Assessment of Swelling-Activated Cl^- Channels Using the Halide-Sensitive Fluorescent Indicator 6-Methoxy-*N*-(3-Sulfopropyl)quinolinium

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ABSTRACT This study describes a quantitative analysis of the enhancement in anion permeability through swelling-activated Cl^- channels, using the halide-sensitive fluorescent dye 6-methoxy-*N*-(3-sulfopropyl)quinolinium (SPQ). Cultured bovine corneal endothelial monolayers perfused with NO_3^- Ringer's were exposed to I^- pulses under isosmotic and, subsequently, hyposmotic conditions. Changes in SPQ fluorescence due to I^- influx were significantly faster under hyposmotic than under isosmotic conditions. Plasma membrane potential (E_m) was −58 and −32 mV under isosmotic and hyposmotic conditions, respectively. An expression for the ratio of I^- permeability under hyposmotic condition to that under isosmotic condition (termed enhancement ratio or ER) was derived by combining the Stern-Volmer equation (for modeling SPQ fluorescence quenching by I^-) and the Goldman flux equation (for modeling the electrodiffusive unidirectional I^- influx). The fluorescence values and slopes at the inflection points of the SPQ fluorescence profile during I^- influx, together with E_m under isosmotic and hyposmotic conditions, were used to calculate ER. Based on this approach, endothelial cells were shown to express swelling-activated Cl^- channels with ER = 4.9 when the hyposmotic shock was 110 ± 10 mosM. These results illustrate the application of the SPQ-based method for quantitative characterization of swelling-activated Cl^- channels in monolayers.

INTRODUCTION

Cell volume control is a homeostatic phenomenon. After swelling in response to hyposmotic shock, most epithelial cells are capable of returning to their original cell volume in a process referred to as regulatory volume decrease (RVD) (Hoffmann and Dunham, 1995). A principal mechanism underlying RVD entails the efflux of K^+ and Cl^- through their respective conductive pathways, leading to an obligatory loss of water and a concomitant volume decrease (Hoffmann and Dunham, 1995). Under resting conditions, epithelial cells possess substantial permeability to K^+, so that an activation of Cl^- channels can be expected to facilitate RVD. Accordingly, several investigations have been successful in identifying the opening of swelling-activated Cl^- channels as an acute response to hyposmotic swelling. These channels are characterized by 1) outward rectification; 2) inhibition by 4,4′-diisothiocyanatostilben-2,2′-disulfonic acid (DIDS), 5-nitro-2-(3-phenyl-propylamino)-benzoic acid (NPPB), and tamoxifen; 3) higher permeability to I^- and NO_3^- compared to Cl^-; and 4) permeability to organic osmolytes (e.g., taurine, myoinositol; Strange et al., 1996; Brochiero et al., 1995; Nilius et al., 1996; Woll et al., 1996).

Previously, activation of swelling-activated Cl^- channels has been assessed by $^{36}Cl^-$ fluxes (e.g., Rugolo et al., 1992), electrophysiological protocols (i.e., measurement of transepithelial short-circuit current; e.g., McEwan et al., 1992; Koslowsky et al., 1994), and a fluorescence technique based on the halide-sensitive fluorescent dye 6-methoxy-*N*-(3-sulfopropyl)quinolinium (SPQ) or its analogs (Dho and Foskett, 1993; Ehring et al., 1994; Brochiero et al., 1995; Woll et al., 1996). These procedures have been supplemented with patch-clamp techniques to characterize the pharmacology, single-channel conductance, ion selectivity, and gating (Ehring et al., 1994; Brochiero et al., 1995; Woll et al., 1996) of swelling-activated Cl^- channels. As an alternative to using radioisotopes, the fluorescence technique offers higher temporal resolution and sensitivity as well as ease of use. In addition, where the measurement of transepithelial short-circuit current is inapplicable (e.g., nonconfluent cultured epithelial cells or cells in suspension) or tedious (e.g., in monolayers of leaky epithelium), a quantitative fluorescence protocol would be useful. Thus far, however, use of SPQ has been limited to qualitative indications of the increase in membrane anion permeability upon activation of swelling-activated Cl^- channels (Brochiero et al., 1995; Dho and Foskett, 1993; Ehring et al., 1994; Woll et al., 1996). A quantitative assessment, on the other hand, requires consideration of the nonlinear dependence of SPQ fluorescence on changes in $[Cl^-]_i$ and competitive quenching of SPQ fluorescence by unknown intracellular organic anions and proteins (collectively referred to as the intracellular quenchers), among other factors. (The presence of intracellular quenchers is apparent in the significantly low quenching constant for Cl^- noted during $[Cl^-]_i$ measurements. The quenching constant for Cl^- is ~15 M^{-1} in cells (Foskett, 1990; Verkman, 1990; Bonanno et al., 1995; Brochiero et al., 1995) compared to 120 M^{-1} in vitro (Verkman, 1990).) For example, the concentration of intracellular quenchers is dependent on cell volume, and there-

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fore volume changes per se affect SPQ fluorescence. In other words, the apparent quenching constant for $[Cl^-]_i$ is a function of cell volume (Srinivas and Bonanno, 1997a). When these factors are considered, SPQ can be used for quantitative characterization of swelling-activated Cl^- channel activity in undissociated confluent or nonconfluent cell populations, and serve as a useful supplement to patch-clamp protocols.

The objective of this study was to reexamine the use of SPQ to assess activation of swelling-activated Cl^- channels from a quantitative standpoint. We express the increase in anion permeability in the form of a ratio, referred to as the enhancement ratio (ER), given by I^- permeability under hyposmotic conditions compared to that under isosmotic conditions. For the purpose of demonstrating the applicability of the approach, we have examined swelling-activated Cl^- channels in cultured bovine corneal endothelial cells, which possess a swelling-activated anion conductance that is sensitive to NPPB, DIDS, and tamoxifen (Srinivas and Bonanno, 1996).

MATERIALS AND METHODS

Solutions

All experiments were conducted with HCO_3^--free Ringer's solutions (pH 7.5) equilibrated with air, which was bubbled through NaOH to remove traces of CO_2. Two types of Ringer's were used. Type I consisted of (mM) 145 Na^+, 4 K^+, 0.6 Mg^{2+}, 1.4 Ca^{2+}, 145 NO_3^-, 1 HPO_4^{2-}, 10 $HEPES^-$, 3.5 gluconate, 5 glucose. Type II was the same, except that Na^+ and NO_3^- were reduced to 85 mM. Corresponding Cl^- and I^- Ringer's solutions were obtained by replacing NO_3^- with Cl^- or I^-. For both types of Ringer's, isosmotic solutions were adjusted to 300 ± 5 mosM with sucrose or mannitol. Type I hyposmotic Ringer's was made by dilution with deionized water. Type II hyposmotic Ringer's was made by omission of sucrose or mannitol. Osmolality was measured with a vapor pressure osmometer (model 5500; Wescor, Logan, UT).

Experimental protocol for the estimation of the enhancement ratio

We define the enhancement ratio (ER) as the ratio of I^- permeability under hyposmotic conditions (P_H) to that under isosmotic conditions (P_I). Its estimation was carried out in two independent experimental steps. This required factoring of ER into ER_U and R_E (see Results). For a specific hyposmotic shock, the factor ER_U, referred to as uncorrected ER, was determined based on SPQ fluorescence measurements. Then, the factor R_E, which is dependent on swelling-induced changes in E_m, was obtained for the same osmotic shock through a patch-clamp protocol in separate cells.

ER was determined with I^- as the model anion because it is able to permeate through swelling-activated Cl^- channels (Strange et al., 1996; Nilius et al., 1996) and offers the experimental convenience of being able to quench SPQ fluorescence efficiently (Verkman, 1990). (SPQ quenching constants of SPQ in Ringer's (Verkman, 1990) are 118 M^{-1} (for Cl^-), 175 M^{-1} (for Br^-), 276 M^{-1} (for I^-), 4 M^{-1} (for HCO_3^-), 7 M^{-1} (for gluconate), and 0 M^{-1} (for NO_3^-).) In addition, I^- is not a substrate for Cl^--dependent carriers, and its fluxes across the plasma membrane, therefore, occur largely through conductive pathways (see Discussion). Furthermore, to elicit SPQ fluorescence changes selectively for I^- influx alone, cells were first depleted of Cl^- by a prolonged exposure to Cl^--free Ringer's (NO_3^- substituted for Cl^-) during the SPQ loading procedure, as explained below. Although NO_3^- is permeable through swelling-activated Cl^- channels (Strange et al., 1996; Nilius et al., 1996), it does not quench the fluorescence of SPQ ($K_{NO3-} \approx 0.5\ M^{-1}$). Therefore, changes in SPQ fluorescence result from I^- influx alone.

For the calculation of ER_U, relative changes in SPQ fluorescence were measured by exposing the cells, under NO_3^- perfusion, to I^- Ringer's for a brief period of time (60–120 s) under isosmotic and, subsequently, hyposmotic conditions. The resulting changes in SPQ fluorescence were then measured and employed to calculate maximum rates of change of SPQ fluorescence. This was computed based on the first time derivative of the SPQ fluorescence profile, determined with the Savitzky-Golay convolution algorithm (Table 2D Software; Jandel Scientific, San Rafael, CA).

Measurement of SPQ fluorescence

The bovine corneal endothelial cells were cultured to confluence as a monolayer on glass coverslips (Bonanno et al., 1995; Bonanno and Srinivas, 1997; Srinivas and Bonanno, 1997a). The cells were loaded with SPQ at room temperature by a 6-min exposure to hyposmotic Ringer's (180 mosM NO_3^- Ringer's) containing 20 mM SPQ (Bonanno and Srinivas, 1997; Srinivas and Bonanno, 1997a). After loading, the cells were allowed to recover from the hyposmotic shock for 30–40 min in isosmotic NO_3^- Ringer's, also containing 20 mM SPQ. Subsequently, the adhering dye was washed off with isosmotic NO_3^- Ringer's, and the coverslip was mounted in a perfusion chamber (volume = 80 μl) designed for fluorescence measurement with an inverted microscope (Bonanno and Srinivas, 1997; Srinivas and Bonanno, 1997a). The flow of the perfusate, >3 vol/min, was achieved by gravity through PE-50 tubing. Ringer's solutions were placed in hanging syringes in a cylindrical Plexiglas chamber maintained at 37°C, and the perfusate was selected by means of an eight-way valve.

Excitation for SPQ fluorescence, centered at 365 ± 10 nm, was reflected to the microscope objective (40× Fluor; 1.3 N.A., Nikon) by a dichroic mirror centered at 400 nm. Neutral density filters (2.5–3.5 OD) were included in the excitation path to minimize photobleaching. The fluorescence emission from the cells was collected by the objective and passed through the dichroic and a barrier filter (450 ± 25 nm) to an intensified CCD camera (model C2400, 8 bits/pixel; 540 × 480 pixels resolution; Hamamatsu Photonics, Bridgewater, NJ). The fluorescence images, acquired at 4-s intervals with a frame grabber (Image-LC; Matrox Electronics Systems, Dorval, QC, Canada), were averaged over eight frames to yield average pixel intensities for predefined regions of interest (ROIs), using MetaFluor Imaging software (Universal Imaging Systems, Chester, PA). Generally, four or five ROIs, each consisting of 10–20 cells, were selected for each field of view. To minimize photobleaching, the cells were exposed to the excitation light synchronous with sampling by the use of a shutter (Lambda-10; Sutter Instruments, Novato, CA). The background fluorescence was determined by quenching SPQ fluorescence with 150 mM SCN^- (Bonanno et al., 1995; Bonanno and Srinivas, 1997; Srinivas and Bonanno, 1997a). Although the microscope objective was of high numerical aperture, its depth of focus was large enough that the fluorescence response collected by the objective integrated the complete thickness of the cells (~5 μm). This ensured that the fluorescence responses are not influenced by the potential loss of focus upon cell swelling. This was tested in independent experiments in which it was found that fluorescence from 440-nm excitation of cells loaded with 2′,7′-bis-2-carboxyethyl-5-carboxyfluorescein (BCECF) remained unaltered in response to changes in cell volume (shrinkage or swelling). Because emission from 440-nm excitation is insensitive to changes in cytosolic pH, lack of change in the signal indicates that the objective could not optically section the cells.

Measurement of membrane potential (E_m)

Cells were seeded onto coverslips and cultured to form confluent colonies consisting of 10–100 cells within 2 days. The culture conditions were the same as described earlier (Bonanno and Srinivas, 1997), except that the medium was without growth factor. The coverslip with the cell colonies was held at the bottom of a Lucite perfusion chamber maintained at 35°C.

Perfusion was achieved by gravity at a rate of 1 ml/min, and the fluid level in the perfusion chamber was controlled by continuous suction. Quantitative changes in E_m were measured by following the zero-current potential (V_0) of a colony of cells with the whole-cell patch-clamp technique (Watsky and Rae, 1991; Segawa and Hughes, 1994). Zero-current potential was recorded with an Axopatch 200 patch clamp amplifier (Axon Instruments, Foster City, CA) in current clamp mode. The output was filtered at 1 kHz and acquired at 10 Hz with the Fetchex program (version 6.0; Axon Instruments). The external solutions were the same as described above. Pipette internal solution contained 2 or 4 mM K_2ATP, 30 mM KCl, 83 mM K-gluconate, 5.5 mM EGTA-KOH, 0.5 mM $CaCl_2$, 4 mM $MgCl_2$, and 10 mM HEPES (280 ± 5 mosM; pH 7.2). Pipettes were pulled from 7052 glass (Garner Glass, Claremont, CA) with a multistage programmable puller (model P-87; Sutter Instruments, San Rafael, CA). Before their use, pipette tips were heat-polished so that tip resistance was between 3 and 5 MΩ when they were filled with the internal solution and placed in the isosmotic external solution.

Liquid junction potentials arising between the bathing and pipette solutions and at the interface between the bathing solution and the agar bridge were calculated with the software program JPCalc (Barry, 1993). Membrane potentials were corrected for the tip potential (TP) (+13 mV) and the liquid junction potential at the agar bridge, produced by changing the bath solution from 85 mM NaCl Ringer's to 85 mM $NaNO_3$ Ringer's (ΔLJP_{bridge}; −2 mV) as follows: $E_m = V_0 - TP - \Delta LJP_{bridge}$. For the case in which the bath solution was changed from 85 mM NaCl to 85 mM NaI, the calculated liquid junction potential was less than 1 mV and was ignored.

Chemicals

SPQ was obtained from Calbiochem (San Diego, CA) or Molecular Probes (Eugene, OR). Cell culture supplies were obtained from GibcoBRL (Grand Island, NY). All other chemicals were obtained from Sigma (St. Louis, MO).

RESULTS

Theory for the estimation of ER

We have defined ER as

$$\mathrm{ER} = \frac{P_H}{P_I} \tag{1}$$

where P_H and P_I are the permeability coefficients for I^- under hyposmotic and isosmotic conditions, respectively, governing I^- flux through conductive pathways. As a first approximation, these fluxes are assumed to follow the Goldman equation (Hallows and Knauf, 1994):

$$J = P\left(\frac{zFE_m}{RT}\right)\frac{\left[C_o^I(t) - C_i^I(t)\exp\left(\frac{zFE_m}{RT}\right)\right]}{\left[1 - \exp\left(\frac{zFE_m}{RT}\right)\right]} \tag{2}$$

where J is the flux of I^-, whose external concentration is $C_o^I(t)$ and whose intracellular concentration is $C_i^I(t)$ (also denoted by $[I^-]_i$), P is the permeability coefficient for I^-, E_m is the membrane potential, R is the gas constant, T is absolute temperature, F is the Faraday constant, and z is the valence of I^-. To relate J to changes in $[I^-]_i$, quenching of SPQ fluorescence (denoted by f) is modeled by an extended form of the Stern-Volmer equation (Srinivas and Bonanno, 1997a):

$$\frac{f_o - f_{ex}}{f - f_{ex}} = 1 + K_P C^P + K_I C_i^I \tag{3}$$

where f_{ex} is the background fluorescence, f_0 is the fluorescence in the absence of quenchers, C^P represents an apparent concentration of endogenous intracellular quenchers (ICQs), K_P represents an apparent quenching constant for the ICQs, and K_I is the quenching constant of I^-. In Eq. 3, it is assumed that ICQs also quench SPQ fluorescence by a collisional mechanism (Chao et al., 1989; Srinivas and Bonanno, 1997a).

To calculate ER with Eqs. 2 and 3, two simplifying assumptions are made:

1. During I^- pulses, C^P remains constant. This assumption removes the requirement for an explicit knowledge of K_p and C^P, which are unknown (Srinivas and Bonanno, 1997a). Because ICQs are entrapped in the cells (Srinivas and Bonanno, 1997a), changes in C^P would be inversely proportional to changes in cell volume. However, because NO_3^- and I^- have approximately equal permeabilities through swelling-activated Cl^- channels (Strange et al., 1996; Nilius et al., 1996), it can be assumed that I^- pulses do not perturb cell volume significantly.

2. We assume that $C_i^I \approx 0$ during I^- pulses, to remove nonlinear terms in the numerator of Eq. 2. This assumption is reasonable, because the I^- pulses are short (60–120 s), and the intracellular concentration of I^- is generally less than 5% of its external concentration (see Discussion). Because I^- quenches SPQ fluorescence very efficiently, even small changes in I^- concentration are measurable with high signal-to-noise ratio.

Using these assumptions, Eqs. 1, 2, and 3 can be combined to express ER as a function of two factors (see Appendix):

1. The uncorrected enhancement ratio, denoted by ER_U, which can be estimated from SPQ fluorescence measurements alone; and

2. R_E, a correction factor dependent on E_m during isosmotic and hyposmotic conditions. The resulting equation for ER is

$$\mathrm{ER} = R_E \mathrm{ER}_U \tag{4}$$

where

$$R_E = \left[\frac{\Phi_I}{\Phi_H}\right] \tag{5}$$

and

$$\mathrm{ER}_U = \left(\frac{\Pi_I}{\Pi_H}\right)\left[\frac{(f - f_{ex})_I^2}{(f - f_{ex})_H^2}\right]\left[\frac{\left(\frac{\partial f}{\partial t}\right)_H}{\left(\frac{\partial f}{\partial t}\right)_I}\right] \tag{6}$$

Π_I and Π_H are the osmolarities of the isosmotic and hyposmotic Ringer's. Similarly, Φ_I and Φ_H are functions of E_m under isosmotic and hyposmotic conditions.

Experimental determination of uncorrected ER (ER_u)

Fig. 1 *A* shows a typical SPQ fluorescence profile in response to I^- pulses during isosmotic and hyposmotic conditions. At point A, the cells perfused with isosmotic NO_3^- Ringer's were exposed to isosmotic I^- Ringer's. The influx of I^- led to decreased SPQ fluorescence. After ~120 s (i.e., at point B), NO_3^- Ringer's was returned, leading to a relatively small and slow increase in fluorescence due to I^- efflux. Efflux of I^- was comparatively slow, partly because the chemical gradient for efflux ($0\ mM < \Delta[I^-] < 12\ mM$; $[I^-]_i = 12$ mM, assuming that I^- becomes passively distributed with $[I^-]_o = 120$ mM, and $E_m = -60$ mV) was relatively small compared to that imposed for influx (at point A, $\Delta[I^-] = 120$ mM with Type I Ringer's and $\Delta[I^-] = 80$ mM with Type II Ringer's). Because I^- is not a substrate for the anion exchanger or the Na^+-K^+-$2Cl^-$ cotransporter (O'Grady et al., 1987), the influx can be taken to occur through anion channels active under resting conditions. (To determine whether I^- could substitute for Cl^- in Cl^-/HCO_3^- exchange, we examined changes in pH_i in HCO_3^- Ringer's upon substitution of Cl^- with gluconate and then subsequently with I^-. Substitution of Cl^- by gluconate led to substantial reversible alkalinization (0.2 pH units) consistent with previous observations (Bonanno et al., 1995). The alkalinization caused by gluconate substitution could not be reversed when gluconate was substituted by I^-, indicating that I^- is not a substrate for the anion exchanger in the cultured bovine corneal endothelial cells.)

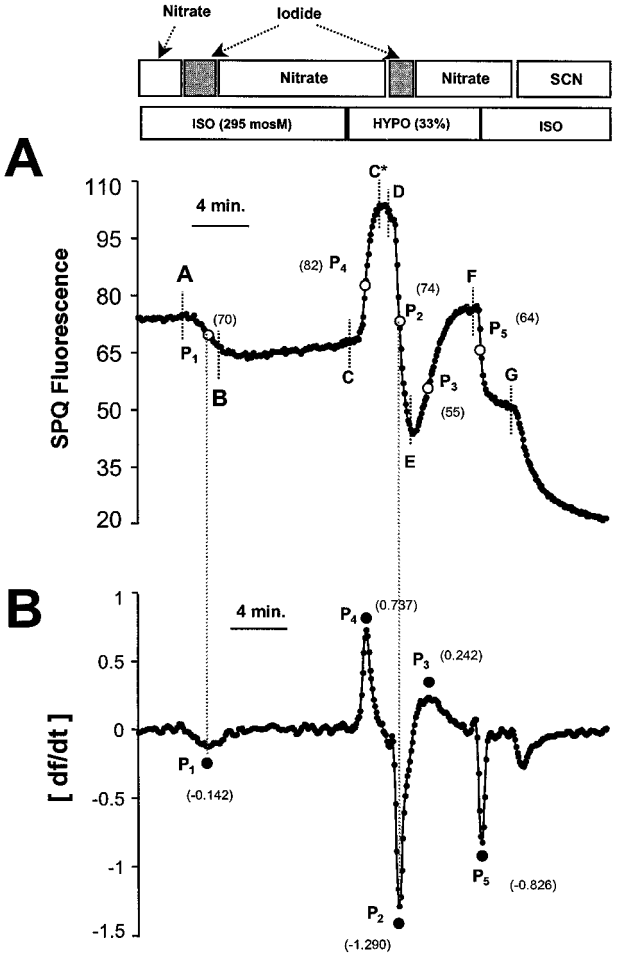

FIGURE 1 Measurement of uncorrected enhancement ratio (ER_U). (*A*) Typical SPQ fluorescence profile in response to a short duration (60 s) I^- pulse. At point A, the endothelial cells perfused with NO_3^- Ringer's were exposed to a pulse of isosmotic I^- Ringer's. This procedure was repeated under hyposmotic conditions (~185 mosM) at point D. Upon returning to isosmotic conditions (at point F), the cells were exposed to SCN^- to quench SPQ fluorescence completely. The results shown are typical of seven independent experiments. (*B*) First time derivative of the SPQ fluorescence profile shown in *A*, obtained using the Savitzky-Golay convolution algorithm with a window size of 2%. P_1–P_5 represent the inflection points of the SPQ fluorescence profile. P_1 and P_2 represent maximum rates of change of SPQ fluorescence during I^- influx under isosmotic and hyposmotic conditions, respectively. P_3 represents the maximum rate of SPQ fluorescence change during I^- efflux. The corresponding point during the isosmotic conditions is not noticeable. P_4 and P_5 represent the inflection points during volume increase and decrease in response to hyposmotic and return to isosmotic conditions, respectively.

We specify the evaluation of $[\partial f/\partial t]$ in Eq. 6 at the inflection points of the fluorescence profile (i.e., at the maximum rate of change of fluorescence) during I^- pulses. A plot of $[\partial f/\partial t]$ against time will therefore produce distinct peaks, which are easily identified (Fig. 1 *B*). This enables an objective estimate of both the slope ($[\partial f/\partial t]$) and fluorescence (f) values at a common time point, as required by Eq. 6. This procedure avoids estimating initial slopes subjectively or by nonlinear curve fitting, both of which are confounded by fluid mixing delays. Fig. 1 *B* shows the first derivative of the fluorescence profile from Fig. 1 *A*. The inflection point marked P_1 occurs during isosmotic I^- influx. Similarly, the inflection point marked P_2 occurs during hyposmotic I^- influx. The inflection point marked P_3 represents the maximum rate of I^- efflux under hyposmotic conditions because of removal of I^- and a return to NO_3^-. A similar inflection point under isosmotic conditions is not distinctly noticeable, indicating a relatively small and slow

efflux rate of I^-. The other inflection points, P_4 and P_5, are associated with volume increase and decrease in response to the hyposmotic shock, and returning to isosmotic conditions, respectively. For the typical experiment shown in Fig. 1 *A*, ER_U was calculated using Eq. 6 to be 13.6. For two other ROIs selected on the same coverslip, ER_U was 13.7 and 13.2. An average value of 8.9 ± 2.9 was found for ER_U from seven experiments on separate coverslips (comprising a total of 26 ROIs), with no significant difference between Type I and Type II Ringer's.

Upper and lower bounds on ER

To obtain an estimate of ER from ER_U, changes in E_m between isosmotic and hyposmotic conditions are needed to calculate the factor R_E (Eq. 5). These changes in E_m were measured with the whole-cell patch-clamp recording technique (see Materials and Methods; also see Watsky and Rae, 1991). The baseline E_m in Type I Cl^--rich Ringer's was found to be −58 ± 8 mV (n = 47; range: −46 mV to −79 mV). Fig. 2 *A* shows a representative graded voltage response of a colony of cells to 15%, 20%, and 25% hyposmotic shocks. In some cases, the response was close to saturation, even for 15% shocks, as shown in Fig. 2 *B*. These membrane depolarizations indicate that cell swelling causes E_m to approach the equilibrium potential of Cl^- (E_{Cl}), which is ~−28 mV ($[Cl^-]_i$ = 40 mM; Bonanno et al., 1995; Bonanno and Srinivas, 1997). Larger osmotic shocks often disrupted the gigaseal or led to formation of blebs on the patched cell surface. In any case, the response generally saturated with a 25% shock (Fig. 2 *B*). The magnitude of depolarization produced by graded osmotic shocks was 18 ± 6 mV (n = 7), 18 ± 6.1 mV (n = 6), and 23 ± 5.0 mV (n = 14) for 15%, 20%, and 25% hyposmotic shocks, respectively. The extent of depolarization produced by hyposmotic shock was similar when external Cl^- was replaced by NO_3^- in Type II Ringer's (Fig. 3). For swelling experiments with NO_3^- (Type II) Ringer's in the bath, $[E_m]_I$ was −57 ± 7 mV (95% confidence interval: −64 mV < $[E_m]_I$ < −50 mV), whereas $[E_m]_H$ was −32 ± 5 mV (95% confident interval: −37 mV < $[E_m]_H$ < −27 mV), indicating a depolarization by ~25 mV (n = 6).

To obtain upper and lower bounds of ER from ER_U based on these independent measurements of E_m, a range for R_E was calculated based on the 95% confidence intervals for E_m under isosmotic and hyposmotic conditions as the bounding values. Fig. 4 illustrates this calculation. The ordinates corresponding to points B and C form the lower and upper bounds, respectively, of R_E, based on confidence intervals for $[E_m]_I$ and $[E_m]_H$. When these bounding values of R_E are substituted into Eq. 4, we obtain 3.6 < ER < 6.4. Based on the mean values of $[E_m]_I$ and $[E_m]_H$, R_E would be 0.55 (see *point A* in Fig. 2) and the corresponding ER would be 4.9.

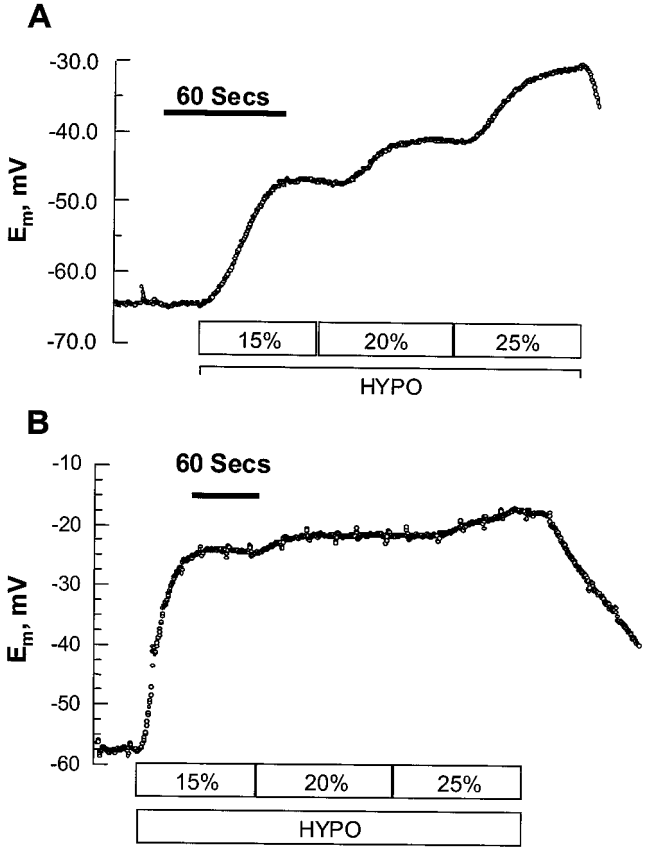

FIGURE 2 Swelling-induced depolarization of the membrane potential in small colonies of CBCE cells: representative profiles of membrane potential recorded with whole-cell patch pipettes under current-clamp in response to graded hyposmotic shocks (Type I Ringer's containing Cl^-) from two different coverslips. The profiles shown are typical of six independent experiments. (*A*) Response showing increasing extent of depolarization to graded hyposmotic shocks. (*B*) Response showing little change beyond 15% osmotic shock.

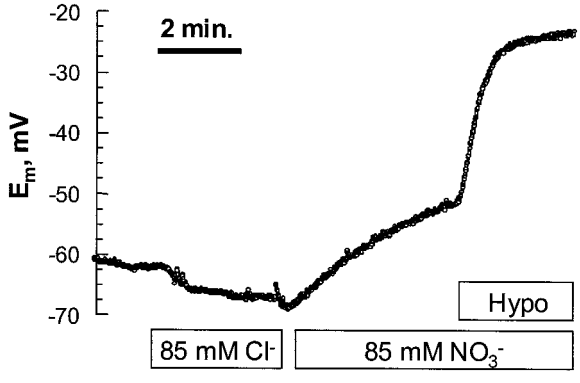

FIGURE 3 Swelling-induced depolarization in the presence of NO_3^-: a representative profile of the zero-current potential recorded with whole-cell patch pipettes in response to hyposmotic shocks with Type II Ringer's, and NO_3^- substituting for Cl^-. Cells were perfused with Type I Ringer's (145 NaCl) and then switched to Type II (85 NaCl), which produced a small hyperpolarization. NO_3^- was then substituted for Cl^-, yielding a small depolarization. The profile shown is typical of six independent experiments.

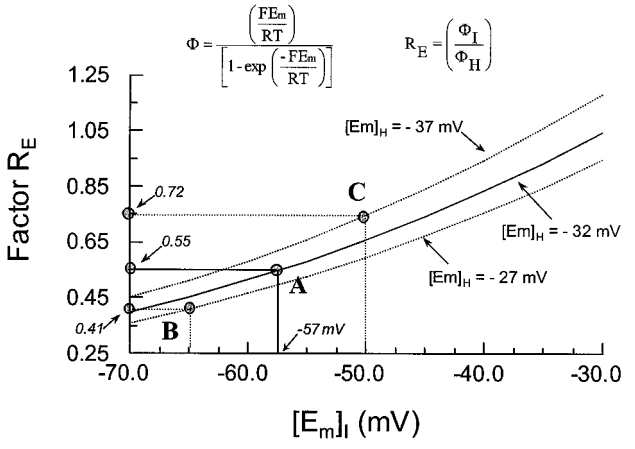

FIGURE 4 Dependence of factor R_E on $[E_m]_I$ and $[E_m]_H$: Plot of factor R_E against $[E_m]_I$ (E_m under resting isosmotic conditions) calculated at three values of depolarizing E_m under hyposmotic conditions (i.e., $[E_m]_H$). R_E was calculated, using Eq. 5 and the definition for Φ, at 37°C. The abscissa corresponding to point A represents the mean values of $[E_m]_I$ obtained experimentally (see Results). Similarly, the abscissa corresponding to points B and C forms the 95% confidence interval for the mean shown corresponding to point A. An upper bound for R_E is shown by point C and corresponds to the intersection of the lower estimate of $[E_m]_I$ and the smaller estimate of depolarization (i.e., $[E_m]_H = -37$ mV). Similarly, the lower bound for R_E is shown by point B and corresponds to the intersection of the upper estimate of $[E_m]_I$ and the higher estimate of depolarization (i.e., $[E_m]_H = -27$ mV).

DISCUSSION

SPQ and its analogs currently offer the only approach to estimating intracellular halide levels by fluorometry. This approach, based on collisional quenching of SPQ fluorescence by halides, is not ratiometric and is therefore prone to errors accruing from photobleaching and dye loss (Verkman, 1990). These factors, together with the nonlinearity inherent in the Stern-Volmer equation, confound a facile interpretation of changes in halide fluxes. When SPQ is employed to assess swelling-activated Cl^- channels, changes in cell volume further complicate the interpretation of the measured fluorescence in at least two significant ways. First, as demonstrated in our previous study (Srinivas and Bonanno, 1997a), SPQ fluorescence is sensitive to changes in cell volume because the concentration of intracellular quenchers is altered. Second, cell swelling enhances dye leakage, leading to limited duration of the experiments and increasing the importance of the background fluorescence in the calculation of the halide fluxes. In light of these complexities, the current study is focused on the quantitative consideration of an existing SPQ fluorescence protocol (Dho and Foskett, 1993; Ehring et al., 1994; Brochiero et al., 1995; Woll et al., 1996) developed for estimating relative anion permeability through swelling-activated Cl^- channels. Although we have used SPQ, the underlying principles are applicable to SPQ analogs as well, because halide sensitivity of different the analogs (e.g., MQAE, diH-MEQ) is dependent on the mechanism of collisional quenching (Verkman, 1990; Ehring et al., 1994; Brochiero et al., 1995; Woll et al., 1996). We have shown that the swelling-induced increase in anion permeability can be expressed in the form of a ratio, referred to as the enhancement ratio (ER), which is given by I^- permeability under hyposmotic conditions compared to that under isosmotic conditions. We have also demonstrated that a true estimate of ER requires simultaneous assessment of both I^- influx and E_m. However, because concurrent E_m measurements are not practical, independent measurements of I^- influx and E_m obtained under similar conditions are necessary. To a first approximation, ER_u, the enhancement ratio uncorrected for changes in E_m, is a useful measure for screening cell populations for the presence of swelling-activated Cl^- channels. Furthermore, the ER_u calculation is simplified by the choice of the inflection point for evaluating the slope of the fluorescence change. Thus ER_u, by itself, is a significant improvement over previous attempts to use SPQ fluorescence to estimate relative anion permeability through swelling-activated Cl^- channels.

Validation of Eqs. 4, 5, and 6

Using SPQ to determine absolute anion permeabilities under isosmotic and hyposmotic conditions presents several formidable challenges, because it requires knowledge of the cell surface-to-volume ratio, $[I^-]_i$, as well as C^P and K_P (Eq. 3). Therefore, we have chosen to obtain a measure of the relative increase in anion permeability in the form of the ratio ER. Our definition of ER removes the need for estimation of C^P, K_P, and changes in cell volume. Further rationales for the validity of Eqs. 4, 5, and 6 under the experimental conditions that we have specified are as follows:

1. Because representation of I^- influx through conductive pathways requires a consideration of the influence of E_m, it was modeled as an electrodiffusive flux by use of the Goldman flux equation. Thus this study extends the work of previous SPQ-based fluorescence studies of swelling-activated Cl^- channels (Dho and Foskett, 1993; Ehring et al., 1994; Brochiero et al., 1995; Woll et al., 1996) by characterizing the influence of E_m on the relative anion fluxes.

2. To remove terms containing $[I^-]_i$ in equations for ER, it was imperative to evaluate fluorescence changes when C_i^I was insignificant (0 mM was assumed). This was achieved by imposing short I^- pulses. Furthermore, the inflection points employed to calculate ER occurred within the first half of the I^- pulses (30–60 s, as shown in Fig. 1 *B*). This suggests that slopes employed in the calculation of ER are not smeared by C_i^I being significantly greater than 0 mM. Compared to C_o^I (140 mM in Type I and 80 mM in Type II Ringer's), $[I^-]_i$ can be conservatively estimated to be less than 4 mM after 1 min in swollen cells (assuming $K_{I-} = 28$ M^{-1} in the intracellular milieu under swollen conditions), which is less than 5% of $[I^-]_o$ for Type I Ringer's and less than 4% for Type II ($[I^-]_O = 120$ mM). (K_I in Ringer's is 280 M^{-1} (Verkman, 1990). Because the Cl^- quenching constant is reduced by 10 times intracellularly, a similar

reduction in K_I suggests a value of 28 M^{-1} inside the cells.) Thus the assumption that $C_i^I \approx 0$ at the inflection point (<1 min after the pulse in swollen cells) introduces only a small error and hence forms a reasonable approximation.

3. The assumption that cell volume remains constant during the I^- pulses requires that NO_3^- and I^- have equal permeabilities. This was demonstrated in previous studies (reviewed in Strange et al., 1996; Nilius et al., 1996). However, the short duration of pulses also ensures that the total influx is small, so that volume changes, if any, resulting from differences in permeability would be negligible. In addition, another route for volume change is through RVD itself, but the extent of RVD over the first 1–2 min under HCO_3^--free conditions in cultured bovine corneal endothelial cells is negligible (Srinivas and Bonanno, 1997a).

4. Calculated ER from this protocol is, at best, an underestimate. This is because isosmotic permeability (P_I) may be overestimated, as the fraction of I^- influx through nonconductive pathways would be relatively higher under isosmotic conditions than under hyposmotic conditions.

Estimation of ER in cultured bovine endothelial cells

Estimation of ER from ER_U requires calculation of R_E from the changes in E_m under iso- and hyposmotic conditions. Fig. 4 shows that R_E is significantly different from unity for changes in E_m that would be expected when channels are activated at resting E_m. Our whole-cell recording of zero-current potential indicates that cultured bovine corneal endothelial cells have a resting E_m between −46 and −79 mV (mean $E_m = -58 \pm 8$ mV), consistent with previous measurements employing microelectrodes (Jentsch et al., 1984; Coroneo et al., 1989) and patch pipettes (Watsky and Rae, 1991; Rae and Watsky, 1996). In response to 25% hyposmotic shocks, E_m was found to depolarize by 23 mV toward E_{Cl-} (−28 mV, assuming an E_m of −60 mV and $[Cl^-]_i = 40$ mM; Bonanno and Srinivas, 1997; Bonanno et al., 1995). Similarly, Coroneo et al. (1989) demonstrated that for cultured bovine corneal endothelial cells, a 20% hyposmotic shock depolarized E_m by 20 mV from an initial value of −50 mV within 3 min. This is consistent with a swelling-induced increase in the NPPB-, DIDS-, and tamoxifen-sensitive anion conductance in cultured bovine corneal endothelial cells (Srinivas and Bonanno, 1996). Confidence limits (95%) for depolarization of E_m in response to hyposmotic shock suggested $0.4 < R_E < 0.7$, and the corresponding bounds on ER were found to be $3.7 < ER < 6.4$. These estimates of increased anion permeability are similar to previous reports, using various techniques, of swelling-activated Cl^- channel activity in other cell types (Nilius et al., 1996).

The large increase in anion permeability after hyposmotic shock can be taken to suggest that RVD in cultured bovine corneal endothelial cells would not be limited by Cl^- conductance. This is consistent with our previous observation that RVD can be enhanced by exposure to gramicidin (2 μM) in the presence of low $[Na^+]$ or by exposure to 0.4 μM valinomycin, thereby facilitating a cooperative K^+ and Cl^- loss (Srinivas and Bonanno, 1997a,b). Although cell swelling often results in the activation of Ca^{2+}-dependent K^+ channels (Hoffmann and Dunham, 1995), the robust depolarization seen in CBCE cells suggests that this is not occurring. Depolarization induced by hyposmotic shock could be observed in Type II Ringer's as well, indicating that dilution of the intracellular ionic composition is not responsible for the response (Fig. 3).

Choice of anions

The choice of NO_3^- and I^- as model anions was motivated by the following factors:

1. NO_3^- does not quench SPQ fluorescence; therefore fluorescence changes are due to unidirectional I^- fluxes alone.

2. NO_3^- and I^- have approximately the same permeability through swelling-activated Cl^- channels (Ehring et al., 1994; Brochiero et al., 1995; Woll et al., 1996), indicating that only small changes in cell volume and E_m can be expected during I^- pulses.

3. The quenching constant for I^- is large compared to that for Cl^- ($K_I = 280\ M^{-1} > K_{Cl} = 120\ M^{-1}$ in Ringer's solutions), so that the responses are very sensitive to small changes in $[I^-]_i$. This makes it possible to use short pulse periods to capture the response faster, so that changes in cell volume (due to RVD) or dye leakage during the I^- pulse period are negligible.

4. Neither I^- nor NO_3^- is a substitute for Cl^- in Na^+-K^+-$2Cl^-$ cotransport, and I^- is not a preferred substrate for the Cl^-/HCO_3^- exchanger in cultured bovine corneal endothelial cells. Furthermore, the large increase in I^- influx during swollen conditions cannot be explained by an increase in the activity of the Na^+-K^+-$2Cl^-$ cotransporter or Cl^-/HCO_3^- exchanger. In fact, these transport systems are not activated during RVD (Hoffmann and Dunham, 1995). Thus I^- fluxes are largely conductive.

5. I^- does not appear to be toxic to cultured bovine corneal endothelial cells within the time frame of our experiments. If I^- had been toxic, SPQ loss would have been extremely rapid, even under isosmotic conditions. Similarly, the loss of the pH-sensitive fluorescent dye BCECF in the presence of >80 mM I^- in the Ringer's was no greater than in Cl^--rich Ringer's. In several experiments (not shown), SPQ could be loaded even after 40 min of exposure to >80 mM I^- in the Ringer's. These observations are consistent with other studies, which have made use of I^- in the Ringer's as a maneuver to accentuate the sensitivity of the measured SPQ response, to assess the activity of Cl^- channels or Cl^- transport systems (Hwang et al., 1997; Stern et al., 1995).

Limitations of the SPQ fluorescence method

The principal disadvantage of this procedure is possible interference with SPQ fluorescence by the fluorescence and absorption properties of drugs sometimes used to examine the pharmacological properties of swelling-activated Cl^- channels. NPPB, a known inhibitor of swelling-activated Cl^- channels in many cell types, has an absorption spectrum that overlaps the emission spectrum of SPQ (Woll et al., 1996); NPPB at 100 μM causes a significant decrease in the measured SPQ fluorescence (data not shown). Similarly, niflumic acid (100 μM), which absorbs at 360 nm, also led to a reduction in SPQ fluorescence (data not shown). DIDS, a known inhibitor of swelling-activated Cl^- channels, is fluorescent, and its excitation and emission spectra overlap those of SPQ (Woll et al., 1996). However, interference by these compounds does not preclude use of the SPQ technique if drug concentrations can be kept low relative to intracellular SPQ concentration, and/or when appropriate correction factors can be employed.

In addition to the influence of intracellular quenchers, SPQ and related dyes pose the problems of dye leakage and background fluorescence. Both the extent and rate of dye leakage influence the absolute level of fluorescence and the derivative calculation (see Eq. A6). The leakage problem assumes particular significance during swollen conditions because of the enhanced dye leakage (Srinivas and Bonanno, 1997a). The current protocol, however, reduces the influence of dye leakage by using short I^- pulses. Background fluorescence becomes significant because it is squared during the flux calculation (see Eq. A10). This is especially true when SPQ loading is poor or when dye leakage dominates. Thus, to obtain accurate estimates of ER, careful measurement of background fluorescence (i.e., f_{ex}) is needed.

Despite the disadvantages associated with the SPQ fluorescence method, our results indicate that a careful selection of experimental maneuvers, combined with independent assessment of E_m, can result in information that cannot be obtained by patch-clamp protocols alone. For example, the fluorescence method is applicable to the assessment of swelling-activated Cl^- channels in intact monolayers and potentially from intact tissues. It is also useful in identifying the apical-basal polarity of swelling-activated Cl^- channels (Brochiero et al., 1995). In addition, the analysis presented in this study should be useful even when changes in cell volume are negligible, but channel activation (e.g., CFTR) is being investigated. Because fluorescence imaging can be adopted to study individual cells, responses from heterogeneous cell populations are easily examined. Thus it is possible to employ the SPQ fluorescence method quantitatively to screen and characterize swelling-activated Cl^- channel activity in heterogeneous cell populations after overexpression of channels or blocking of channel expression by anti-sense oligonucleotides.

APPENDIX A: CALCULATION OF THE ENHANCEMENT RATIO

To correlate unidirectional fluxes with changes in $[I^-]_i$, Eq. 2 will be expressed as

$$J = \frac{1}{A}\frac{\mathrm{d}([V_c - b]C_i^I(t))}{\mathrm{d}t} \tag{A1}$$

where V_c is the cell volume, b is the nonsolvent cell volume (Hallows and Knauf, 1994), A is the surface area, and $C_i^I(t)$ is the $[I^-]_i$. Assuming that V_c remains constant during the I^- pulse, substitution of Eq. A1 into Eq. 2 gives

$$\left(\frac{\partial C_i^I}{\partial t}\right) = \left(\frac{A}{[V_c - b]}\right)P(\Phi(t))C_o^I(t) \tag{A2}$$

where

$$\Phi(t) = \frac{\left(\frac{FE_m}{RT}\right)}{\left[1 - \exp\left(\frac{-FE_m}{RT}\right)\right]} \tag{A3}$$

When changes in E_m are small during the I^- pulses (explained by the fact that I^- and NO_3^- have roughly equal permeability through swelling activated Cl^- channels; Nilius et al., 1996; Strange et al., 1996), $\Phi(t)$ becomes independent of time. With this assumption, solving for P from Eq. A2 and substituting the results into Eq. 1, we get

$$\frac{P_H}{P_I} = \mathrm{ER} = \left(\frac{(V_c - b)_H}{(V_c - b)_I}\right)\left(\frac{\left(\frac{\partial C_i^I}{\mathrm{d}t}\right)_H}{\left(\frac{\partial C_i^I}{\mathrm{d}t}\right)_I}\right)\left(\frac{\Phi_I}{\Phi_H}\right) \tag{A4}$$

where Φ_I and Φ_H are functions of E_m (see Eq. A3) evaluated under isosmotic and hyposmotic conditions, respectively. The ratio $[\Phi_I/\Phi_H]$ will be denoted by R_E. We can then substitute for partial derivatives in Eq. A4 from Eq. 3 as

$$\frac{P_H}{P_I} = \left(\frac{(V_c - b)_H}{(V_c - b)_I}\right)\left(\frac{\Phi_I}{\Phi_H}\right)\left[\frac{(f - f_{ex})_I^2}{(f - f_{ex})_H^2}\right]\left[\frac{\left(\frac{\partial f}{\partial t}\right)_H}{\left(\frac{\partial f}{\partial t}\right)_I}\right] \tag{A5}$$

If the change in cell volume after the hyposmotic shock is assumed to follow the Boyle Van't Hoff law (Hallows and Knauf, 1994), then the first term in Eq. A5 can be substituted in terms of osmolarities at points close to the osmotic shock as

$$\mathrm{ER} = \left(\frac{\Pi_I}{\Pi_H}\right)(R_E)\left[\frac{(f - f_{ex})_I^2}{(f - f_{ex})_H^2}\right]\left[\frac{\left(\frac{\partial f}{\partial t}\right)_H}{\left(\frac{\partial f}{\partial t}\right)_I}\right] \tag{A6}$$

where Π_I and Π_H represent the osmolarity of the perfusate under isosmotic and hyposmotic conditions.

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