Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 3919–3923, May 1989
Physiological Sciences

Activity of ion channels during volume regulation by clonal N1E115 neuroblastoma cells

(stretch-activated cation channels/delayed-rectifier K^+ channels/anion channels/regulatory volume decrease/patch clamp)

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Communicated by Bertil Hille, February 27, 1989 (received for review October 4, 1988)

ABSTRACT **When exposed to a hypotonic bathing solution, clonal N1E115 neuroblastoma cells initially swell and then undergo a regulatory volume decrease (RVD). Using cell-attached patch-clamp recording, we have found that the activity of a stretch-sensitive, nonselective cation [C^+(SA)] channel increases shortly after the onset of osmotically induced cell swelling; this depolarizes the cells as much as 30 mV. Shortly thereafter, and roughly coincident with the onset of RVD, two types of voltage-dependent channels open at the new resting potential; these are (*i*) a delayed-rectifier type K^+ [K^+(DR)] channel and (*ii*) a large-conductance anion channel. We suggest that opening of the C^+(SA) channel may contribute to the volume "sensor" mechanism, while the depolarization-induced opening of the K^+(DR) and anion channels may constitute a significant K^+ salt exit pathway, operating in RVD.**

Volume regulation is a feature common to many vertebrate cells (1). When placed in a hypotonic bathing solution, these cells initially swell but then, over several minutes, shrink back to near their resting volumes. The regulatory volume decrease (RVD) is usually accompanied by the loss of intracellular K^+ and anions, including Cl^-. Recently, attention has turned to stretch-activated ion channels, which appear to be ubiquitous (2–8), as possible candidates for "sensor" mechanisms and K^+ and anion exit pathways for cell-volume regulation (6–8). Here, we report that neuroblastoma cells of the clone N1E115, which display RVD, express numerous stretch-activated nonselective cation [C^+(SA)] channels. The activity of the C^+(SA) channel increases shortly after the onset of osmotically induced cell swelling and then decreases during RVD. Cell depolarization caused by the opening of C^+(SA) channels opens voltage-dependent, delayed-rectifier type K^+ [K^+(DR)] channels and seems to be responsible for the activation of multiple-conductance-state anion channels. These three types of channels may serve as a volume "sensor" and volume "effector" pathways for RVD. Some of this work has been presented in abstract form (9).

METHODS AND MATERIALS

Experiments were performed on 20- to 50-μm-diameter N1E115 cells grown for 3–15 days on glass coverslips at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal calf serum, 1–2% dimethyl sulfoxide, 0.5% penicillin, and 0.5% streptomycin in the presence of 5% CO_2/95% air. Though the cell population was quite pleomorphic, with some cells resembling hemiellipsoids and others having complex neuritic processes, all cells examined displayed nearly identical ionic channel currents as well as action potential activity in response to depolarization or anodal break. The standard extracellular-like bathing solution, ES, whose osmolarity was 285 mosM and whose pH was 7.3, contained 130 mM NaCl, 5.5 mM KCl, 2.5 mM $CaCl_2$, 1.25 mM $MgCl_2$, 20 mM NaOH-adjusted Hepes buffer, and 10 mM glucose. Bath osmolarity was reduced to 225 milliosmolar (mosM) or 185 mosM by diluting ES with distilled water, hence reducing the NaCl concentration to 98 mM or 65 mM, respectively, and the KCl concentration to 4.15 mM or 2.75 mM, respectively, without altering solution pH. For some experiments, ES contained 1 mM EGTA and no $CaCl_2$ (low-Ca^{2+} ES).

Cell-volume regulation was monitored during osmotic perturbations by following changes in the cross-sectional areas of cells plated on glass coverslips. A microscopic field containing many noncontiguous cells was photographed at intervals through phase-contrast optics (at ×320) at a fixed focal plane. Cross-sectional areas of 6–10 similarly sized cells lacking elongated processes were calculated from cell circumferences traced on photographic prints with a digitizing tablet. The area of each cell was normalized to that at the beginning of the experiment ($t = 0$), and the normalized areas were averaged to generate points for plotting (A_t/A_0).

Single-channel currents were recorded and analyzed by techniques that are standard for our laboratory (10). Pulses of suction were generated by microprocessor activation of an on/off valve connected to suction by a suction regulator (Puritan-Bennett, Saint Louis) and were applied to the side port of the pipette holder. Data were filtered at 0.5–0.9 kHz and sampled at 1.0–2.0 kHz. Analysis of channel current amplitude and activity was done by using digitized data and an interactive graphics display. The average number of channels open in a patch (I/i) was measured from segments of raw data by using interactively specified half-amplitude level crossings to determine the fraction of time when zero, one, two, three, or more channels were open; the results from a 4-sec to 2-min segment of record were then averaged and plotted. The clamping voltage (V_c) is defined as the negative of the inside-pipette potential with respect to the bath as ground. Hence, in the case of the cell-attached patch, a V_c value of +20 indicates a depolarization of the patch of membrane by 20 mV from the voltage at rest (V_{rest}); in the case of the inside-out excised membrane patch, a V_c value of +20 mV represents the absolute membrane potential.

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Abbreviations: V_c, clamping voltage; γ, maximum slope (single channel) conductance; E_{rev}, reversal potential (zero-current potential); I/i, average number of channels open; RVD, regulatory volume decrease; C^+(SA), stretch-activated nonselective cation channel; K^+(DR), delayed rectifier-type K^+ channel; V_{rest}, voltage at rest; mosM, milliosmolar.

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RESULTS

Volume Regulation by N1E115 Cells in Hypotonic Solutions. When exposed to hypotonic bathing solution, N1E115 cells swell within the first several minutes and then slowly shrink toward their original size. Further rapid shrinking is observed when the isotonic bathing solution is restored after RVD. As illustrated by a typical experiment (Fig. 1), reduction of the osmotic pressure of ES from 285 to 185 mosM resulted in a >30% increase in A_t/A_0 within 5 min. In the presence of either normal or very low Ca^{2+}, A_t/A_0 subsequently declined, reaching control values in 30–50 min corresponding to a phase of RVD. Reducing the osmotic pressure of ES from 285 to 225 mosM resulted in a smaller (≈15%) increase in A_t/A_0; subsequent recovery of A_t/A_0 was slower.

A C^+(SA) Channel Activated During Cell Swelling. Fig. 2 presents basic features of a C^+(SA) channel regularly seen in patches of N1E115 plasma membrane. After formation of a stable gigaohm pipette-to-membrane seal, application of pipette suction of >5 mmHg evoked pulses of current of uniform amplitude (see Fig. 2*a*). Stepwise increments in suction from 5 to 12.5 mmHg increased the steady-state frequency and duration of these inward currents, while further increases in suction to >15 mmHg caused gaps to appear in channel activity, reducing I/i from 0.25 at 15 mmHg to 0.17 at 17.5 mmHg. The suction or "stretch" sensitivity of the channels was maintained over several such runs spanning many minutes, provided that suction of >25 mmHg was not maintained for more than several seconds. In trial experiments, the channel was also activated by pulses of positive pressure of similar magnitude; mechanical instability of the patch, which usually developed after several pulses, discouraged quantification of this behavior.

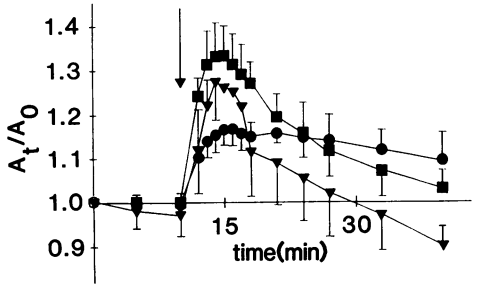

FIG. 1. Typical time courses of A_t/A_0 for N1E115 cells exposed to bath solutions of reduced osmolarity. At the arrow, the osmotic pressure was reduced by replacing the bath with one of the following solutions: ■, 185 mosM ES containing 2 mM Ca^{2+}; ▼, 185 mosM ES containing 0.5 mM EGTA; ●, 225 mosM ES containing 2 mM Ca^{2+}. Comparisons were made between cells of the same age and plating condition; the same six cells were followed throughout an osmotic-perturbation run. Data are presented as means with bars representing SDs.

In cell-attached patches, the channel showed only slight voltage dependence over the range $V_c = \pm 30$ mV (see Fig. 2*b Left*). With ES in the pipette and bath, the current/voltage (*I–V*) curve of the stretch-activated channel had an interpolated reversal potential (E_{rev}; zero-current potential) of 60 mV and a maximum single-channel (slope) conductance (γ) of 22 pS (see Fig. 2*c*). Stretch-activation of this channel was maintained after patch excision; Fig. 2*b* shows the similar increase in channel activity produced by pulses of suction in the

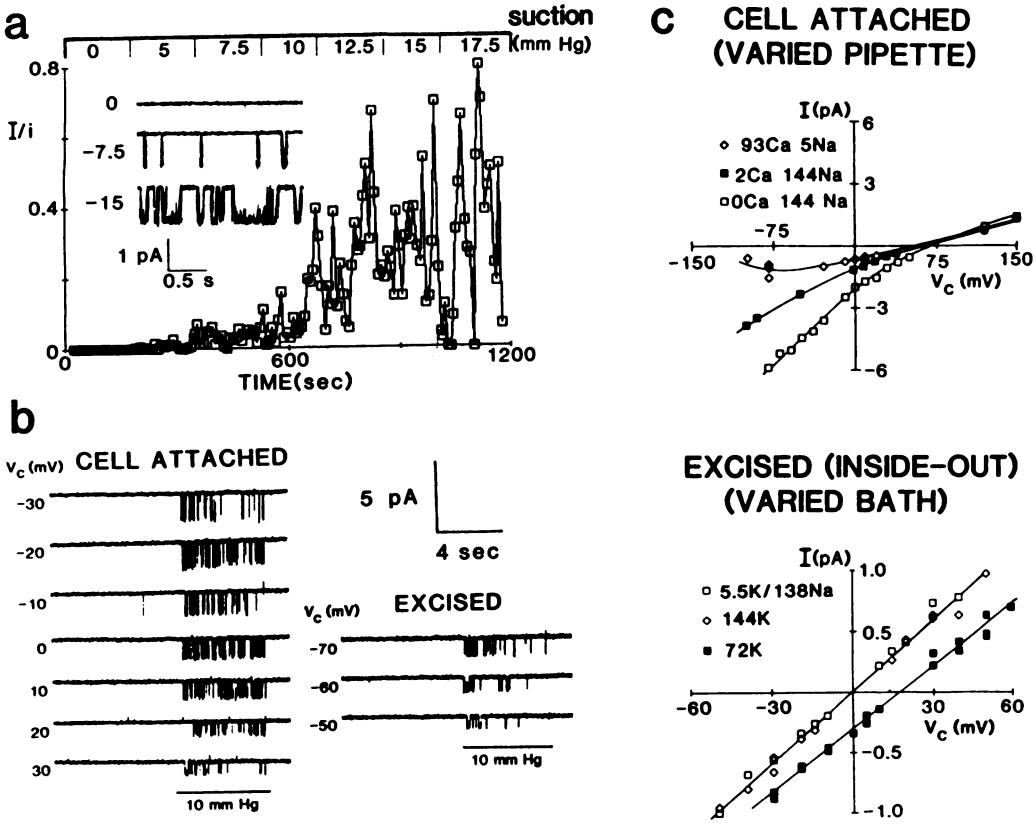

FIG. 2. C^+(SA) channel activated by pipette suction, with ES-containing pipette and bath unless otherwise indicated. (*a*) Time course of channel activity in the cell-attached patch during a progressive increase in steady-state suction applied to the patch pipette ($V_c = 0$ mV). (*Inset*) Sample traces recorded at $V_c = 0$ mV and the noted pipette suction (in mmHg). (*b*) Comparison of C^+(SA) channel activity in response to pulses of suction (10 mmHg) applied at various V_c values in the cell-attached patch (*Left*) and then immediately after patch inside-out excision into low-Ca^{2+} ES (*Right*). (*c*) (*I–V*) curves of C^+(SA) channel in different ionic conditions. (*Upper*) Cell-attached patches with different pipette solutions and constant-ES bath solution. (*Lower*) Inside-out excised patch with various bath solutions and constant ES in the pipette. (Variant solutions were buffered with 20 mM Hepes, pH 7.3.)

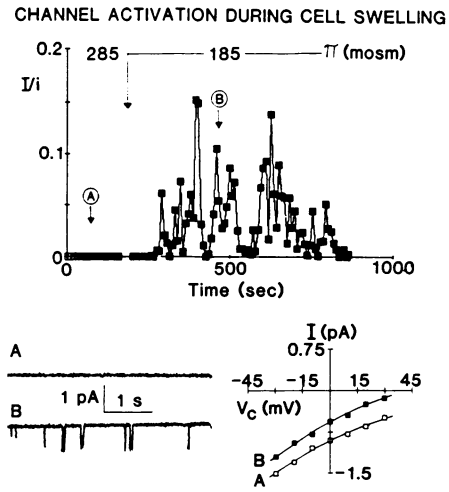

FIG. 3. (*Upper*) C^+(SA) channel activity during reduction in the osmotic pressure (π_{osm}) of the bath solution (ES-containing pipette; V_c = 0 mV). (*Lower*) Representative current traces (*Left*) and *I–V* curves (*Right*) of the channel before (trace A) and during (trace B) cell swelling. *I–V* curve of channel prior to swelling (curve A) was obtained by using short pulses of suction to activate the channel.

cell-attached patch and after excision of the patch (inside-out) into low-Ca^{2+} ES.

The channel's selectivity to ions was examined. In the inside-out excised patch, mole for mole replacement of NaCl by KCl in the bath had no effect on E_{rev} (= 0 mV) or γ, whereas diluting KCl by half shifted E_{rev} positively by ≈15 mV (Fig. 2*c Lower*). This established that the channel was cation selective. In the cell-attached patch, inward current with a similar E_{rev} persisted when the NaCl content of a Mg^{2+}-free ES-containing pipette was isoosmotically replaced with $CaCl_2$ although γ was reduced to 7 pS, whereas eliminating Ca^{2+} from a Mg^{2+}-free ES-containing pipette increased γ from 22 to 55 pS (Fig. 2*c*). These results suggest that the channel is both permeable to and partially blocked by Ca^{2+}.

Activity of the C^+(SA) channel was enhanced during exposure of the cell to hypotonic ES solutions. Fig. 3 depicts a time course of C^+(SA) channel activity, in a cell-attached patch of membrane selected for its prominent inward channel current activity during a pulse of suction. Within several minutes of reducing the osmotic pressure of the ES bath from 285 to 185 mosM, C^+(SA) channel openings were seen with no applied suction. Their frequency increased with a time course similar to cell swelling and was transiently enhanced by a pulse of suction. During cell swelling, E_{rev} of this channel was shifted negatively by at least 15 mV. This is consistent with depolarization of the remainder of the cell membrane because of the opening of many nonselective cation channels. (This "observed" shift in E_{rev} may underestimate the "actual" shift in membrane potential. The initial water influx occurring with osmotic equilibration should reduce intracellular univalent cation concentration from nearly 145 mM to nearly 90 mM. This would shift E_{rev} positively by nearly 12 mV.)

On closer inspection of records of channel activity, two other types of single-channel currents were observed at or near V_c = 0 during cell swelling. Short-duration, small-amplitude, outward current steps appeared after the onset of C^+(SA) channel activity; we identified these as K^+(DR)

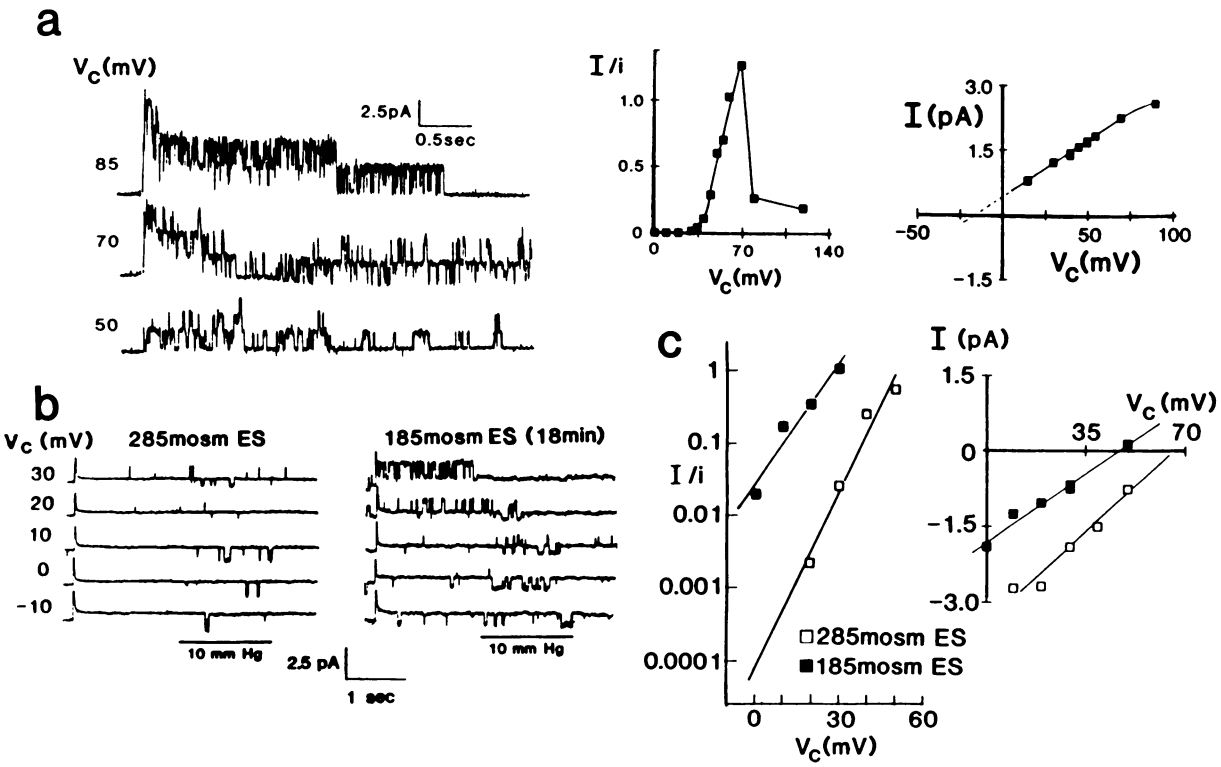

FIG. 4. K^+(DR) channel and its activity during RVD, with cell-attached patch and ES-containing pipette and bath unless otherwise indicated. (*a Left*) Single-channel traces illustrating typical activity were observed in response to a rapid depolarization from a holding potential of 0 mV to the designated test potentials (capacity transients have been subtracted). (*a Right*) Graphs illustrate the *I–V* and *I/i* vs. V_c relationships during a "staircase" depolarization from V_c = 0 to 120 mV (10-mV steps at 100-sec intervals). (*b*) Sample traces recorded from a patch containing both K^+(DR) and C^+(SA) channels in isotonic ES at 225 mosM and 18 min after reduction of osmolarity to 185 mosM. Note that K^+(DR) currents are outward and C^+(SA) channels are inward in these traces. (*c*) K^+(DR) channel current and average activity vs. clamping potential before (□) and during (■) a reduction of osmolarity from 285 mosM to 185 mosM. The pipette contained 150 mM KCl buffered with 20 mM Hepes/KOH, pH 7.3.

channel currents. Within several minutes, large-amplitude, long-duration, multiple-conductance-level inward currents appeared; we identified these as voltage-dependent anion-selective channels.

Characteristics of K^+(DR) Channels in Resting Cells. In cell-attached N1E115 patches formed with ES in the pipette and bath, we observed steps of outward current during steps of depolarization from a holding potential of 0 mV to a test potential $\geq$ +20 mV (Fig. 4*a Left*). Peak channel activity, seen early after a depolarizing step, increased with larger depolarization; at later times inactivation set in. The extrapolated E_{rev} of this channel was roughly 20 mV hyperpolarized from V_{rest}, and γ was 20–22 pS (Fig. 4*a Right*). These features identify this channel as a K^+(DR) channel, similar to the FK channel previously described by Quandt (11). As many as 20 such channels may be seen in a single membrane patch. In rare patches, a smaller conductance (11–13 pS) variety of this channel was also seen; this channel probably corresponds to Quandt's SK channel. Another important feature of the K^+(DR) channel is its steady-state activity during prolonged depolarization. Fig. 4*a Center* shows that with sustained (100-sec) depolarization, average channel activity increased e-fold per 6–8 mV over the V_c range 35–70 mV. The K^+(DR) channel was not directly activated by membrane stretch induced by pipette suction up to 25 mmHg.

Activity of the K^+(DR) Channel During Cell Swelling. Fig. 4*b* shows the effect of reduction in the osmolarity of ES on K^+(DR) channel activity in the cell-attached patch. In 285 mosM ES, outward K^+(DR) channel currents were first activated at depolarizations > 20 mV from V_{rest}. During exposure to 185 mosM ES, K^+(DR) currents were seen at or near V_c = 0 mV; their extrapolated E_{rev} was also shifted negatively by 20 mV. Inward C^+(SA) channel currents were also seen in this patch; their activity was similarly enhanced in 185 mosM ES, where openings were seen even in the absence of applied suction. The similar shifts in the E_{rev} values of the K^+(DR) and C^+(SA) channels further suggested that the plasma membrane was depolarized by at least 20 mV during cell swelling.

To better quantify the shift in E_{rev} and the increase in average channel activity near V_{rest} during cell swelling maneuvers, we performed several experiments in which the pipette was filled with 144 mM KCl (buffered with 20 mM Hepes/KOH), thereby shifting E_{rev} of the K^+(DR) channel positively by $\approx$60 mV and increasing γ to nearly 50 pS. This facilitated the observation of small currents around V_{rest}. From a series of four experiments similar to that shown in Fig. 4*c*, it is clear that both E_{rev} and the probability of a channel opening at a given voltage are shifted negatively by 15–40 mV during cell swelling.

Identification of an Anion Channel Active After Cell Swelling. In 6 of nearly 30 cell-attached patches, large inward current steps were seen after reducing bath osmotic pressure from 285 mosM to 185 mosM (Fig. 5*a Lower*). γ varied between 300 and 400 pS in different patches. Their appearance usually followed by several minutes the appearance of C^+(SA) channel activity (Fig. 5*a Upper*). The activity of this large-conductance channel ceased with restoration of the osmotic pressure to 285 mosM, as did the activity of C^+(SA) channels.

The large currents persisted after the patch of membrane was excised inside-out. In this configuration, reducing NaCl of the ES bath from 130 to 55 mM shifted E_{rev} of the channel from 0 to −23 mV (see Fig. 5*b Upper*). By using the Goldman–Hodgkin–Katz equation, P_{Cl}/P_{Na} (the ratio of permeabilities of the channel to Cl^- and Na^+) was estimated as 30:1. When the NaCl content of the ES bath was replaced by sodium acetate, E_{rev} was shifted negatively by 15 mV, and γ was $\approx$150 pS (Fig. 5*b Lower*). After correction for liquid junction potentials, $P_{Cl^-}/P_{CH_3COO^-}$ was 2.4:1, suggesting that the channel is permeable to small organic anions as well as to Cl^-. We attempted to characterize the voltage dependence of the anion channel in the inside-out excised patch. Channel activity was present but rare at −30 mV and increased upon depolarization to 10 mV (Fig. 5*c Top*). Channel closure occurred with subsequent repolarization to negative V_c values (Fig. 5*c Middle*) or depolarization to more positive V_c values (Fig. 5*c Bottom*). Inspection of records of repeated voltage pulses suggested a steep increase in channel activity between −40 and −20 mV (i.e., the range of values of V_{rest} anticipated for N1E115 cells during osmotic swelling) as well as peak channel activation near V_c = 0–10 mV.

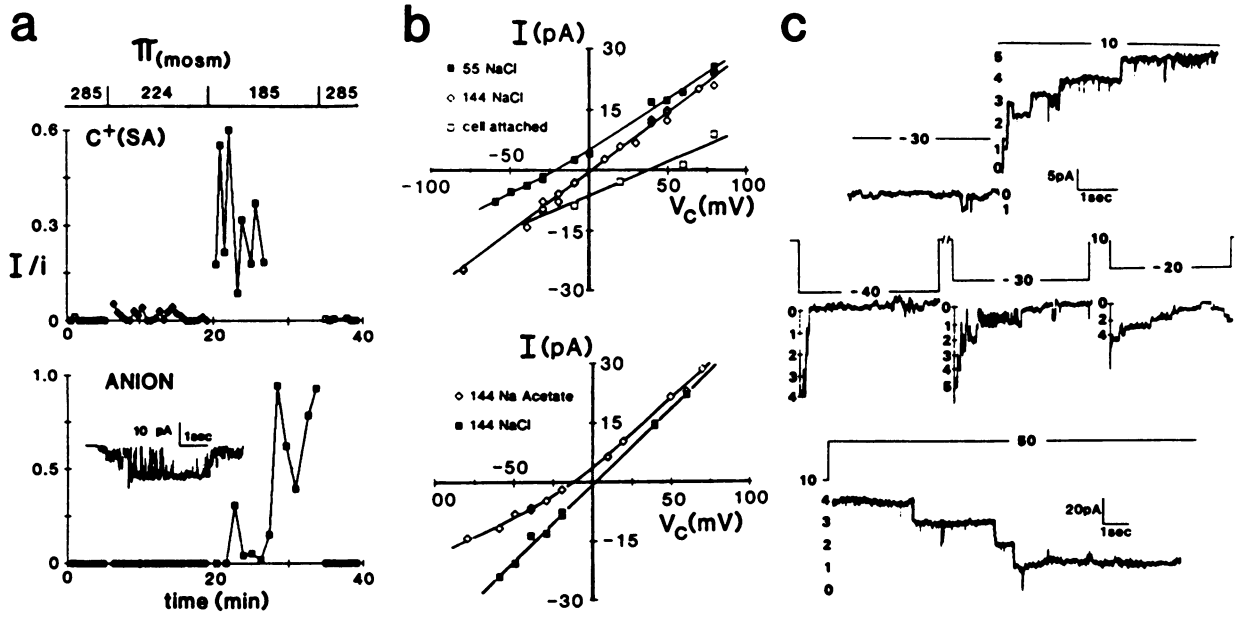

FIG. 5. Characterization of the anion-selective channel and activity during RVD. (*a*) Time course of activity of C^+(SA) and large multiple-conductance-level anion channels during sequential osmotic pressure (π_{mosM}) changes in the bath (cell-attached patch; V_c = 0 mV; ES-containing pipette). (*b*) *I–V* curves of the anion channel in the cell-attached patch and after inside-out excision of the patch into the indicated bath solutions, which were buffered with 20 mM Hepes/NaOH, pH 7.3. (*c*) Voltage dependence of the anion-channel activity in the inside-out excised patch.

DISCUSSION

We suggest that in clonal N1E115 neuroblastoma cells, hypotonicity-induced cell swelling and ion-channel activity may be related in the following way. Cell swelling opens C^+(SA) channels; a plausible link is the alteration in cell membrane forces transmitted to the channel by the cytoskeleton. This results in a 20- to 30-mV membrane depolarization, causing the opening of K^+(DR) channels, which are not directly sensitive to pipette suction. Membrane depolarization, perhaps in conjunction with other swelling-induced changes, also results in the opening of large-conductance anion channels. The activation of both K^+- and anion-selective pathways permits the exit of K^+ salts. This should facilitate H_2O exit, the restoration of normal cell volume, and a low level of membrane channel activity and ultimately should result in membrane repolarization.

While ion-exchange carriers, cotransporters, and amino acid exit mechanisms might still play an important role in volume regulation in N1E115 as they do in other cells (e.g., see ref. 1), it is intriguing that three rather commonplace ionic channels might form a joint volume sensor and effector system. The C^+(SA) channel described here is quite similar to stretch-activated channels found in a variety of other vertebrate cells (2–6). The K^+(DR) channel of neuroblastoma is strikingly similar to that found, not only in a variety of excitable cells but also in circulating blood cells such as lymphocytes, monocytes, and macrophages (12–14), which actively regulate their volumes. New evidence by Lee *et al.* (14)—that mutant lymphocyte clones that do not regulate volume also lack K^+(DR) channels—is among a body of work that suggests a nonexcitatory role for this ubiquitous class of K^+ channels (12–14). Finally, similar large, multiple-conductance-state, voltage-dependent, anion-selective channels have been seen in excised patches from a wide variety of cells, including lymphocytes, skeletal and smooth muscle cells, and epithelial and glial cells (13, 15–18). The observation that larger organic anions (e.g., acetate) also permeate the channel supports its possible role in volume regulation; a large fraction of the cell's nonprotein anions are probably organic acids that have to exit the cell for RVD to occur.

From our data, it is still unclear whether a Ca^{2+}-dependent pathway contributes to volume regulation in N1E115 cells, as it does in other cells (1, 7). (*i*) The time course of RVD is not slowed by reducing extracellular Ca^{2+} to submicromolar levels. (*ii*) C^+(SA) channels of N1E115 are permeated by Ca^{2+} (see Fig. 2*c*) and large-conductance Ca^{2+}-activated K^+ channels are present in these cells. However, the large-conductance Ca^{2+}-activated K^+ channels are active in <20% of cell-attached patches and then only at very large depolarizing voltages, even after addition of ionomycin to an ES bath. The latter maneuver should raise intracellular Ca^{2+} concentrations to near micromolar levels. (*iii*) In inside-out excised patches, the large-conductance anion channels are not obviously dependent on intracellular Ca^{2+} levels over the range of <1 μM to >2 mM. This does not, however, rule out intracellular Ca^{2+}-dependence of anion-channel activity in intact cells.

A more complete understanding of the contribution of these three ion channels to RVD in neuroblastoma should be based on a more precise quantification of ion-channel activity and more detailed knowledge of the forces and intracellular messengers that modulate gating. This should require, among other approaches, (*i*) identification of selective blockers of the C^+(SA), K^+(DR), and anion channels; (*ii*) monitoring of "whole-cell" membrane potential and currents in the presence and absence of these blockers during volume regulation; and (*iii*) correlation of the time courses of these currents with cell size under various conditions.

We thank Alex Mauro for the gift of neuroblastoma cultures, Rich Robb and Tom Woolsey for use of the digitizing tablet, Ulises Alvarez for maintaining the cultures, and Betty Ytzaina and Helen Odle for preparing the typescript. This work was supported by a grant from the National Institutes of Health (DK37380).

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