ESTIMATION OF INTRACELLULAR CHLORIDE ACTIVITY IN ISOLATED PERFUSED RABBIT PROXIMAL CONVOLUTED TUBULES USING A FLUORESCENT INDICATOR

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ABSTRACT The methodology has been developed to measure cell chloride activity by fluorescence microscopy using the chloride-sensitive dye, 6-methoxy-1-(3-sulfonatopropyl)quinolinium (SPQ). SPQ was loaded into cells of the in vitro microperfused rabbit proximal convoluted tubule by a 10 min luminal perfusion with 20 mM SPQ at 38°C. Fluorescence was excited with a broad band excitation filter (340 and 380 nm) and detected with a 435 nm cut-on filter. The signal to background (autofluorescence) ratio was 4.6 ± 0.6. The halftime for SPQ leakage from cells at 38°C was 8.6 ± 1.1 min. In suspended tubules, SPQ did not affect O_2 consumption significantly. Intracellular SPQ calibration was performed using the ionophores nigericin and tributyltin, high external potassium concentrations, and varying extracellular chloride concentrations. Cell fluorescence was related to intracellular chloride by a Stern-Volmer relation with a quenching constant of 12 M^{-1}. Apparent chloride concentration in tubules perfused with solutions characteristic for the late proximal convoluted tubule was 27.5 ± 5 mM (activity 20.6 mM). The halftime of the transient in cell chloride activity upon bath chloride addition was ~3 s (38°C). Applications and limitations of this new fluorescence method to study cell chloride transport are discussed.

INTRODUCTION

The currently available techniques for measuring chloride transport in viable epithelial cells have several technical limitations. ^{36}Cl tracer methods have poor sensitivity because of the low specific activity of this isotope. Microelectrodes are technically difficult to use and have restricted chloride selectivity and response rate. However, microelectrodes are the current standard to examine regulation of intracellular chloride activity.

In analogy to the widespread application of pH and Ca-sensitive fluorophores, measurement of intracellular chloride with fluorescent indicators would provide a noninvasive method to study chloride transport in cells and intact tissues. Several quinoline derivatives have been shown to have chloride-sensitive fluorescence properties (Wolfbeis and Urbano, 1982; Wolfbeis and Urbano 1983; Krapf et al., 1988). Chloride quenches fluorescence of these substances by a collisional mechanism. One of these compounds, 6-methoxy-1-(3-sulfonatopropyl)quinolinium (SPQ) has been used successfully to study chloride transport mechanisms in isolated membrane vesicles by cuvette fluorometry (Illsley and Verkman, 1987; Chen et al., 1988; Chen and Verkman, 1988). Based on these findings, we have used SPQ and fluorescence microscopy to measure intracellular chloride activity in an intact epithelial tissue, the in vitro microperfused rabbit proximal convoluted tubule (PCT).

The purposes of this study were (*a*) to establish the feasibility and technique of measuring SPQ fluorescence in the rabbit PCT; (*b*) to calibrate SPQ intracelluarly; (*c*) to obtain an estimate of intracellular chloride activity; and (*d*) to record the time course of cell chloride activity in response to addition and removal of extracellular chloride. The results show that SPQ can be loaded into cells of the rabbit PCT without apparent toxicity. Intracellular chloride concentration in PCT perfused and bathed with solutions characteristic for the late proximal tubule was 27.5 ± 5 mM (± SEM). The corresponding cell chloride activity (20.6 mM) is significantly higher than the predicted cell chloride activity if chloride were at electrochemical equilibrium (~15 mM). The halftime for the transient in cell chloride activity when bath chloride was added (bath chloride changed from 0 to 128.6 mM) was ~3 s (38°C). The current limitations of this new fluorescence method and guidelines for its further development and applications are discussed.

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METHODS

SPQ Synthesis and Characterization

SPQ was synthesised as described previously (Wolfbeis and Urbano, 1982; Krapf et al., 1988*b*). 6-methoxyquinoline and 1,3-propanesultone (molar ratio: 0.46) were heated to 100°C for 30 min. The product was recrystallized three times from water-ethanol (1:1, vol/vol). Product purity was confirmed by reverse phase thin-layer chromatography using a methanol:chloroform (1:35 vol/vol) solvent system. Product structure was confirmed by infrared spectroscopy (KBr pellet), nuclear magnetic resonance (1% solution in D_2O), and mass spectroscopy. Maximal solubility in water was 0.09 M (Krapf et al., 1988*b*) at 23°C. The optical characteristics of the compound have been reported previously (Illsley and Verkman, 1987, Krapf et al., 1988*b*). In aqueous solutions, SPQ has peak excitation wavelengths at 318 and 350 nm and a single peak emission wavelength at 442 nm. The molar extinction coefficient at 350 nm is 4,700 M^{-1}. The Stern-Volmer constants[1] for several anions are (in M^{-1}): chloride 118, bromide 175, iodide 276, thiocyanate 211, citrate 15. The dye is not quenched significantly by other physiological anions including bicarbonate, sulfate, and phosphate (Illsley and Verkman, 1987, Krapf et al., 1988*b*), or by changes in ionic strength and pH (Illsley and Verkman, 1987). The response time of SPQ fluorescence to changes in chloride concentration is <1 ms (Illsley and Verkman, 1987).

Fluorescence Microscopy

Fluorescence measurements were made with an inverted fluorescence microscope (Fluovert, Leitz-Wetzlar, West Germany) using a Leitz 25x, long-working distance objective (numerical aperture: 0.35). This objective was chosen because it had minimal autofluorescence at the wavelengths used in this study (see below). A 100 W mercury arc lamp was used as a light source. Fluorescence was excited with a 340 to 380 nm broad band pass filter in series with a UG 11 black glass (Schott Glaswerke, Mainz, West Germany). The filter cube contained a 400 nm dichroic mirror (Leitz-Wetzlar). Fluorescence emission was filtered using a GG 435 nm cut-on filter (Schottt) and detected by a photomultiplier (Leitz) using 500 V. The photomultiplier was interfaced to an IBM PC/XT computer.

Tubule Experiments

Isolated segments of rabbit PCT were dissected and perfused in vitro as described previously (Burg et al., 1966). Briefly, kidneys from New Zealand white rabbits, sacrificed by decapitation, were removed quickly and cut into thin (~1 mm) coronal slices. Cortical PCT (S2 segments) were dissected in a cooled (4°C) solution which was identical to the bath control solution of the respective experiment. Late PCT as defined by their attachment to straight tubules were not used. The tubules were transferred into a bath chamber with a volume of 150 μl. Bath fluid was exchanged continuously at ~10 ml per minute, permitting a complete bath fluid exchange within 1 s, as reported previously (Krapf et al., 1987). Bath solutions were preheated to 38° and equilibrated with a gas mixture of 7% CO_2/93% O_2. A specially designed heater (10 cm long glass tubing surrounded by a coiled heating wire with a resistance of 15Ω) was placed 5 cm proximal to the bath chamber. Bath temperature could be held constant at 38 ± 0.5°C. The fluid in the perfusing pipette was exchanged continuously at a rate of 2 ml per minute through hydrostatic pressure and a constantly open efflux valve, as reported previously (Krapf et al., 1987; 1988*a*). The time lag between the change from a control to an experimental luminal solution and its appearance at the most proximal portions of the perfused tubule was ~10 s. At a perfusion rate of 10 ml per minute the bath lag time was 4 s. Thus, a coordinated fluid exchange (bath fluid changed 6 s after luminal fluid exchange) permitted synchronization of luminal and bath fluid exchanges (Krapf et al., 1987; 1988*a*). The luminal and bath fluid exchange times were calibrated before the experiments by recording the time course of the fluorescent signal when luminal or bath fluids were exchanged from a nonfluorescent solution to one containing SPQ, respectively. The three types of solutions used in this study are listed in Table I.

TABLE I
PERFUSION SOLUTIONS (mmol/l)

	1	2	3
Na^+	147	149.5	147
K^+	5	5	5
Ca^{++}	1.8	1.8	9.4
Mg^{++}	1	1	1
Cl^-	128.6	145.1	
HCO_3^-	25	5	25
Gluconate$^-$			143.8
SO_4^-	1	1	1
HPO_4^-	1	4	1
Alanine	5		5
Glucose	5		5
Urea	5	5	5
CO_2/O_2/%	7/93	7/93	7/93

After allowing the tubules to equilibrate at 38°C for 10 min in symmetrical zero chloride solution (Solution 3, Table I), tubules were loaded with 20 mM SPQ from the lumen over 10 min. To maintain osmotic balance the loading solution was reduced by equiosmolar amounts of NaGluconate. After loading, luminal fluid was exchanged to the zero chloride solution not containing SPQ for 2 min before the first fluorescence measurements. An adjustable measuring diaphragm was placed over a tubule segment just distal to the tip of the perfusion pipette and opened to ~40 μm square. The tubule length exposed to the bath fluid was ~400 μm. Autofluorescence of the tubules was measured before loading the tubule with the dye. The measuring diaphragm was left in the same place for the entire experiment.

To quantitate the changes in fluorescence, the total quenchable signal of the dye was determined in each experiment. The total quenchable signal is the difference between the fluorescence signal when tubules are perfused and bathed with zero chloride solutions (at the beginning of the experiment) and the signal obtained after total quenching (at the end of the experiment). Total quenching was accomplished by perfusing the tubules symmetrically with 150 mM potassium thiocyanate in the presence of 5 μM valinomycin to facilitate cellular uptake of potassium thiocyanate. Since thiocyanate has a much higher Stern-Volmer constant than chloride in free solution (211 versus 118, Krapf et al., 1988*b*) it would be expected to quench the dye >97% at this concentration.[2] Quenching of SPQ by thiocyanate was completely reversible and unaffected by changes in extracellular chloride concentration from 0 to 128.6

[1]According to the Stern-Volmer equation, collisional quenching is described by the reaction, $F_o/F = 1 + K_q \cdot [Q]$, where F_o and F are the fluorescence intensities in the absence and presence of the quencher, respectively, $[Q]$ is the concentration of the quencher and K_q is the Stern-Volmer constant.

[2]According to the Stern-Volmer equation (see footnote 1) the remaining magnitude of the fluorescence signal after addition of 150 mM potassium thiocyanate (K_q 211 M^{-1}) can be calculated: $F_o/F = 1 + 0.211[150] = 32.6$. Thus, fluorescence after thiocyanate (F) is reduced to ~3% of the total quenchable signal (F_o). However, K_q is probably much lower intracellularly (see Results). If the K_q for KSCN were also 10 times lower intracellularly as the K_q for chloride, F_o/F would be 4.16. Thus, 150 mM KSCN would decrease SPQ fluorescence to ~25% of the total quenchable signal.

mM. The fluorescence signal (ΔF) was therefore expressed as the ratio,

$$\Delta F = \frac{F_{Cl} - F_{SCN}}{F_{Cl=0} - F_{SCN}} \quad (1)$$

where F_{Cl} is the fluorescence intensity at a given extracellular chloride concentration, $F_{Cl=0}$ is the fluorescence intensity when tubules were perfused with symmetrical 0 chloride solutions and F_{SCN} is the fluorescence signal after addition of potassium thiocyanate.

To define the relation between SPQ fluorescence intensity and cell chloride activity, SPQ was calibrated intracellularly. Tubules were perfused symmetrically with buffered solutions (50 mM Hepes) titrated to pH 7.4 containing 7 μM nigericin (a K/H antiporter), 10 μM tributyltin (a Cl/OH antiporter), and 66 mmol/l of potassium (estimated to approximate cell potassium activity in the rabbit proximal tubule, Biagi et al., 1981) at various chloride concentrations. In this setting, nigericin abolishes transcellular H and OH ion activities gradients. When intra- and extracellular OH ion activity are the same, cell chloride activity approximately equals extracellular chloride activity by the action of tributyltin. The validity of this approach is supported by the observation from experiments with renal brush border vesicles that final fluorescence was the same when either Triton-X100 or the combination nigericin/tributyltin (in the same concentration as in this study) were used to equilibrate extra- and intravesicular chloride (Chen et al., 1988). Tubules were first perfused symmetrically with zero chloride (Solution 3, Table I). Lumen and bath solutions were then changed to the calibration solution containing the ionophores but no chloride. Subsequently, fluids were changed to solutions containing different chloride concentrations. At the end of a calibration procedure, SPQ fluorescence was quenced with potassium thiocyanate. Addition of the ionophores did not affect the fluorescence signal when tubules were perfused with symmetrical zero chloride solutions.

Toxicity Studies

To evaluate dye toxicity, oxygen consumption was measured in suspended renal proximal tubules. Tubule suspensions were prepared by modification of the techniques of Chung et al. (1982) and Sakharani et al. (1984) without collagenase digestion as described previously (Meyer and Verkman, 1987; Verkman and Wong, 1987). The suspensions contained >90% PCT as judged by light microscopy.

150 μl of tubule suspensions were preincubated with 1.8 ml of buffer (composition in mM: NaCl 147, KCl 5, $MgSO_4$ 1, Na_2HPO_4 1, $CaCl_2$ 1.8, D-glucose 5, L-lactate 2 and L-succinate 5) in the presence and absence of 20 mM SPQ for 15 min at 38°C. 10 mM NaCl was removed from the buffer containing SPQ. The rate of oxygen consumption of the tubule suspensions was measured using a Clark-type oxygen electrode and an oxymeter (model 53 Oxygen monitor; Yellow Springs Instrument Co., Yellow Springs, OH) to monitor the disappearance of oxygen from a closed, thermostatically controlled 2 ml chamber (38°).

The protein content of the samples was determined by the Lowry method. Bovine serum albumin was used as the protein standard.

All values represent means ± standard error. The intracellular calibration curve was fitted using linear regression analysis.

RESULTS

Dye Loading

Tubules were loaded with 1, 5, 10, 20, or 30 mM SPQ at 38°C from the lumen or the bath. At any SPQ concentra-

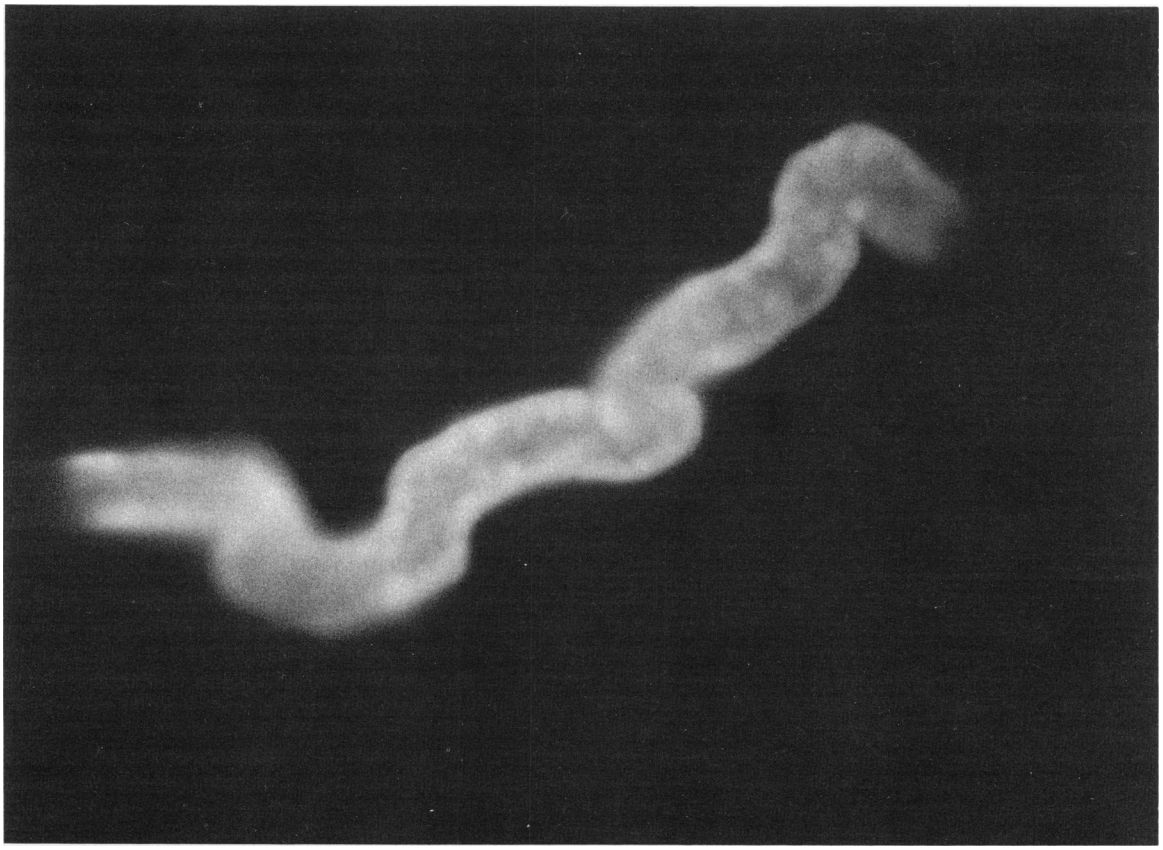

FIGURE 1. Photograph of intracellular SPQ fluorescence in a perfused PCT (250×). The fluorescence signal is homogenously distributed throughout the cell. Differences in intensities among cells are due to the convoluted nature of the PCT; only a limited number of cells are within the focal plane at one time.

tion, the fluorescence signal was maximal after 10 min and was not different whether tubules were loaded from the bath or the lumen.[3] Loading from lumen and bath simultaneously did not enhance the total signal (5 and 20 mM SPQ). With loading from the lumen, the signal to autofluorescence ratio at a SPQ concentration of 1 mM was 1.8 ± 0.4 (n = 3), at 5 mM 2.6 ± 0.5 (n = 3), at 10 mM 3.4 ± 0.3 (n = 4), at 20 mM 4.6 ± 0.6 and at 30 mM 4.5 ± 0.5 (n = 3). Subsequently, all tubules were loaded from the lumen with 20 mM SPQ for 10 min.

As illustrated by Fig. 1 all cells exhibited homogenous, blue fluorescence after SPQ was washed out of the lumen. The tubular lumen was dark and nonfluorescent. No morphological alteration of the PCT cells could be detected by phase contrast light microscopy (400x) after exposure to SPQ under experimental conditions.

When no neutral density filter was used to reduce the excitation light intensity, the apparent intracellular halftime of the dye was 7.2 ± 1.3 min (n = 5). With reduction of light intensity by a neutral density filter of 0.5 O.D. units the intracellular halftime of the dye was 8.6 ± 1.1 min in the absence and 8.8 ± 1.4 min in the presence of continued light exposure (n = 5 tubules, NS). This decrease in fluorescence therefore represents SPQ leakage from tubule cells without significant photobleaching. The decrease in fluorescence signal was a linear over the first 5 min. of recording. Thereafter, decrease of signal per unit time was progressively lower. All fluorescence measurements were restricted to the linear phase.

Intracellular Dye Calibration

SPQ was calibrated intracellularly at concentrations of 0, 10, 20, 30, 45, and 60 mM chloride.[4] Because the linear phase of dye leakage was reduced to an average of 3.5 min in the presence of tributyltin/nigericin, calibration had to be limited to three data points per tubule. As shown by Fig. 2, there is a linear correlation between intracellular chloride concentration and changes in fluorescence (r = 0.97). Data were fitted to the Stern-Volmer equation with an intracellular quenching constant K_q of a 12 M^{-1}. A 1 mM change in intracellular chloride concentration is associated with a 1.2% change in fluorescence signal. To obtain chloride activity from solution chloride concentration, multiply by the activity coefficient for NaCl at 0.15 M and 38°C, 0.749.

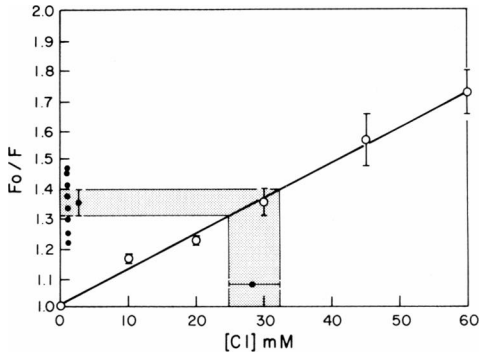

FIGURE 2 Intracellular calibration of SPQ. Fluorescence in the absence of chloride divided by fluorescence in the presence of chloride (F_o/F) is plotted against the chloride concentration (intracellular Stern-Volmer plot). The data shown as open symbols (mean ± SEM) were obtained by the calibration procedure (see Methods) and represent results from eight tubules at 10 and 20 mM chloride, from six tubules at 30 mM chloride and from five tubules at 45 and 60 mM chloride. Closed symbols represent the individual results from eight tubules, when fluorescence was recorded after perfusates were changed from bilateral zero chloride solutions to solutions containing 146.1 mM chloride in the lumen and 128.6 mM chloride in the bath. Plotting the mean of fluorescence ± SEM in the calibration curve yields a cell chloride concentration of 27.5 ± 5 mM (activity 20.6 mM) (*shaded area*).

Estimation of Intracellular Chloride Activity

Two independent methods were used to estimate cell chloride activity. First, tubules were initially perfused symmetrically with zero chloride (solution 3, Table I) and fluorescence was recorded continuously. Solutions were then changed to the same solutions characteristic for the late PCT as above (solution 1 bath, solution 2 lumen, Table I). Thereafter, solutions were changed to 150 mM KSCN to quench SPQ. The change in fluorescence induced by the chloride containing solutions was then calculated by Eq. 1 (see Methods) and plotted into the calibration curve obtained from other tubules (see above). Fig. 2 shows the fluorescence changes and the corresponding intracellular chloride concentration in eight tubules. The mean intracellular chloride concentration was 27.5 ± 5 mM (chloride activity 20.6 mM).

Second, tubules were perfused with solutions physiological for the late PCT (solution 1 bath, solution 2, high chloride, lumen, Table I). Luminal and bath perfusates were then changed to a calibration solution (see Methods) containing chloride solutions anticipated to be either above or below cell chloride concentration (null-method). After it was established that cell chloride concentration was between 0 and 60 mM (see typical study, Fig. 3), chloride concentrations were progressively increased (10, 15, 22.5 mM) and lowered (45, 37.5, and 30 mM), respectively. With this method, cell chloride concentration was bracketed consistently between 22.5 and 30 mM (activity 16.8 to 22.5 mM) in six tubules (see typical study, Fig. 4), in

[3]When tubules were inspected with high power magnification (400×), a few tubules loaded from the bath showed some fluorescence signal concentrated on interstitial material sometimes not fully removed by micro-dissection. We believe that SPQ adheres to this peritubular, interstitial tissue. The phenomenon was never seen on the apical membrane with luminal loading.

[4]Unless stated otherwise chloride concentrations rather than activities are used to describe experimental conditions.

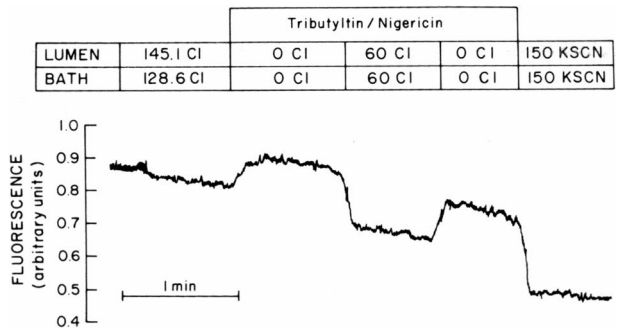

FIGURE 3 Estimate of cell chloride activity: null method. Bath and lumen perfusates are changed from solutions characteristic for the late PCT to 0 and 60 mM chloride calibration solutions containing the ionophores nigericin and tributyltin. At the end of the procedure SPQ is quenched by KSCN in the presence of 5 μM valinomycin.

agreement with the data obtained by the direct calibration method.

Measurement of the Transient in Cell Chloride Activity in Response to Changes in Bath Chloride Concentration

Because the response time of SPQ to changes in chloride concentration is very fast (<1 ms, Illsley and Verkman, 1987), intracellular SPQ fluorescence recordings are well suited to examine the kinetics of chloride influx or efflux when bath chloride is changed. Fig. 5 shows a typical study of the effects of repeated addition and removal of 128.6 mM bath chloride in the absence of luminal chloride. It is clear from Fig. 5 that chloride influx and efflux rates are very fast. The initial rate of chloride influx after a change in bath chloride from 0 to 128.6 mM was 5.3 ± 0.2 mM/s. This value was calculated from the initial slopes of the downward deflections (fluorescence units/time), the total quenchable fluorescence signal and the Stern-Volmer constant 12 M^{-1} using equations reported previously (Chen et al., 1988).[5] It is notable that the initial curve slopes of the upward deflections (bath chloride changed from 128.6 to 0 mM chloride, chloride efflux) are lower than those of the downward deflections because of the hyperbolic calibration between SPQ fluorescence and cell chloride activity.

O_2-Consumption

In the absence of SPQ, oxygen consumption of tubule suspensions was 34.8 ± 1.9 nmol O_2/mg protein/min

[5]It was calculated from Fig. 5 that the initial rate of Cl influx in response to an increase in bath Cl from 0 to 128.6 mM was 5.3 mM/s. The halftime for the transient can be estimated by dividing an estimated steady-state cell Cl concentration in the presence of 128.6 mM bath and 0 lumen Cl by the initial Cl flux (5.3 mM/s). A halftime of 2.8 s is obtained for this calculation if a cell chloride concentration of 15 mM, a value midway between 0 and the cell chloride concentration of 27.5, is assumed.

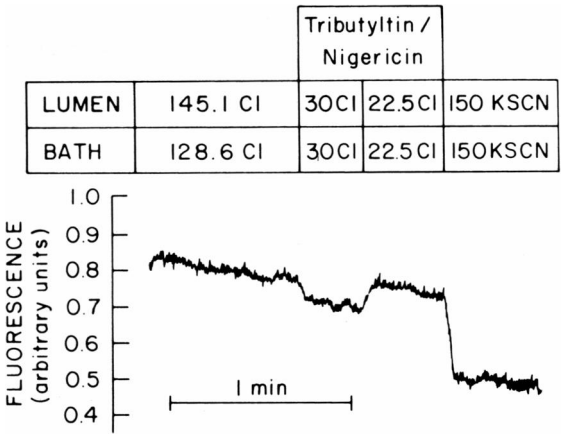

FIGURE 4 Estimate of cell chloride activity: null method. By extrapolation, the fluorescence signal for the chloride activity associated with physiological perfusates falls between the fluorescence signal associated with the calibration solutions containing 30 and 22.5 mM chloride, respectively. At the end of the procedure, SPQ is quenched by KSCN in the presence of 5 μM valinomycin.

(n = 4). In the presence of 20 mM SPQ, the tubules consumed oxygen at a rate of 36.3 ± 2.2 nmol/mg protein/min (n = 4, NS).

DISCUSSION

These studies show that the chloride-sensitive fluorophore SPQ can be loaded into cells of an intact epithelial tissue, the rabbit PCT. SPQ is a zwitterionic molecule with a high water solubility (0.09 M), low water:octanol partition coefficient (0.01; Krapf et al., 1988*b*) and thus a relatively low diffusability through lipid bilayers. High SPQ concentrations (20 mM) are required to load tubules optimally at 38°C within 10 min. Light microscopic inspection (maximal magnification 400×) of the tubules after washout of the dye showed that the lumen was free of fluorophore and that the dye was distributed evenly throughout the cytoplasm (see Fig. 1). This suggests that the dye is not compartmentalized within the cells. Given the limitations of optical resolution we cannot be certain that SPQ does not bind to intracellular organelles or structures of the cytoskeleton (see below). It is not known definitively how SPQ penetrates into PCT cells. However, because cell staining is homogeneous and SPQ permeability in the intact tubule is comparable with that in brush border membrane vesicles from rabbit renal cortex (Chen et al., 1988) it is likely that SPQ uptake occurs by diffusion rather than endocytosis.

Direct, functional proof for the conclusion that SPQ fluorescence is a measure of intracellular chloride activity is provided by the calibration procedure (see original tracings in Figs. 3 and 4). When extracellular solutions were changed from physiologically high chloride concentrations (128.6 mM in the bath, 146.1 in the lumen) to the calibration solutions with much lower extracellular chlo-

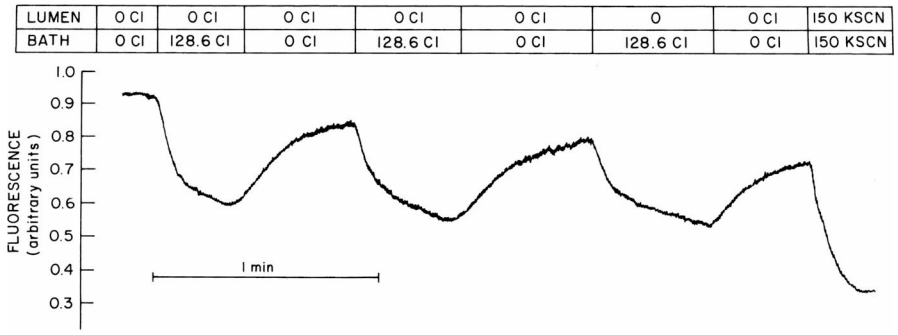

FIGURE 5 Effect of changes in bath chloride on intracellular SPQ fluorescence. 128.6 mM chloride is added and removed repeatedly from the bath with 0 chloride in the lumen. Leakage progressively decreases the absolute change in fluorescence induced by the bath chloride change. At the end of the experiment, SPQ is quenched by KSCN in the presence of 5 μM valinomycin.

ride concentrations (60 mM in the tracing of Fig. 3 and 30 mM in the tracing of Fig. 4) the fluorescence signal decreased, corresponding to increased SPQ quenching. Addition of a solution with an extracellular chloride activity above the physiological cell chloride activity lead—through the action of the ionophores tributyltin and nigericin—to a rapid increase in intracellular chloride activity and, therefore, quenching of SPQ. It is evident, that the reverse would have occurred if the fluorescence changes reflected changes in chloride activity on the extracellular surface of the PCT cells.

Using the calibration method described in this study, we found a linear relationship between SPQ fluorescence and cell chloride concentration between 0 and 60 mM (cell chloride activity 0–44.9 mM, Fig. 2) as has been described for SPQ quenching in free solution (Illsley and Verkman, 1987). The Stern-Volmer constant for SPQ quenching by chloride is 118 M^{-1} in free solution, whereas, intracellularly it is 12 M^{-1}. Thus, SPQ is ~10 times less sensitive to chloride within the cell than in free solution. The decreased intracellular chloride sensitivity of SPQ could be artifactual if the ionophores did not fully equilibrate intra- and extracellular chloride activities. This explanation is unlikely, because SPQ fluorescence rapidly increased or decreased when the calibration solutions were changed to those with chloride activity closely above or below cell chloride activities and because the intracellular calibration curve was linear. In addition, studies using renal brush border vesicles have shown that final fluorescence was the same when either Triton-X100 or the combination of nigericin/tributyltin were used to equilibrate intra- and extravesicular chloride activities (see Methods, Chen et al., 1988).

Three mechanisms might account for the reduced intracellular sensitivity to SPQ to chloride. (*a*) Interactions of intracellular SPQ with organic anions including proteins could cause a decrease in SPQ lifetime and a consequent decrease in SPQ chloride sensitivity. We found that addition of 20% albumin to the buffer solutions decreased the Stern-Volmer constant for chloride by 38%. To determine whether a change in *intracellular* anionic charge could affect SPQ fluorescence, it was shown in 3 tubules that in the absence of chloride, SPQ fluorescence was unaffected by external pH (6.8, 7.4 and 8.0, in the presence of nigericin/tributyltin). While the results of albumin in free solutions suggest a role for an interaction of SPQ with charged proteins, the effects of pH on intracellular anionic charges are probably too small to affect fluorescence. (*b*) Intracellular viscosity could limit chloride diffusion and thus the rates of collisional quenching. When 1.6 M sucrose were added to the buffer solutions to increase viscosity 10-fold, we found that the Stern-Volmer constant for quenching of SPQ by chloride decreased by 40%. (*c*) A physical barrier for chloride access to SPQ due to intracellular SPQ binding could also limit sensitivity. Such a possibility is supported by the in vitro observation that SPQ bound to triethylsilane-covered glass had a reduced Stern-Volmer constant for chloride (Urbano et al., 1984). Based on these observations we conclude that the mechanism for the reduced intracellular sensitivity of SPQ to chloride is likely to be multifactorial. Interactions of SPQ with intracellular anions, effects of intracellular viscosity, intracellular SPQ binding, all may contribute to this phenomenon.

Estimates of cell chloride activity when PCT were perfused with solutions characteristic for the late proximal tubule (high luminal chloride concentration) with two independent methods gave very similar results. Using the null-method, cell chloride concentration could be bracketed consistently between 22.5 and 30 mM (activities 16.8 and 22.5 mM, Fig. 4). When the changes in fluorescence signal associated with these perfusates (relative to the fluorescence signal associated with zero chloride and potassium thiocyanate solutions, respectively) were plotted into the calibration curve obtained from a different group of tubules, the resultant cell chloride concentration was 27.5 ± 5 mM (activity 20.6 mM, Fig. 2). These cell chloride activities are above electrochemical equilibrium for chloride.[6] Two groups have found comparable cell

[6]The electrochemical equilibrium is calculated according to the Nernst equation, $\Delta\psi = RT/F \ln [CL_i]/[Cl_o]$, where the subscripts i and o denote

chloride activities in the rabbit proximal straight tubule using microelectrodes. Sasaki et al. (1987) reported activities of ~20 mM and Kondo and Froemter found a cell chloride activity in this segment between 25 and 30 mM (personal communication). In both studies, the cell chloride activities were also above electrochemical equilibrium.

Our study thus demonstrates that the halide-specific fluorophore SPQ can be used to estimate cell chloride acitivity and to record rapid changes in cell chloride activity in the PCT. In addition, the SPQ has no apparent cell toxicity as determined by its lack of influence on cell respiration. However, the present study defines several limitations which will require further technical modifications: (*a*) SPQ is only moderately diffusible across cell membranes and leaks out significantly after 5–10 min. (*b*) Peak excitation at 350 nm makes autofluorescence of cells (NADH) and microscope optics quantitatively important. In addition, excitation at 350 nm causes interference with widely used inhibitors (i.e., disulfonic stilbenes and amiloride). However, this latter problem can be eliminated by using protonated stilbene derivatives or amiloride analogues which are non-fluorescent. (*c*) The absolute fluorescence signal depends on the intracellular dye concentration. Since the linear phase of dye leakage at 38°C was only 5 min, the time for reproducible measurements of chloride acitivity is quite limited. (*d*) The marked reduction of chloride-sensitivity of SPQ within the intracellular environment (1.2% change in fluorescence for 1 mM change in intracellular chloride concentration or 0.75 mM change in cell chloride activity limits sensitivity to small changes in cell chloride activity. This low intracellular sensitivity makes it difficult to study specific transport systems by comparison of steady state cell chloride acitivities, because microelectrode studies suggest that changes in cell chloride activity are quite small (<5 mM) when an isolated chloride transport system is examined (Sasaki et al., 1987, Ishibashi et al., 1987).

To overcome these current limitations, future developments should be directed at increasing the membrane permeability and the intracellular halftime of the dye, by addition of acetoxymethyl groups to the quinoline backbone. In addition, a ratiometric determination of the fluorescence signal would be helpful. It has been suggested that this could be accomplished by conjugation of a chloride-insensitive chromophore, such as dansyl, to the chloride-sensitive molecule (Verkman, et al., 1988). Work from our laboratory has established a set of empiric guidelines for the synthesis of these tailored chloride-sensitive fluorescent indicators (Krapf et al., 1988*b*).

In conclusion, these studies show the feasibility of loading and calibrating SPQ in intact cells of the rabbit proximal convoluted tubule. Cell chloride activity can be estimated accurately and rapid transients in cell chloride activity can be measured. The easy applicability of the method described may make it useful to estimate cell chloride activity and measure chloride transients in cultured cells and in other intact tissues. Improved chloride-sensitive indicators are expected to facilitate greatly the examination of chloride transport in intact cells.

We thank Dr. Jill Eveloff for assistance in the determination of oxygen consumption. Dr. Verkman is an established investigator of the American Heart Association.

This work was supported by National Institutes of Health grants DK39354, AM26142 and DK35124, a grant from the National Cystic Fibrosis Foundation and a grant-in-aid from the American Heart Association with partial support provided by the Long Beach American Heart Association Chapter. Dr. Krapf is the recipient of a generous grant from the Swiss Foundation for biological and medical research. The authors are grateful to Dr. F. C. Rector, Jr., for advice and support during the performance of these studies.

Received for publication 23 September 1987 and in final form 3 December 1987.

REFERENCES

Alpern, R. J., K. J. Howlin, and P. A. Preisig. 1985. Active and passive components of chloride transport in the rat proximal convoluted tubules. *J. Clin Invest.* 76:1360–1366.

Biagi, B. A., M. Sohtell, and G. Giebisch. 1981. Intracellular potassium activity in the rabbit proximal straight tubule. *Am. J. Physiol.* 241.

Burg, M., J. Grantham, M. Abramow, and J. Orloff. 1966. Preparation and study of fragments of the single rabbit nephron. *Am. J. Physiol.* 210:1293-1298.

Chen, P.-Y., N. P. Illsley, and A. S. Verkman. 1988. Renal brush border chloride transport mechanisms characterized using a fluorescent indicator. *Am. J. Physiol.* 254: F114–F120.

Chen, P.-Y., and A. S. Verkman. 1988. Sodium-dependent chloride transport in basolateral membrane vesicles isolated from rabbit proximal tubule. *Biochemistry.* 27:655–660.

Chung, S. D., R. B. Cooper, A. C. Young, C. J. Martin, and B. Ancher-Johnson. 1982. Characterization of primary rabbit kidney cultures that express proximal tubule functions in a hormonally defined medium. *J. Cell. Biol.* 95:118–126.

Illsley, N. P., and A. S. Verkman. 1987. Membrane chloride transport measured using a chloride-sensitive fluorescent probe. *Biochemistry.* 26:1215–1219.

Ishibashi, K., S. Sasaki, N. Yoshiyama, T. Shiigai, and J. Takeuchi. 1987. Intracellular chloride activity of rabbit proximal straight tubules. *Kidney Int.* 31:437. (Abstr.)

Krapf, R., R. J. Alpern, F. C. Rector, Jr., and C. A. Berry. 1988. Basolateral membrane Na/base cotransport is dependent on CO_2/HCO_3 in the proximal convoluted tubule. *J. Gen. Physiol.* 90:833–853.

Krapf, R., R. J. Alpern, C. A. Berry, and F. C. Rector, Jr. 1988*a*. Regulation of cell pH by ambient pH, HCO_3 and P_{CO2} in the rabbit proximal convoluted tubule. *J. Clin. Invest.* 81:381–389.

Krapf, R., N. P. Illsley, H. C. Tseng, and A. S. Verkman. 1988*b*. Structure-activity relationships for chloride-sensitive fluorescent dyes for biological applications. *Anly. Biochem.* 169:142–150.

inside and outside and ψ, R, T and F have their usual meaning. Assuming $\Delta\psi = -55$ mV (Kuwahara, M., F. C. Rector, Jr., and C. A. Berry, manuscript submitted for publication.) and an activity coefficient for both intra- and extracellular chloride of 0.75, the electrochemical equilibrium for chloride is 12.4 mM for an extracellular chloride concentration of 128.6 mM and 14.0 mM for an extracellular chloride concentration of 145.1 mM.

Meyer, M. M., and A. S. Verkman. 1987. Evidence for water channels in renal proximal tubule cell membranes. *J. Membr. Biol.* 96:107–119.

Sakharani, L. M., B. Badie-Dezfooly, W. Trizna, N. Mikhail, A. G. Lowe, M. Taub, and L. G. Fine. 1984. Transport and metabolism of glucose in renal proximal tubular cells in primary culture. *Am. J. Physiol.* 246 (Renal Electrolyte Fluid Physiol. 15):F757–764.

Sasaki, S., K. Ishibashi, N. Yoshiyama, T. Shiigai, and J. Takeuchi. 1987. Cl/HCO_3 exchange at basolateral membrane of rabbit proximal tubule. *Kidney Int.* 31:415. (Abstr.)

Urbano, E., H. Offenbacher, and O. S. Wolfbeis. 1984. Optical sensor for continuous determination of halides. *Analy. Chem.* 56:427–429.

Verkman, A. S., and K. R. Wong. 1987. Proton nuclear magnetic resonance measurement of diffusional water permeability in suspended renal proximal tubules. *Biophys. J.* 51:717–723.

Verkman, A. S., P.-Y. Chen, B. Davis, P. Fong, N. P. Illsley, and R. Krapf. 1988. Development of chloride-sensitive fluorescent indicators. *In* Cellular and Molecular Basis of Cystic Fibrosis. G. Mastella and P. M. Quinton, editors. San Francisco Press. 471–478.

Wolfbeis, O. S., and E. Urbano. 1982. Synthesis of fluorescent dyes. XIV. Standards for fluorescence measurements at near neutral pH-range. *J. Heterocycl. Chem.* 19:841-843.

Wolfbeis, O. S., and E. Urbano. 1983. Eine fluorimetrische, schwermetallfreie *Methode zur Analyse* von Chlor, Brom and Iod in organischen *Materialien. Fresenius Z. Anal. Chem.* 314:577–581.