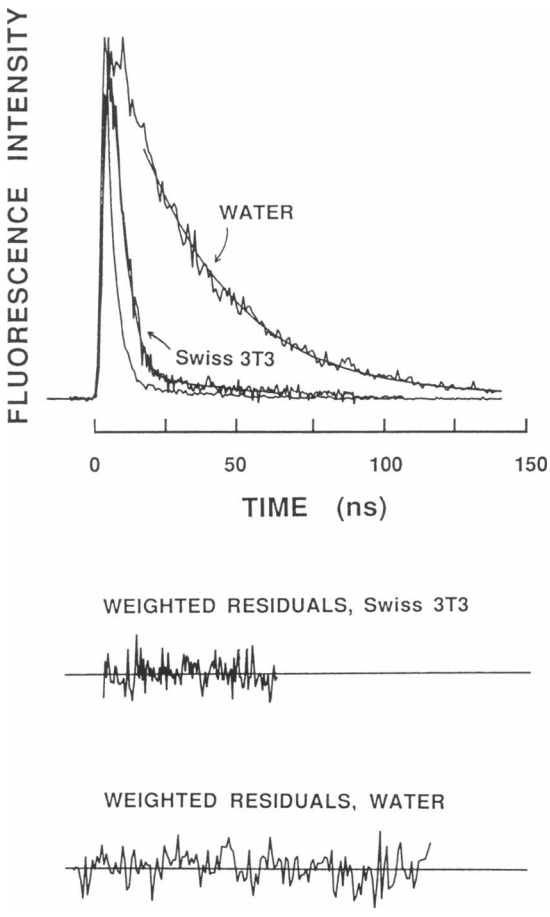

FIGURE 5 Nanosecond SPQ lifetime in a single fibroblast and in free solution. SPQ-loaded fibroblasts were incubated in solution 16 (zero Cl) for 30 min. Free solution SPQ consisted of 100 μM SPQ in H_2O. The decay of fluorescence was measured as described in Methods. Fitted lifetimes and X^2 values were 26.0 ± 0.2 ns and 1.5 (free solution) and 3.2 ± 0.2 ns and 1.1 (fibroblast).

[1]To test this assumption, fibroblasts loaded with 0 Cl buffer were incubated for 3 min at 37°C in the high K buffer containing 2 μCi/1.5 ml ^{36}Cl and ^{3}H-*O*-methyl glucose and Cl concentrations of 10, 40, and 114 mM. Calculated intracellular Cl concentrations at 30 min were 12, 42, and 116 mM in one set of experiments typical of two. Therefore the use of high K buffers in the absence of ionophores is a valid approach for calibration of intracellular Cl in fibroblasts.

[2]In the presence of Cl and an "unknown" intracellular quencher X, the Stern-Volmer relation is $F_o/F = 1 + K_{Cl}[Cl] + K_x[X]$, where K_{Cl} and K_X are Stern-Volmer quenching constants. This relation can be written in the form $(F_o/F)/(1 + K_x[X]) = 1 + K_{Cl}[Cl]/(1 + K_x[X])$. If [X] is constant, then a Stern-Volmer plot of apparent F_o ($=F_o/\{1 + K_x[X]\}$) divided by F vs. [Cl] would be linear with slope $K_{Cl}/(1 + K_x[X])$. Therefore the presence of a non-Cl intracellular quencher results in a decrease in the apparent Stern-Volmer constant for quenching of SPQ by Cl. The presence of a non-Cl quencher that cannot be eliminated must be proven by lifetime measurements.

was quenched dynamically by non-Cl intracellular components. These results show that the intracellular milieu has a strong influence on the SPQ sensitivity and indicate that the intracellular sensitivity of Cl indicators should be evaluated in every cell type by a direct ionophore calibration procedure under the conditions of the experiment.

Mechanisms of Cl transport in fibroblasts

It has been reported that a major Cl transport pathway in human skin fibroblasts is Cl/anion exchange (Lin and Gruenstein, 1988). To determine whether Swiss 3T3 fibroblasts have a Cl/HCO_3 transporter, we subjected cells to Cl gradients in the presence of inward and outward HCO_3 gradients. The effect of the stilbene inhibitor of anion exchange, H_2DIDS, was also tested. To avoid CO_2 gradients, all solutions were bubbled with 5% CO_2/95% O_2 for at least 1 h. Fig. 6 (*top*) shows that an outward HCO_3 gradient (~40 mM inside, 5 mM outside) reversibly enhanced Cl entry and that HCO_3-gradient driven Cl entry was strongly inhibited by H_2DIDS. To prove that the gradient driven Cl entry was due to anion exchange rather than to parallel HCO_3 and Cl conductances, similar experiments were carried out in the presence of a high K buffer and valinomycin to depolarize membrane potential to nearly 0 mV (Fig. 6, *bottom*). There was no significant effect of this "voltage-clamp" on the HCO_3-gradient driven Cl influx. Taken together, these results establish the presence of Cl/HCO_3 exchange in 3T3 fibroblasts.

To compare rates of Cl influx quantitatively, we calculated Cl influx in units of millimolar per second from the initial fluorescence vs. time slope, the Stern-Volmer constant of 13 M^{-1}, and the fluorescence values at 0 Cl and after KSCN addition by equations reported previously (Chen et al., 1988). In three sets of experiments, the initial rate of Cl influx in response to a 30 mM inward Cl gradient was 0.06 ± 0.01 mM/s (SEM) in the absence, and 0.26 ± 0.03 mM/s in the presence of the outward HCO_3 gradient. The initial rate of Cl influx in the presence of the outward HCO_3 gradient decreased to 0.07 ± 0.02 mM/s with addition of 0.2 mM H_2DIDS. In two sets of experiments in the presence of the K/valinomycin voltage-clamp, the initial rate of Cl influx increased from 0.07 ± 0.02 mM/s, with the inwardly directed HCO_3 gradient, to 0.25 ± 0.04 mM/s with the outwardly directed HCO_3 gradient.

The presence of Na-dependent Cl transport was examined from the effects of Na removal and furosemide

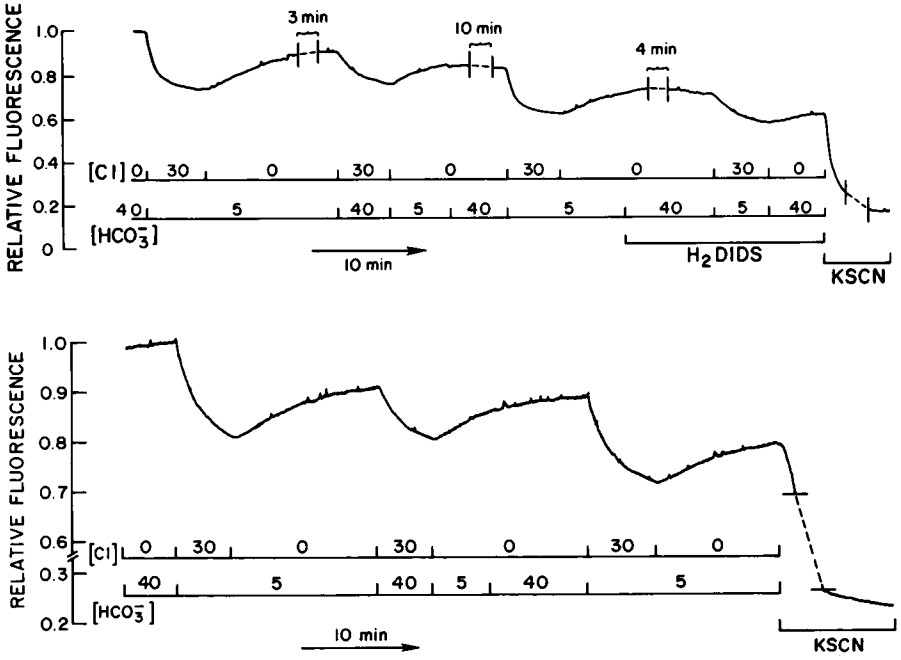

FIGURE 6 Cl/HCO_3 exchange in Swiss 3T3 fibroblasts measured by SPQ fluorescence. Fibroblasts were perfused continuously at 37°C. (*Top*) Perfusion fluid was altered from solutions 8 or 9 (containing 0 Cl) to solutions 10 or 11 (containing 30 mM Cl) to induce Cl influx at different transmembrane HCO_3 gradients. Where indicated, 0.2 mM H_2DIDS was present in the perfusion solutions. (*Bottom*) The same studies were performed in membranes depolarized by high K/valinomycin. Perfusion fluid was altered from solutions 12 or 13 (containing 0 Cl) to solutions 14 or 15 (containing 30 mM Cl). All solutions contained 5 μM valinomycin.

addition on Cl influx in the absence of HCO_3 (Fig. 7). In two sets of experiments, Na removal caused a >90% decrease in the initial rate of Cl influx, indicating that the major route for Cl entry in the absence of HCO_3 required Na. Furosemide has been shown to inhibit Na/K/2Cl electroneutral cotransport in a variety of cells (Geck and Heinz, 1986). Addition of 0.5 mM furosemide in the presence of Na gave a >90% decrease in the rate of Cl influx, providing strong evidence for presence of a Na/K/2Cl cotransporter. Therefore in the absence of HCO_3, >90% of the Cl influx occurs by this mechanism.

It has been proposed that skin fibroblasts contain a conductive Cl pathway that is regulated by cAMP (Bear, 1988; Lin et al., 1988) and that the cAMP regulation may be abnormal in cystic fibrosis (Rugolo et al., 1986; Lin and Gruenstein, 1987). To examine whether 3T3 fibroblasts contained a regulated Cl conductance, effects of Ca and dibutyryl-cAMP on Cl entry were studied. Fig. 8 (*top*) shows that Ca is an activator of cell Cl conductance. Experiments were performed in the absence of Na and HCO_3 to eliminate Cl influx via Cl/HCO_3 exchange and Na/K/2Cl cotransport. Under these conditions the rates of Cl influx for the first Cl addition (<0.02 mM/s, n = 3), and in the presence of ionomycin and low Ca (<0.02 mM/s), were very low. The addition of ionomycin in the presence of Ca resulted in a large increase in the rate of Cl influx (0.19 ± 0.04 mM/s). A similar approach was used recently to demonstrate regulation of the epithelial Cl channel by Ca in tracheal epithelia (Willumsen and Boucher, 1989).

To examine whether fibroblast Cl conductance could be modulated by a cAMP-dependent pathway, effects of dibutyryl-cAMP and IBMX were examined. In three sets of experiments performed in the absence of HCO_3 and Na as in Fig. 8 (*top*) there was no measurable stimulatory effect of 1 mM dibutyryl-cAMP or 0.5 mM dibutyryl-cAMP + 0.1 mM IBMX (not shown). Additional experiments were performed in the presence of Na to examine whether Cl efflux could be stimulated by dibutyryl-cAMP. This protocol was used effectively to show an approximate fivefold increase in Cl conductance by isoproterenol in primary cultures of dog tracheal cells (Chao et al., 1990). Fig. 8 (*bottom*) shows that dibutyryl-cAMP with IBMX does not result in a measurable increase in Cl influx or efflux rates. These results are different than data in skin fibroblasts showing a small (~30%) cAMP-dependent increase in Cl conductance (Lin and Gruenstein, 1988).

^{36}Cl uptake studies

Experiments were carried out in fibroblasts to determine the intracellular Cl concentration under physiological conditions and to demonstrate adequate Cl equilibration between intracellular and extracellular compartments in calibration experiments. The intracellular Cl concentra-

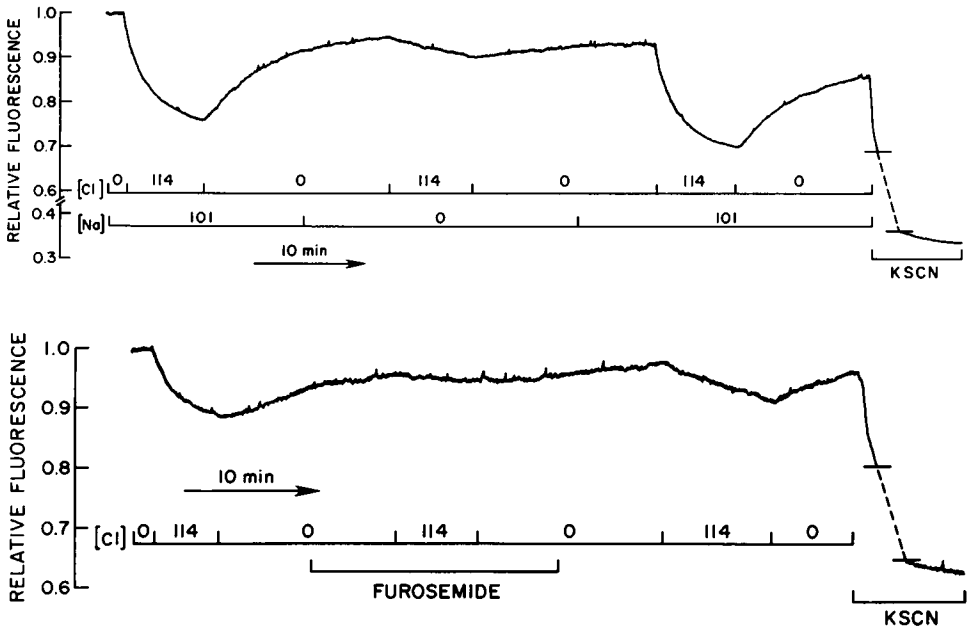

FIGURE 7 Na/K/2Cl cotransport in fibroblasts measured by SPQ fluorescence. Fibroblasts were perfused continuously at 37°C. (*Top*) Cl was added and removed in the presence and absence of Na using solutions 16–19. (*Bottom*) Cl was added and removed in the presence of Na and in the absence or presence of 0.5 mM furosemide.

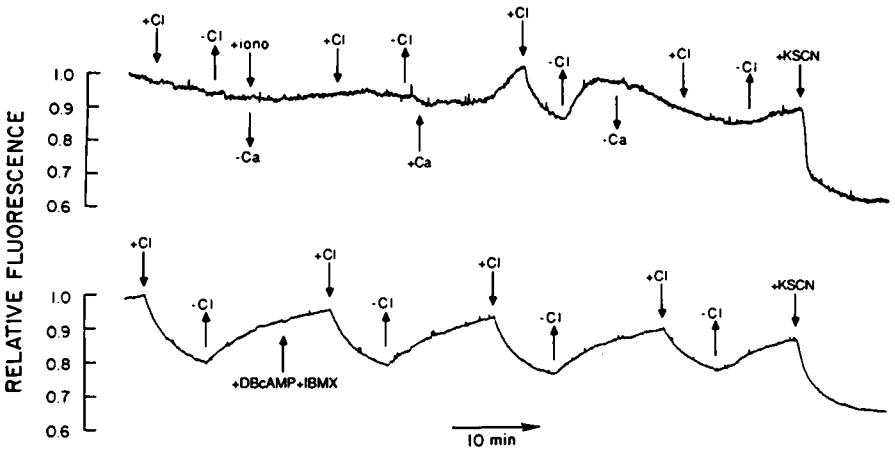

FIGURE 8 Regulation of Cl conductance in fibroblasts. (*Top*) Cl was added and removed in the absence of Na with high K (solutions 20 and 21), and in the presence of 1 μM ionomycin containing 0 or 2 mM Ca. (*Bottom*) Cl was added and removed in the presence of Na with low K (solutions 16 and 17). Where indicated, 0.5 mM dibutyryl-cAMP and 0.1 mM IBMX were added. An artifactual increase in fluorescence that occurred immediately with addition of ionomycin was subtracted from the data curve.

tion was measured by a double label technique in which ^{3}H-*O*-methyl glucose was used as a marker for cell water (see Methods). In six sets of experiments, there was 1.4 ± 0.2 μl of cell water per 35-mm-diameter cell culture dish. From equilibrium double label experiments, intracellular Cl concentration was 76 ± 7 mM (n = 4). Assuming an intracellular Cl activity coefficient of 0.75, the intracellular Cl activity becomes 57 ± 5 mM, which is similar to the Cl activity measured by the SPQ technique as given in the legend to Fig. 3 (50 mM).

To test whether high K solutions containing nigericin and tributyltin effectively caused Cl equilibration, cells were incubated with a zero Cl solution containing ^{3}H-*O*-methyl glucose (see Methods). The solution was then replaced by a buffer containing nigericin and tributyltin, 40 or 120 mM Cl, and 2 μCi ^{36}Cl and ^{3}H-*O*-methyl glucose. In one set of experiments typical of two, the intracellular Cl concentration was 0, 29, 37, 43, and 41 mM at 0, 1, 2, 10, and 30 min after addition of ^{36}Cl in the buffer containing 40 mM Cl. Intracellular Cl concentration was 0, 94, 110, 118, and 115 mM at 0, 1, 2, 10, and 30 min after addition of ^{36}Cl in the buffer containing 120 mM Cl. Therefore equilibrium of extracellular and intracellular Cl is >90% complete within 2 min under the conditions of the calibration experiment.

DISCUSSION

The purpose of the studies performed here was to establish the methodology to measure intracellular chloride activity in cultured cells by epifluorescence microscopy. For use in intact cultured cells, the important characteristics of a Cl-sensitive indicator are (*a*) high Cl sensitivity and specificity, (*b*) rapid response, (*c*) low toxicity, (*d*) rapid loading and slow leakage, and (*e*) bright fluorescence. As described below, our results show that SPQ satisfies these criteria for use as an intracellular Cl indicator.

At the start of our study, the major potential difficulty was the development of an indicator and loading method to ensure rapid loading and slow leakage from cells. In vesicle and liposome studies, SPQ was loaded by prolonged incubation or by entrapment during liposome formation; once loaded, SPQ leaked out from vesicles or liposomes slowly (typically <20% in 60 min at 23°C). In intact cells of the kidney proximal tubule, SPQ was loaded at high concentration (15 mM) for 10 min at 37°C by luminal perfusion; SPQ leaked out of cells with a half-time of 8 min at 37°C. Two approaches were taken to design a Cl indicator/loading method which would give rapid cell loading and slow leakage. The first was to modify the structure of SPQ chemically to increase lipid solubility and add cleavable ester functions for cell entrapment; the synthetic organic chemistry has been partially completed to accomplish this goal (Krapf et al., 1988*b*; Verkman et al., 1989*a*). The second approach was to develop a transient cell permeabilization procedure to load cells with a small, impermeable Cl indicator. After testing several permeabilization methods and Cl indicators, it was found that a brief exposure of cells to hypotonic solution in the presence of SPQ provided good loading in all cells tested with maintenance of membrane integrity upon return of the cell bathing solution to isosmolality.

After loading, the SPQ leakage rate from both the 3T3

fibroblasts and LLC-PK1 cells was <10% in 60 min at 37°C. This is much slower than the rate of SPQ leakage from cells in the perfused proximal tubule (50% leakage in 8 min), but comparable to SPQ leakage rates across isolated membrane vesicles. This difference is probably due to the ~30-fold higher surface-to-volume ratio of proximal tubule cells compared with cultured cells used in this study, however we cannot rule out the presence of an SPQ transport system in membranes of the intact proximal tubule.

Cells loaded with SPQ have brightness comparable with that of cells loaded with fura-2, but less than fivefold the brightness of cells loaded with a fluorescein or rhodamine compound. This is due to the low molar absorptivity of SPQ (~3,000 $M^{-1}cm^{-1}$ at 360 nm). Despite this small fluorescence signal, it was possible to measure intracellular SPQ fluorescence transients with signal-to-noise ratios exceeding 100:1 with very low illumination intensity by use of a 100-W tungsten-halogen lamp in series with an O.D. 2 neutral density filter (100-fold light reduction), a UG-1 black glass filter (approximately twofold light reduction) and a 360 ± 5 nm interference filter (~40% efficiency at 360 nm). No photobleaching or apparent photodynamic cell injury occurred with this illumination scheme. Removal of the O.D. 2 filter resulted in ~5% reduction in signal per minute due to photobleaching.

Cells loaded with SPQ appeared to be stained uniformly. The uniformity of cytoplasmic staining was confirmed quantitatively by ratio imaging using BCECF as an assumed fluid phase cytoplasmic marker. The high polarity of SPQ probably makes its binding to intracellular lipids and transport into intracellular organelles unlikely. In cells grown for 48 h in 5 mM SPQ, there was no apparent cell toxicity. Fluorescence microscopy after 48 h of loading showed marked nonuniformity in cell staining with heavy accumulation of SPQ in the perinuclear region. It is not known whether this staining effect is due to SPQ binding to proteins or to chemical degradation of SPQ. In studies of the kidney endosomal Cl channel, rabbits were infused with 250 mg of SPQ without apparent ill effects (Bae and Verkman, 1990).

The sensitivity of SPQ to intracellular Cl activity was similar for LLC-PK1 cells and 3T3 fibroblasts. When expressed as a Stern-Volmer quenching constant, the intracellular chloride sensitivity was 13 M^{-1} for both cell types, similar to the value of 12 M^{-1} for SPQ quenching by Cl in the proximal tubule, but an order of magnitude less than the Stern-Volmer constant (118 M^{-1}) for SPQ quenching by Cl in aqueous solution. Possible reasons for this large difference in sensitivity are effects of cytoplasmic viscosity, SPQ quenching by cytoplasmic proteins, and restricted access of Cl to intracellular SPQ (Krapf et al., 1988*a*). Lifetime microscopy studies proved that the mechanism was quenching of SPQ by intracellular non-Cl compounds such as proteins. Despite this reduction in sensitivity, intracellular SPQ sensitivity is adequate to monitor Cl transients in the physiological range of intracellular Cl activity (0–60 mM). More sensitive Cl indicators have been synthesized recently with Stern-Volmer constants for quenching by Cl of ~200 M^{-1} in aqueous solution (Verkman et al., 1989*a*).

One strategy for use of an intracellular Cl indicator to establish Cl transport mechanisms involves measurement of the effect of ion gradients and inhibitors on cell Cl transients. Experiments in Swiss 3T3 fibroblasts showed the presence of a Cl/HCO_3 exchanger, in agreement with results reported in human skin fibroblasts using intracellular pH and ^{36}Cl flux measurements (L'Allemain et al., 1985; Lin and Gruenstein, 1988). Cl/HCO_3 exchange was demonstrated from (*a*) enhancement of inward Cl influx by an outwardly directed HCO_3 gradient, (*b*) inhibition of the HCO_3-gradient driven Cl influx by the stilbene H_2DIDS, and (*c*) independence of the HCO_3 effect on membrane depolarization with K and valinomycin.

In the absence of HCO_3, the major route of Cl entry was Na dependent and inhibited by furosemide. Although the coupling stoichiometry was not quantitated in this study, it is likely that these findings indicate presence of Na/K/2Cl cotransport. In the absence of Na and HCO_3, the remaining route for Cl entry is probably conductive. This conductive entry was stimulated by an elevation in intracellular Ca, but not stimulated by the permeable cAMP analogue dibutyryl-cAMP.

There are several limitations in the use of SPQ to study cell Cl transport. SPQ has low fluorescence and requires ultraviolet excitation associated with significant cell and instrument background autofluorescence, and potential photodynamic cell injury. The use of quartz optics and low illumination intensity minimized these concerns in this study. SPQ loading into cells required a permeabilization procedure which could introduce unknown damage to cell membranes that cannot be evaluated easily. Cell toxicity and nonuniformity in SPQ distribution are potential concerns but appear to be of minor importance for LLC-PK1 cells and 3T3 fibroblasts. The lack of a second reference wavelength in the SPQ spectrum makes it difficult to determine accurately cell Cl activity if significant SPQ leakage occurs. However the leakage rate was low in these studies. The synthesis of a dansyl-conjugated SPQ via a spacer chain may provide a ratiometric approach for determination of cell Cl (Verkman et al., 1988).

Bearing these difficulties in mind, the use of an intracellular Cl indicator provides important information on cellular Cl transport and regulatory mechanisms. Compared to Cl-sensitive microelectrode methods, the fluores-

cence method is technically easier and can be used to study Cl transport in one or a group of cells, or using quantitative image analysis, in many individual cells simultaneously.

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