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Monitoring Cl⁻ Movement in Single Cells Exposed to Hypotonic Solution

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Abstract. Self-referencing ion - selective electrodes (ISEs), made with Chloride Ionophore I-Cocktail A (Fluka), were positioned 1–3 μm from human embryonic kidney cells (tsA201a) and used to record chloride flux during a sustained hyposmotic challenge. The ISE response was close to Nernstian when comparing potentials (V_N) measured in 100 and 10 mM NaCl (ΔV_N = 57 ± 2 mV), but was slightly greater than ideal when comparing 1 and 10 mM NaCl (ΔV_N = 70 ± 3 mV). The response was also linear in the presence of 1 mM glutamate, gluconate, or acetate, 10 μM tamoxifen, or 0.1, 1, or 10 mM HEPES at pH 7.0. The ISE was ~3 orders of magnitude more selective for Cl^- over glutamate or gluconate but less than 2 orders of magnitude move selective for Cl^- over bicarbonate, acetate, citrate or thiosulfate. As a result this ISE is best described as an anion sensor. The ISE was 'poisoned' by 50 μM 5–nitro-2-(3phenylpropyl-amino)-benzoic acid (NPPB), but not by tamoxifen. An outward anion efflux was recorded from cells challenged with hypotonic (250 ± 5 mOsm) solution. The increase in efflux peaked 7–8 min before decreasing, consistent with regulatory volume decreases observed in separate experiments using a similar osmotic protocol. This anion efflux was blocked by 10 μM tamoxifen. These results establish the feasibility of using the modulation of electrochemical, anion-selective, electrodes to monitor anions and, in this case, chloride movement during volume regulatory events. The approach provides a real-time measure of anion movement during regulated volume decrease at the single-cell level.

Key words: Ion selective electrodes — Self-referencing probes — Regulatory volume decrease — Volume-regulated anion current — Chloride

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Introduction

Mammalian cell volume regulation in response to a hyposmotic challenge requires the movement of anions and cations followed by osmotically obligated water (Nilius et al., 1996; Garber & Cahalan, 1997; Kirk & Strange, 1998; Eggermont et al., 2001). The flow of chloride is thought to be initiated by cell swelling, resulting in the activation of swelling-regulated anion channels. This serves to depolarize the cell sufficiently to activate voltage-dependent K^+ channels, reducing cytoplasmic osmolarity and initiating the flow of osmotically obligated water out of the cell, through the lipid bilayer.

The role of anion flux in volume regulation is critical. A swelling-activated anion current characterized in many cell types is thought to be the primary pathway for acute anion movement across the plasma membrane. This current has been characterized using whole-cell patch-clamp techniques that allow experimental access to both sides of the plasma membrane. The current has been shown to be anion-selective, sensitive to a variety of agents including DIDS, NPPB and tamoxifen. Its activation requires a transmembrane hyposmotic gradient, influenced by ion composition and ion strength (Garber & Cahalan, 1997; Nilius, Eggermont & Droogmans, 2000; Eggermont et al., 2001). The study of volume-dependent ion currents using patch-clamp recording requires breaking the cell membrane. Therefore, the ability to monitor current activity concurrently with volume regulation is necessarily lost. Our understanding of volume regulation has, therefore, been limited by the technical difficulties of monitoring volume-regulatory events and ion-channel activity simultaneously.

Our aim in this study was to define a system in which volume-regulated anion flux could be directly monitored in intact single cells. Many previous studies of the mechanisms of volume regulation em-

ployed intact cell populations, using several different methods to follow ion flux or volume changes. A non-invasive method for directly measuring Cl^-, H^+, K^+ or Ca^{++} flux has been developed, using an ion-selective self-referencing electrode (Doughty, Miller & Langton, 1998; Smith et al., 1999; Trimarchi et al., 2000; Doughty & Langton, 2001; Land & Collett, 2001; Smith & Trimarchi, 2001).

This technique has been successfully used to record Cl^- efflux correlated with the myogenic response in rat cerebral arteries and that found in the microenvironment of the apical lung (Doughty & Langton, 2001; Land & Collett, 2001). This technique can be combined with optical measurements of volume and/or patch-clamp recording. Most importantly, the application of this technique to understanding volume regulation will allow us to follow volume-dependent Cl^- efflux during volume-regulatory events in intact single cells, over real time.

The anion selectivity and pH sensitivity of this self-referencing ionophore electrode was characterized. We used this technique to measure a tamoxifen- and NPPB-sensitive anion efflux from a human kidney cell line (tsA201a) in response to a hypotonic challenge. The change in anion efflux correlates with volume-regulated decrease measured in parallel experiments in this same cell line. The successful application of this technique to volume-regulatory events will also allow the future simultaneous measurement of anion and cation fluxes, something that has not been possible with the techniques previously employed.

Materials and Methods

Cell Culture

Human epithelial tsA201a cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (0.1 mg/ml) in 5% CO_2 maintained at 37°C. Cell cultures were split with 0 25% trypsin-EDTA every 2–3 days. Cell culture reagents were purchased from Fisher Scientific, Pittsburgh, PA.

Solutions

Iso- and hypotonic Ringer's superfusion solution contained (in mM) 100 NaCl, 6 KCl, 1 $CaCl_2$, 2 $MgCl_2$,10 HEPES, pH 7.2. Osmolality of isotonic solution was adjusted to 313 ± 5 mOsm and hypotonic solution to 250 ± 5 mOsm using a 1 M sucrose stock while keeping ion concentrations constant. The extracellular solution for whole-cell recording was composed of (in mM): 150 NMDG-Cl,$^-$ 10 HEPES, 1 $CaCl_2$, 2 $MgCl_2$, pH 7.2 using NMDGOH, adjusted to 313 ± 5 mOsm with 1 M sucrose. The intracellular solution for whole-cell recording was composed of (in mM): 150 NMDG-Glutamate, 10 HEPES, 0.1 $CaCl_2$, 1.1 EGTA, 4 ATP, pH 7.2 using CsOH, adjusted to 413 ± 5 mOsm with 1 M sucrose. Osmolality of all solutions was determined prior to each experiment, using a vapor pressure osmometer (Wescor, Logan, UT). Tamoxifen-citrate and 5-nitro-2-(3phenylpropyl-amino)-benzoic acid (NPPB) were purchased from Calbiochem (La Jolla, CA). Stock solutions of these agents were made with EtOH or DMSO such that the final working concentration of the vehicle was 0.1%. Experiments were performed at room temperature (22°C ± 2).

Anion Electrode and Flux Measurement

Microelectrodes (WPI borosilicate, o.d. 1.5 mm), pulled on a Sutter P97 to a tip diameter of 2–4 μm were back-filled with 100 mM NaCl and front-filled with a 75–100 μm column of Chloride Ionophore I-Cocktail A (Fluka, Buchs, Switzerland). The reference electrode was an Ag-AgCl pellet connected to the bath via a 3% agar bridge containing 3 M NaAcetate. Preparation of anion-selective electrodes has been previously described in Doughty and Langton (2001) and Land and Collett (2001). Electrode glass was prepared, with silanization, as described in Smith et al. (1999). Self-referencing theory, hardware and software followed established procedures published elsewhere (Smith, Sanger & Jaffe, 1994; Smith et al., 1999). Anion flux calculation and electrode performance has been previously described (Doughty & Langton, 2001).

In brief, self-referencing is a modulation technique where an electrochemical microsensor is translated in a square wave motion (0.3 Hz) between two points a known distance apart—usually 10 μm. In the case of the ion-selective electrodes (ISEs), voltage measurements are made at each pole of translation, representing the ion activity at each point. This is termed V_N and represents the Nernst potential of the solution, which in the presence of a flux will vary between the two poles. The system samples at 1 kHz and approximately one third of the data during and after each movement is discarded to account for gradient disturbance and system response time. Chloride ISEs are relatively slow, with response to 50% within 200 ms and to 90% within 1.8 s (*see* Table 1). As the measuring frequency was 0.3 Hz, we can anticipate that the absolute measure of flux will be slightly greater than presented in the results. Signals at each position are averaged for both the near and the far pole. These are then compared, such that the signal from the far pole is subtracted from that of the near pole — the position closest to the source, sink or cell. Thus, by convention, an anionic movement emanating from a source will appear as a cationic movement into a sink. The differential voltage (ΔV) discards background, parasitic voltages and sensor drift, improving signal extraction by orders of magnitude (*see* Smith et al., 1999). ΔV can be converted into a change in concentration by comparison with a pre-calibration slope and when incorporated into Fick's First Equation, along with the relevant diffusion coefficient (D, which for Cl^- is $2.03 \cdot 10^{-5}$ cm^2/s; Hille, 2001), can be used to calculate the ionic flux between the two points. Radial, random walk, diffusion is assumed. All hardware and software used for the self-referencing procedure are products of the BioCurrents Research Center (BRC, Marine Biological Laboratory, Woods Hole, MA 02543).

Anion-selective electrodes were brought within 1–3 μm of a group of 4–8 cells. This position was defined as the "near pole". The distance between the electrodes and cells was maintained visually. A baseline flux was established in isotonic Ringer's solution before exchanging 5 times the bath volume with hypotonic Ringer's solution. Bath perfusion took 60–90 s. Time 0 was designated as the time when perfusion was complete. Background flux measurements were taken ≥ 500 μm above cells. For experiments including tamoxifen, flux under isotonic, control conditions was initially established. Cells were first exposed to isotonic Ringer's including the drug and then exposed to hypotonic Ringer's plus drug.

Anion flux is presented as the difference between the average flux under isotonic conditions, with or without drug, compared to hypotonic conditions, with or without the drug; normalized flux

Table 1. Time response of ISE to decade steps in [NaCl].

Signal	Time response (s)			
	NaCl Concentration step			
	100 to 10 mM	10 to 1mM	1 to 10 mM	10 to 100 mM
50%	0.13 ± 0.04	0.13 ± 0.04	0.17 ± 0.06	0.20 ± 0.06
90%	1.8 ± 0.3	1.8 ± 0.4	1.5 ± 0.3	1.4 ± 0.2
95%	3.5 ± 0.4*	3.3 ± 0.5	2.8 ± 0.3	2.2 ± 0.4*

Electrodes were exposed to solutions using a flow system to insure that time required to change solutions was not limiting. Time response at 50 %, 90 %, and 95 % of maximal signal is shown. Measurements are averages of 4 electrodes ± SEM. There is no significant differnce when moving between concentrations of NaCl at a time response of 50 or 90 %. The 95 % time response when moving from 10 to 100 mM NaCl is significantly smaller than moving from 100 to 10 mM NaCl (asterisks mark significant differences between measurements, as determined by Student's *t*-test, $p < 0.5$).

from each individual run was pooled into 50 s bins. The average and standard error for binned data was determined for each experimental condition. Normalized fluxes were plotted at the middle time point of the bin (e.g., data for $t = 0$ to 50 s was plotted at $t = 25$ s or 0.42 min.). Statistical differences between experimental points were determined using two-tailed, unpaired Student's *t*-test with $p < 0.05$ considered significant.

ISE Selectivity: Separate Solutions Method

Electrode selectivity was determined using the separate solutions method as described by Umezawa et al. (2000), which involves measuring the potentials from solutions of two different cations or anions of equal activity. The selectivity of the electrode, *K*, can then be calculated based on the following equation:

$$K_{AB} = a_{A}^{(1-zA/aB)} e^{(VB-VA)\bullet 2A\bullet F/RT} \quad (1)$$

Where '*a*' is activity, '*z*' is valence and '*V*' is voltage (Umezawa et al., 2000). For our purposes, '*A*' and '*B*' represent the Cl^- ion and the interfering ion, respectively. A simplified version of this method employs the use of 100 mM concentrations of each of the anions tested rather than matching ionic activities. As long as the valences of the ions under comparison are the same, the discrepancy between 100 mM concentrations and identical activity of the anions is ± 8% on the linear scale. The simplified separate solutions method was chosen, as it was used by Fluka Chemical Co. to describe the anionic selectivity of the anion exchanger. Interference by Cl^--channel inhibitors was determined by comparing the electrode's responses to 0.1, 1.0, 10, and 100 mM KCl in the absence and presence of different concentrations of chloride-transport inhibitors. Mannitol (5%) was added to 0.1, 1.0 and 10.0 mM KCl to decrease the large osmotic gradient across the column of ionophore and cocktail, thereby preventing it from being dislodged from the tip.

ISE Time Response

A rapid exchange flow system was used to measure the time response of the electrodes to changes in solution. A syringe pump (Univentor Zejtun, Malta) was used to pass different solutions at 0.83 ml/min through 0.7 mm square, triple-barreled glass tubing (Warner Instruments, Hamden, CT). The measuring electrode remained stationary as the triple-barreled glass was intermittently shifted, using the SF-77B Perfusion Fast-Step (Warner Instruments, Hamden, CT) to expose the electrode to a new solution. The manufacturer specifies a translational speed of 650 μm in 20 ms. Interfacial exchange at the ISE surface will therefore be faster. Data were acquired by passing the 1× unfiltered signal out of the ion-selective electrode amplifier (BioCurrents Research Center, MBL, Woods Hole, MA) into an EG&G preamplifier (Princeton Applied Research, Oak Ridge, TN) before it was digitized with an Axon Instruments 1322A A/D converter and logged with Axoscope software (Axon Instruments, Union City, CA). The time taken for the probe to reach 50%, 90% and 95% response was determined using Microsoft Excel.

Whole-Cell Recording

Macroscopic ionic currents were measured using the whole-cell recording configuration of the standard patch-clamp technique (Hamill et al., 1981). Pipettes were pulled from 7052 glass (Garner Glass Co, Claremont, CA) using a glass microelectrode puller, coated with Sylgard® 184 (Dow Corning, Midland, MI), and fire-polished to give a final resistance of 4–10 MΩ in the recording solutions. Currents were recorded using an EPC9 amplifier (HEKA Electronik, Lambrecht, Germany) and the accompanying acquisition and analysis software. Further analysis employed IGOR (WaveMetrics, Lake Oswego, OR) and Microsoft EXCEL (Microsoft, Redmond, VA). Net junction potential was nulled immediately before seal formation. Pipette and whole-cell capacitance were automatically compensated and recorded. Whole-cell capacitance and series resistance (R_s) were monitored throughout the recording. Swelling-activated anion current was initiated by a transmembrane hyposmotic gradient (413:313 mOsm, intracellular:extracellular osmolarity, respectively) at the time of break-in ($t = 0$). Current amplitude increased over time and reached a maximum level. Current amplitude was normalized to peak current in hypotonic Ringers (normalized current = I/I_{peak}). Current density was calculated by dividing current (nA) by cell capacitance (μF) with typical biological membrane capacitance = 1 μF/cm^2. Series resistance compensation was employed at 75 to 95% compensation with a 100 μs lag. Errors in the measurement of the reversal potential as a result of series resistance error are negligible. Normal cellular current convention is used when referring to the direction of current (i.e., inward Cl^- movement = outward current).

Fluorescent Volume Assay

This assay was modified from Alvarez-Leefmans, Altamirano & Crowe (1995) and Crowe et al. (1995). Cells were plated on poly-L-lysine-coated coverslips and incubated in DMEM containing 2.5 μM calcein-AM (Molecular Probes, Eugene, OR) for 30 min at room temperature. The dye-loading solution was replaced with DMEM supplemented with 10% FBS for 30 minutes at 37°C in 5% CO_2. Cells were washed once with isotonic 100 mM NaCl Ringer's

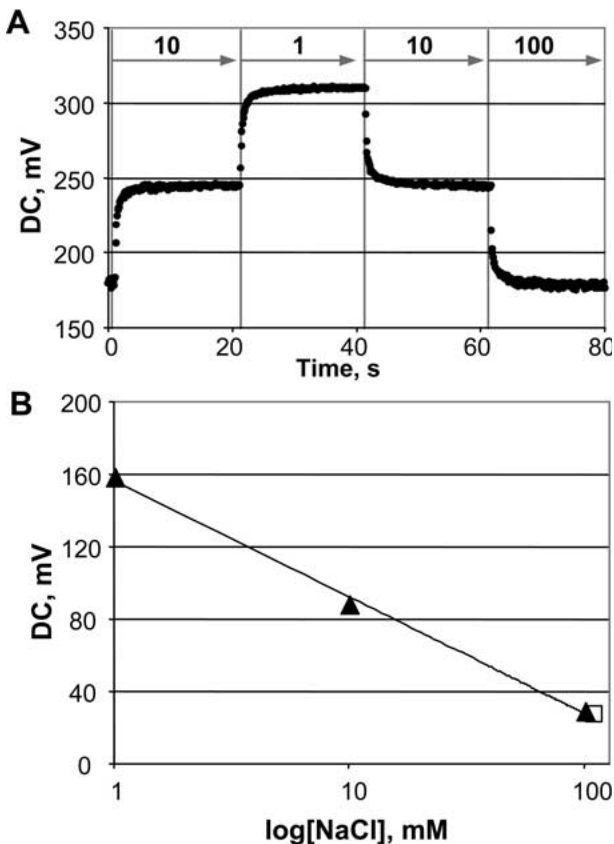

Fig. 1. Potential measured using a chloride ion selective electrode is Nernstian. (*A*) An example of the relative potential (V_N) measurement vs. time by an ISE in 100, 10 and 1 mM NaCl, as indicated. (*B*) Potential measured in standard [NaCl] solutions (▲) was fit by line $y = -64.5x + 159$, with a linear regression coefficient $R^2 > 0.99$ ($n = 31$). V_N value measured in isotonic Ringer's containing 110 mM total Cl^- at pH 7.2 is shown as (□), $n = 4$, and was equivalent to that in 100 mM NaCl. SEMs are smaller than symbols.

solution (313 mOsm) and placed in isotonic 100 mM NaCl Ringer's bath. An SF-77 Perfusion Fast-Step system (Warner Instrument Corporation, Hamden, CT) was used for continuous superfusion of cells.

Calcein-loaded cells were bathed in isotonic 100 mM NaCl Ringer's solution and placed on the stage of an epi-fluorescence microscope. Cells exhibiting uniform fluorescence were chosen for each experiment. A capillary tube filled with isotonic Ringer's solution was lowered into the bath and positioned with the opening toward the cell(s) to be superfused. Once the capillary tube was positioned, a Luer valve was manually opened and superfusion began ≤ 5 s later. A manual stepper motor was used to change the position of the capillary tubing. The entire process of re-positioning the capillary tubes using a manual stepper motor, stopping flow of isotonic 100 mM NaCl Ringer's solution and starting flow of experimental test solution was complete in ≤ 10 s.

Fluorescent images were taken using an inverted, epi-fluorescence microscope (Nikon Diaphot, Tokyo, Japan) and a Hamamatsu C5985 CCD camera (Hamamatsu Photonics, Hamamatsu City, Japan). An HMX-3 mercury lamp (Nikon Instrument Group, Melville, NY) was used as the excitation light source. Exposure time and intensity were limited by a computer-controlled automatic high-speed shutter (Uniblitz, Vincent Associates, Rochester, NY) and neutral density filters (Omega Optical, Brattleboro, VT).

Images were collected at regular intervals throughout superfusion. Mean pixel intensity (MPI) of a cell at each time point was determined from within a small box of fixed size placed in the cell interior. MPI measurements taken during superfusion with experimental test solution (F_t = fluorescence in response to experimental test solution) were normalized with respect to MPI measurements during superfusion with isotonic 100 mM NaCl Ringer's solution (F_i = fluorescence in response to isotonic solution). The percent change in fluorescence was calculated from the normalized fluorescence ratio $F_i/F_t = [((F_i/F_t)-1)/(F_i/F_t)]\cdot 100$ and represents the percent change in cell volume. Data acquisition and analysis were completed using Scion Image 1.57c software.

Results

Ion-Selective Electrode Characterization: Nernstian Response

The Nernstian response (V_N) of the chloride ionophore was tested by placing an ion-selective electrode (ISE) in 1, 10 or 100 mM standard NaCl solutions (Fig. 1). The response time of the ISE when moving the electrode from one solution to another is shown in Table 1. The electrode responded to 50% of the maximal response within 0.02 s, 90% within 1.8 s and 95% within 3.5 s. There was no statistical difference between response times going from 100 to 10, 10 to 1, 1 to 10 or 10 to 100 mM NaCl solutions except the 95% response time in going from 100 to 10 mM was significantly slower than when measured in a solution change from 10 to 100 mM NaCl.

The change in the V_N value of the recorded potential was linear with respect to log[NaCl] (Fig. 1*B*). The slope of the line was m = −65 ± 2 mV. This value was close to that of an ideal Nernstian electrode, which would be expected to give a ΔV_N = 60 mV, for a 10-fold change in concentration. The response was independent of placement or movement of the ISE in the bath (*not shown*). The V_N potential measured in the physiological Ringer's solution (containing 110 mM Cl^- at pH 7.2) was 28.1 ± 0.1 mV (Fig. 1*B*). This value was close to that expected from the Nernstian characteristic of the electrode (27.2 mV). This indicated that the composition of the Ringer's solution does not interfere with the ionophore's ability to monitor the anion.

ISE: Anion Selectivity

The sensitivity of the ISE_{Cl^-} ionophore-filled electrode to anions other than Cl^- is shown in Fig. 2. The effect on the V_N potential in response to adding 1 mM acetate, glutamate or gluconate to the 1, 10 or 100 mM standard NaCl solutions is shown in Fig. 2*A*. The change of the V_N potential *vs.* standard [NaCl] yielded a slope of −62 ΔV_N / (log[NaCl) with a correlation coefficient (R^2) of ≥ 0.99. The addition of the other anions increased this slope to −89, −83, −85

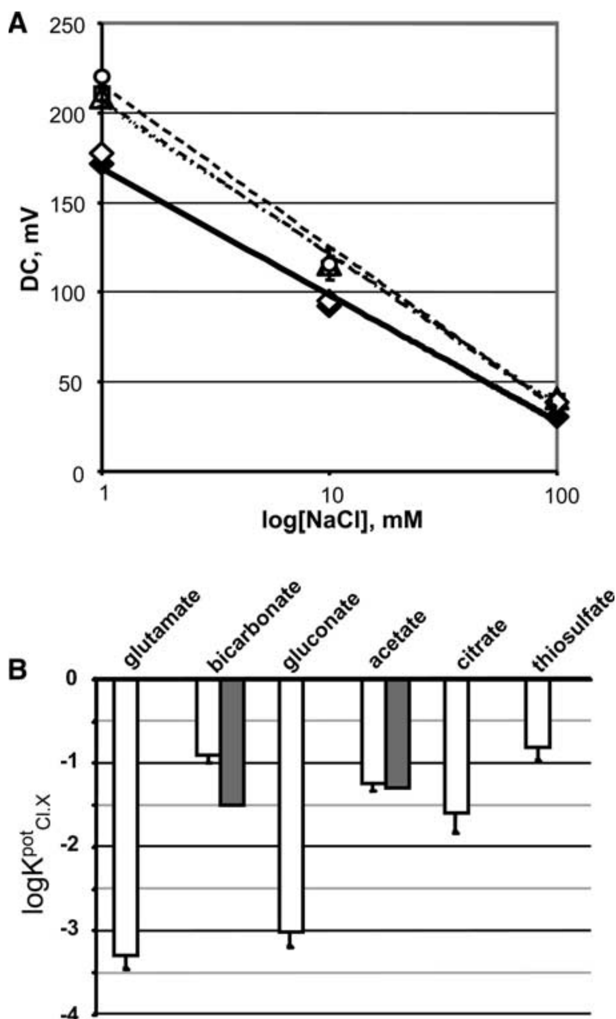

Fig. 2. Anion selectivity of the chloride ISE. (*A*) V_N measurements were made in standard solutions of 1, 10 or 100 mM NaCl containing either 1 mM Acetate (–○–), 1 mM gluconate (-Δ-) or 1 mM glutamate (···◇···). Measurements made with standard 1, 10 or 100 mM NaCl solutions are shown as (♦), where $y = 70.4x - 43$, $R^2 = 0.99$. Slope of the line increased by 29%, 23% and 20%, in NaCl solutions containing 1 mM acetate, 1 mM gluconate or 1 mM glutamate, respectively, as compared to that measured in standard NaCl solutions. SEMS are shown or are smaller than symbols, $n \geq 6$ and $R^2 > 0.99$ in each case. (*B*) Selectivity coefficients for chloride ionophore I - Cocktail A, calculated using the separate solutions method are shown (Umezawa et al., 2000). The ionophore was 3 orders of magnitude more selective for Cl^- than for glutamate or gluconate but was only around 1 order of magnitude more selective for Cl^- than for bicarbonate, acetate, citrate and thiosulfate. SEMS are shown, $n \geq 4$ in each case. The gray bars indicate values previously reported by FLUKA (Huser et al., 1990; Kondo et al., 1989).

ΔV_N / (log[NaCl]), for 1 mM acetate, gluconate and glutamate respectively, with $R^2 \geq 0.99$ in each case.

Selectivity coefficients, calculated by the separate solutions method, for glutamate, bicarbonate, gluconate, acetate, citrate, and thiosulfate are shown in Fig. 2*B*. The anion-selective ionophore is 3 orders of magnitude more selective for Cl^- than for glutamate or gluconate but is only about 1 order of magnitude more selective for Cl^- over bicarbonate, acetate, citrate and thiosulfate. Published selectivity coefficients reported by FLUKA for bicarbonate and acetate are similar to those measured here (Fig. 2*B*).

Thus, the chloride ISE is at best an anion sensor and we will refer, hereafter, to the ISE as 'anion-selective'. As a result of the poor selectivity, Cl^- fluxes from biological specimens in complex medium can only be inferred based on prior physiological knowledge of the specimen. The addition of 1 mM of acetate, glutamate or gluconate, however, had little effect on the V_N value measured in 100 mM NaCl, indicating a small amount of these contaminating anions will have little effect on the ISE's ability to monitor Cl^- flux at this [NaCl]. This is an important consideration in monitoring Cl^- flux from cells in a Ringer's solution containing 100 mM NaCl.

ISE: pH Sensitivity

The pH sensitivity of the ionophore was tested by measuring the V_N potential in 1, 10 or 100 mM NaCl solutions at pH 6.0, 7.0, and 8.0, with [HEPES] = 0.1, 1.0 or 10 mM (Fig. 3). In each case, V_N potential measurements in test solutions were compared to measurements made in NaCl only. Solutions at pH 6.0 showed the least deviation from the standard NaCl solutions, which had a pH = 5.5 (Fig. 3*A*). The dependence of the V_N potential on [NaCl] was slightly steeper at the more alkaline value when buffered with HEPES. The slope was 29%, 17%, or 37% steeper in pH 6.0 solutions of 0.1, 1 and 10 mM HEPES, respectively. The linear coefficient (R^2) was 0.99 or greater in each case.

A similar trend was seen in pH 7.0 solutions (Fig. 3*B*). 100 mM NaCl solutions show little deviation from the standard solutions, whereas the DC potential in 1 mM NaCl, pH 7.0 solutions are 1.5–2-fold greater, depending on the HEPES concentration, than that for an unbuffered standard 1 mM solution. The slope was 60%, 51%, and 63% steeper in pH 7.0 solutions of 0.1, 1 and 10 mM HEPES, respectively, with $R^2 \geq 0.99$ in each case. The greatest deviation from linearity and from the standards was measured in pH 8.0 solutions (Fig. 3*C*). The slope was 66%, 56%, and 11% steeper in pH 8.0 solutions of 0.1, 1 and 10 mM HEPES respectively, with $R^2 = 0.93$, 0.99, and 0.99 respectively. This may be due, in part, to increased competition of $[OH^-]$ with Cl^- as solution pH increased.

Action of Pharmacological Agents

The addition of 10 μM tamoxifen to standard NaCl solutions had little effect on the Nernstian slope of the anion-selective electrode (Fig. 4). The slope was −66 or −76 mV / (log [NaCl]) ($R^2 \geq 0.99$), respec-

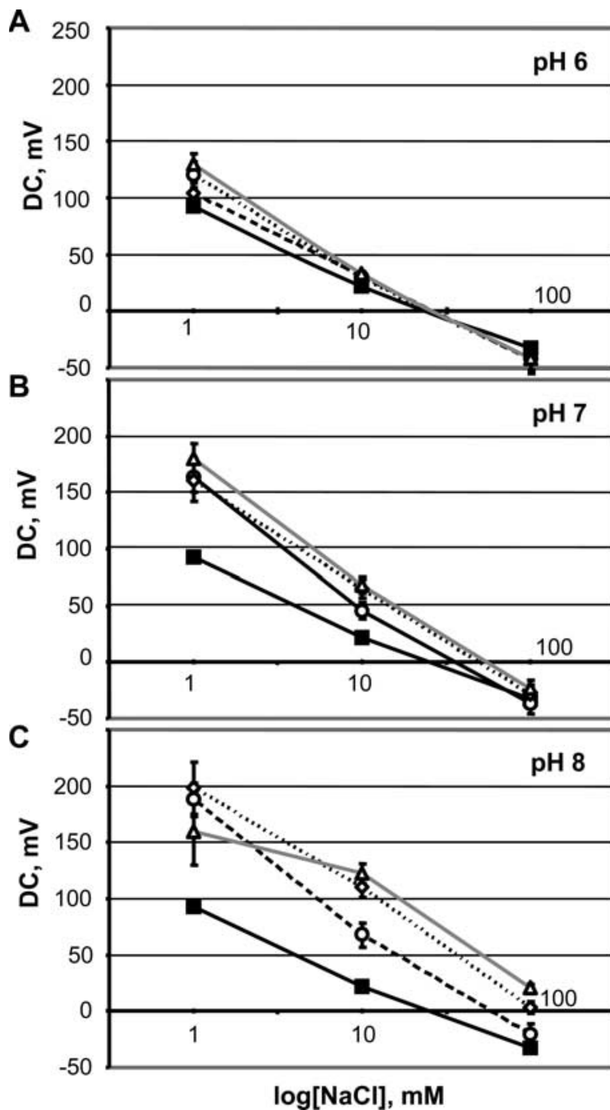

Fig. 3. Dependence of anion electrode on pH and HEPES buffer concentration. V_N measurements were made in 1, 10 or 100 mM NaCl solutions containing 1 (—○—), 10 (···◇···) or 100 (-Δ-) mM HEPES at (*A*) pH = 6.0, (*B*) pH = 7.0 or (C) pH = 8.0. V_N measurements made in standard 1, 10 or 100 mM NaCl-only solutions (-■-) are shown in each panel for reference. The Nernstian relationship of the ionophore in standard NaCl solutions (no buffer or set pH: shown in each panel) is $y = -62.7x + 98$, $R^2 > 0.99$. Addition of buffer increased the Nernstian slope at each pH, but V_N measured at 100 mM NaCl was similar regardless of buffer conditions. SEMS are shown or are smaller than symbols; $n = 4$ in each case.

tively, for standard or tamoxifen-containing NaCl solutions. In contrast, the addition of 50 μM NPPB decreased the sensitivity of the electrode, as the slope decreased to 11 mV / (log [NaCl]) ($R^2 \geq 0.99$; Fig. 4). NPPB clearly destroyed the Nernstian characteristics of the ISE and could not be used in conjunction with this form of measurement. Tamoxifen leaves the Nernst slope and absolute values unaffected and was therefore the blocker of choice during the subsequent experiments.

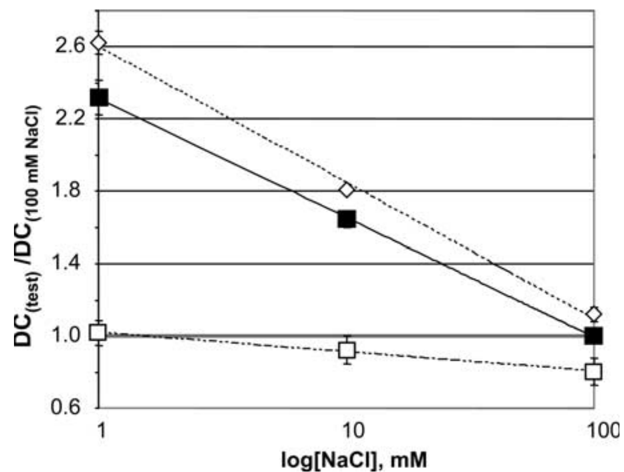

Fig. 4. Effect of tamoxifen and NPPB on anion selectivity of ionophore-filled electrode. V_N measurements, made in standard solutions of 1, 10 or 100 mM NaCl (-●-) and containing either 10 μM tamoxifen (-◇-) or 50 μM NPPB (–□–) are compared. Slopes of the lines are 65.9, 75.1 and 10.6 mV/mM for NaCl, tamoxifen- or NPPB-containing solutions. Addition of NPPB destroyed the Nernstian characteristics of the ISE, but addition of tamoxifen did not. $R^2 > 0.99$, $n \geq 4$ and SEM are shown or are smaller than symbols.

Hypotonic-induced Regulatory Volume Decrease

The tsA201a cells, like many mammalian cells, respond to a maintained hypotonic environment by swelling and then recovering their volume (Fig. 5*A*). Recovery from swelling requires the movement of anions and cations to pull osmotically obligated water from the cell, restoring volume (Eggermont et al., 2001). In response to a sustained hypotonic challenge, tsA201a cells swelled within 5 min, reaching peak volume at 6–7 min before recovering to near initial volume within 20 min. 10 μM tamoxifen blocks volume recovery so that cells continue swelling (Fig. 5*A*). Figure 5*B* shows that while control cells have recovered their original volume after a 20 min exposure to hypotonic Ringer's, cells in the presence of 10 μM tamoxifen or 50 μM NPPB have not (F_i/F_t, = +25 ± 2, +19 ± 1 and −1.6 ± 4.7, for tamoxifen, NPPB and control, respectively).

Hypotonic-induced Anion Current

10 μM tamoxifen blocked 63 ± 6% of the volume-regulated anion current expressed by tsA201a cells (Fig. 6). In this example, the peak current density was near 250 μA/cm^2. The addition of tamoxifen reduced the current density to near 90 μA/cm^2. NPPB blocked 88 ± 4% of this current (Fig. 6*B*). This anion-selective current is activated by cell swelling in response to a transmembrane osmotic gradient and is required for volume-regulatory decrease (Garber & Cahalan, 1997).

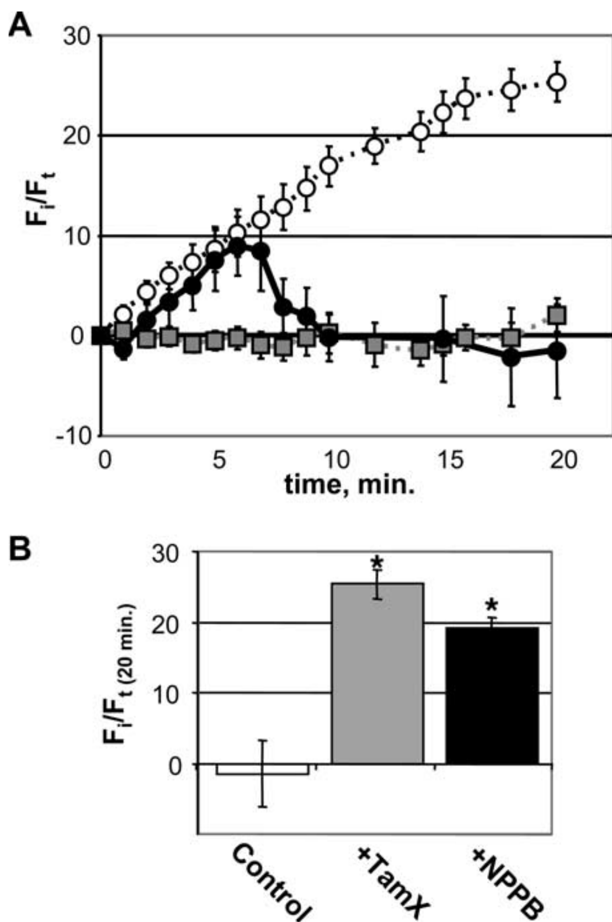

Fig. 5. Tamoxifen- and NPPB-sensitivity of regulatory volume decrease and swelling. (*A*) The tsA201a cells swell and recover volume when continuously exposed to hypotonic Ringer's (●) at $t = 0$, as measured using a fluorescent assay (*see* Methods). In the presence of 10 μM tamoxifen, cells swell, but do not recover (○), indicating that regulatory volume decrease was blocked. Addition of 10 μM tamoxifen to isotonic Ringer's has no effect on isotonic cell volume (■). SEMS are shown, $n \geq 5$. (*B*) Volume recovery was blocked by 50 μM NPPB or 10 μM tamoxifen in hypotonic-treated cells. Cells were exposed to hypotonic solution with or without drug for 20 min. Statistical difference (*) from control was determined by a two-tailed, unpaired Student's *t*-test with $p < 0.0001$. SEM is shown, $n \geq 5$.

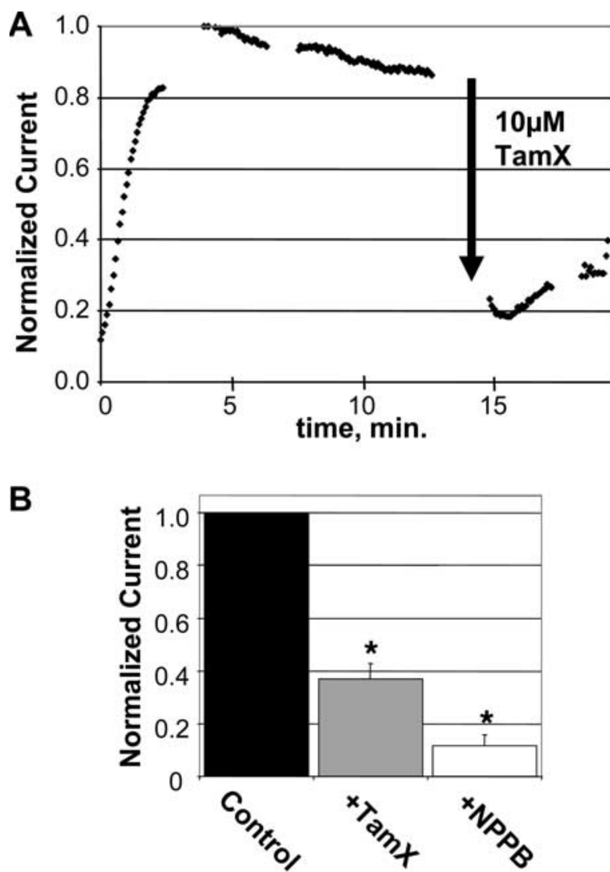

Fig. 6. Tamoxifen and NPPB block volume-regulated anion currents. (*A*) Swelling-activated anion currents, a critical component of regulatory volume decrease, were monitored with whole-cell patch recording. The transmembrane osmotic gradient was initiated with break-in at $t = 0$. 10 μM tamoxifen, added to bath solution at arrow, blocks swelling-activated current. (*B*) Peak amplitude of swelling-activated anion current is decreased 63 ± 6% by 10 μM tamoxifen and 88 ± 4% with 50 μM NPPB. The block of this current prevented regulated volume decrease in this cell line. Statistical difference (*) was determined by a two-tailed, unpaired Student's *t*-test with $p < 0.0001$. SEM is shown $n = 6$, for tamoxifen- or NPPB-treated currents, respectively.

Hypotonic-induced Anion Flux

Anion efflux from tsA201a cells was recorded with an ISE brought within 1–3 μm of a small group of 2–5 cells (Fig. 7*A*). The electrode was calibrated with NaCl standard solutions before and after each experimental recording. Anion flux was first recorded in isotonic Ringer's solution for 1–2 min, establishing a baseline. Bath solution was then changed to hypotonic Ringer's solution. Bath volume was exchanged 5 times within 90 s. Time was determined as $t = 0$ when bath solution exchange was complete. Cells were bathed in hypotonic solution for the remainder of the experiment (~30 min). The relative position of the electrode in relationship to the cells was maintained by visual monitoring as cells swelled and recovered volume in hypotonic solution. A persistent inward current was observed in some recordings over the duration of the recording. The persistent current may be due to differences in the anion composition of the growth medium (DMEM) and Ringer's solutions and/or expression level of chloride conductances at different times during the cell cycle (e.g., Bubien et al., 1990; Chen et al., 2002; Wang et al., 2002).

Figure 7*B* shows an example of an increase in anion efflux with application of hypotonic Ringer's. In this example, efflux peaked within 10–12 min and decreased over the following 20 min. The average change in hypotonic-induced anion flux from 9 separate recordings showed an increased outward

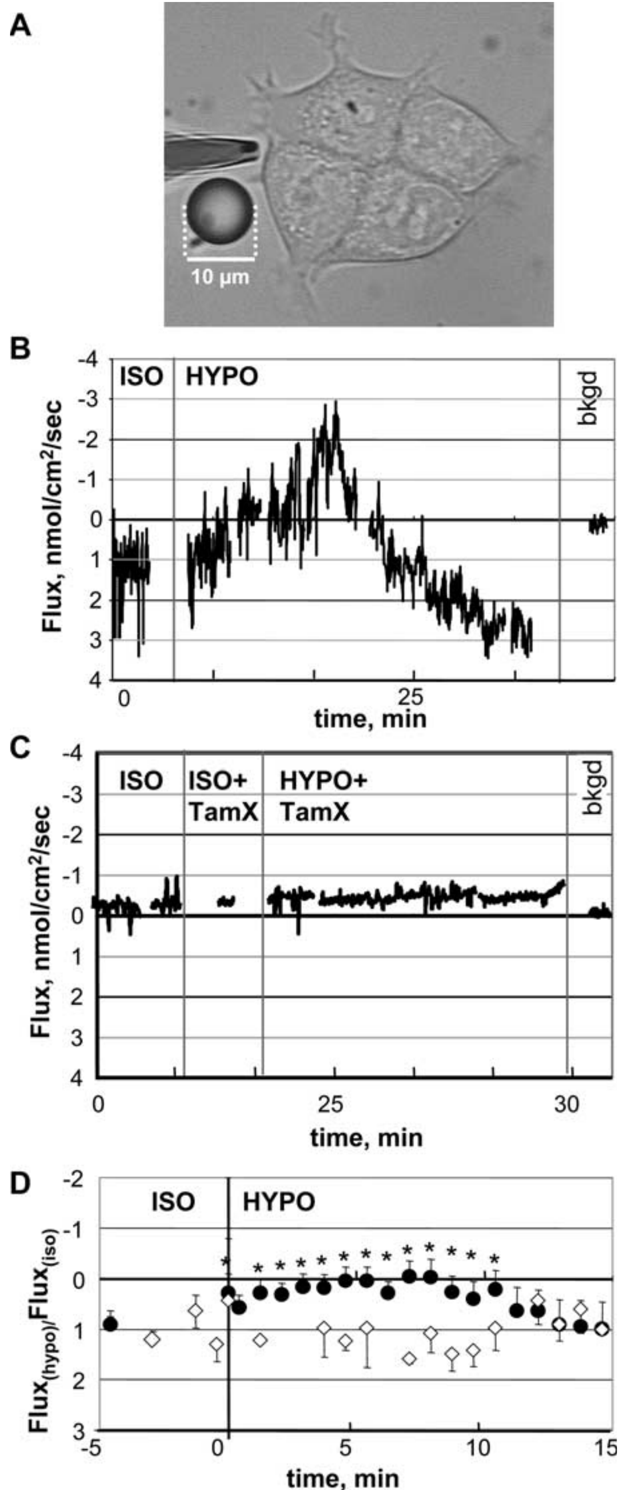

Fig. 7. Anion flux in response to hypotonic Ringer's. (*A*) An ISE is positioned 1–2 μM from a group of 4 cells. A 10 μM diameter is included for size comparison. (*B*) An example of anion flux recorded from cells exposed to isotonic (*ISO*) Ringer's for several minutes to establish a baseline flux. Bath solution was exchanged with hypotonic (*HYPO*) Ringer's as indicated. The upward deflection of the signal indicates anion efflux from the cells. Background (*bkgd*) flux was measured by moving the electrode > 500 μM away from cells. (*C*) Anion flux recorded from a different group of tsA201a cells in the presence of 10 μM tamoxifen. Baseline flux was established in isotonic (*ISO*) Ringer's, before exchanging solution with isotonic Ringer's plus 10 μM tamoxifen (*ISO + TamX*) and then with hypotonic Ringer's plus 10 μM tamoxifen (*HYPO + TamX*). There is no change in efflux in the presence of hypotonic Ringer's plus 10 μM tamoxifen, suggesting that anion efflux via volume-regulated anion currents was blocked. (*D*) Average anion efflux, normalized to average efflux under isotonic solutions alone or plus 10 μM tamoxifen is shown. Under control conditions, anion efflux increased and then decreased with continuous exposure to hypotonic solution (●). Addition of 10 μM tamoxifen (◇) prevented an increase in anion efflux with hypotonic exposure. The time course of anion efflux under control conditions was similar to the time course of volume-regulatory decrease shown in Fig. 5. Statistical differences (*) were determined by a two-tailed, unpaired Student's *t*-test with $p < 0.05$. SEM; $n = 9$, 3 or 6 for control, tamoxifen and NPPB-treated cells, respectively.

movement of anions that peaked near 7–8 min after application of hypotonic solution followed by a subsequent decline in efflux (Fig. 7*D*). The peak anion efflux is consistent with volume changes under similar conditions shown in Fig. 5*A*, although the time of peak efflux was slightly longer than the time of peak swelling shown in Fig. 5*A*.

The peak anion efflux was ~2 nmole/cm^2/s, which was equivalent to a current density of ~190 μA/cm^2. This measurement compared favorably to the peak whole-cell current density of ~250 μA/cm^2 (Fig. 6). The peak anion efflux was not corrected for the time response of the ISE and thus represents a lower limit of the actual flux value. The measured flux is also less than the actual value, as the probe sits 1–3 μm away from the cells.

10 μM tamoxifen blocked the hypotonic-induced increase in anion efflux (Fig. 7*C*, *D*). Recordings made in isotonic Ringer's containing 10 μM tamoxifen showed no significant change in anion flux with application of hypotonic solution + 10 μM tamoxifen ($n = 3$). There was no significant change in the anion flux when the bath solution was changed from isotonic to hypotonic solution in the presence of 10 μM tamoxifen (Fig. 7*C*). These data show that the ISE is capable of monitoring, in real time, anion flux while the cells regulate their volume in response to a maintained hypotonic challenge.

Discussion

Anion-selective Electrode Characteristics in Physiological Solution

We have characterized an anion-selective microelectrode (ISE_{anion}) based on the chloride ionophore 1 in cocktail A available from Fluka in order to determine the feasibility of using this ISE to monitor changes in anion flux across cell membrane during volume-reg-

ulatory events. The response of the ISE was Nernstian (V_N) over a range of [NaCl] from 1 to 100 mM. The value of V_N determined in 100 mM NaCl alone did not change with the addition of 1 mM acetate, 1 mM gluconate or 1 mM glutamate, or at pH 7.0 with 10 mM HEPES. Where available for comparison, the anion selectivities of the ionophore determined in this study were similar to those previously published by Fluka (Kondo et al., 1989; Huser et al., 1990). Most importantly, the response of the ISE when monitoring a change in anion concentration in a background of physiological Ringer's solutions was reliable and comparable to that measured in standard 100 mM NaCl solution. Consequently, we feel confident that we are monitoring anion flux using this ISE operating in a self-referencing format.

The ion-selective electrode was sensitive to pH, buffer and anion composition. There was a greater deviation from the response in standard [NaCl] when the pH was increased. This may, in part, be due to an increase in [OH^-] in solution, acting as an interferent. Indeed, the greatest deviations from V_N values measured with standard NaCl solutions occurred at low [Cl^-], where anionic interferents would be more of a problem.

Anion Flux during Volume Changes

Using this anion-selective electrode, we have measured an increase in outward anion flux with application of hypotonic Ringer's solution. This flux decreased over time, as would be expected during regulated volume decrease. The time course of the change in anion flux and regulated volume decrease, as measured by a fluorescent assay in the same cell line, is similar. The anion efflux time course appears slightly slower than the time course of volume regulation. Concurrent volume and efflux measurements made on individual cells are planned in the future to determine if this temporal difference is physiologically relevant or is due to experimental differences.

Tamoxifen, an agent known to block regulated volume decrease, blocked the hypotonic-induced increase in anion efflux. NPPB, another possible blocker, was shown to 'poison' the ISE. The volume-regulated anion current, which underlies regulated volume decrease in these cells, is blocked by both of these agents. We conclude, therefore, that the anion efflux we are measuring, most likely chloride, is mediated by volume-regulated anion channels activated by a hypotonic challenge.

Conclusion

In this study we have shown that an anion-selective electrode is selective for Cl^- in physiological Ringer's solution. In addition, we have used this electrode to monitor anion flux in an intact cell during regulated volume decrease initiated in the presence of hypotonic solution. The hypotonic-induced anion flux was blocked in the presence of tamoxifen. Thus, we conclude that ISE technology will be useful in understanding the mechanisms underlying volume-regulatory decrease.

This non-invasive technique provides a method of monitoring anion flux during physiological phenomena. It provides the possibility of coordinating cell swelling and recovery with a direct measurement of anion movement. These measurements can be expanded with the use of H^+-, K^+- or Ca^{2+}-selective electrodes (Smith et al., 1999; Smith & Trimarchi, 2001). This technique will prove invaluable in correlating ion flux activity with changes in intracellular [Cl^-] and other osmotically active anions, membrane potential and changes in cell size during swelling and recovery in intact cells.

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