A Novel Function of BCL-2 Overexpression in Regulatory Volume Decrease

ENHANCING SWELLING-ACTIVATED \( \text{Ca}^{2+} \) ENTRY AND CI\(^{-} \) CHANNEL ACTIVITY*

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The cellular function of the oncogene \( \text{bcl-2} \), a key regulator of apoptosis, is still debated. The goal of this study was to explore the relationship between BCL-2 overexpression and cell volume regulation by using two independent models, Madin-Darby canine kidney (MDCK) cells stably transfected with BCL-2 and MDCK clones with inducible BCL-2 expression by the lac operator/repressor. BCL-2 overexpression enhanced the capability of regulatory volume decrease (RVD), a cellular defensive process against hypotonic stress. In various clones of MDCK cells, hypotonic stress induced an outwardly rectified CI\(^{-} \) current that was significantly up-regulated by BCL-2 overexpression. Other fundamental characteristics of this channel were similar among different MDCK clones, such as sensitivity to CI\(^{-} \) channel inhibitor, anion permeability, and time-dependent inactivation at more positive potential. Most importantly, BCL-2 overexpression up-regulates the swelling-activated \( \text{Ca}^{2+} \) transient that is a critical signaling for normal RVD and the activation of swelling-activated CI\(^{-} \) channel in MDCK cells. BCL-2 overexpression also enhances the capacitative \( \text{Ca}^{2+} \) entry that can be differentiated from the swelling-activated \( \text{Ca}^{2+} \) transient by kinetic analysis and sensitivity to GD3. Moreover, neutralization of endogenous BCL-2 by antibody blocks the normal RVD response and the activation of swelling-activated CI\(^{-} \) channel in human cervical cancer HT-3 cells. These results provide a new insight into the novel function of BCL-2 overexpression in the regulation of cell volume and ion flux.

Mammalian cells have to avoid excessive changes of cell volume that jeopardize structural integrity and constancy of the intracellular milieu. Homeostasis of cell volume does not simply indicate a constant volume but rather serves as the integration of events in regulating cell function (1, 2). Most mammalian cells defend themselves against hypotonic stress by losing solutes together with osmotically obligated water, a process termed regulatory volume decrease (RVD). The principal solutes lost during RVD are K\(^{+} \), CI\(^{-} \), and a group of largely electroneutral organic solutes known as organic osmolytes. The predominant pathway for RVD is the opening of separate K\(^{+} \) and CI\(^{-} \) channels (3). Much attention has been focused on the swelling-activated CI\(^{-} \) channel, because it shows a broad sensitivity for different anions and organic osmolytes (3). In addition to volume regulation and osmolyte transport, the swelling-activated CI\(^{-} \) channel participates in several important physiological processes, such as metabolism, hormone release, cell proliferation, differentiation, and migration (1, 4). In some cell types, osmotic swelling increases intracellular \( \text{Ca}^{2+} \) ([\( \text{Ca}^{2+} \)]\(_{i}\)), which plays a critical role in the control of RVD (1, 5).

The gene of BCL-2 is located at chromosome 18q21 and encodes a 25–26-kDa protein (6). Overexpression of BCL-2 is known to convey resistance to apoptosis induced by many agents (7). Despite this fact, the function of BCL-2 on other cellular events is usually overlooked, and very little is known about the involvement of the BCL-2 family in the regulation of cell volume. Because volume constancy is one of the most critical events for cellular homeostasis and survival, it would be interesting to study the association of BCL-2 family with cell volume regulation.

The Madin-Darby canine kidney (MDCK) cell line is one of the best characterized preparations for the study of epithelial ion and water transport and its regulation. We have successfully developed two model systems to dissect the BCL-2 effects on phenotypic or morphological changes of MDCK cells (8–10). One system is MDCK cells with differential expressions of stable BCL-2 transfectant, and the other system is MDCK cells with inducible expression of BCL-2 by lac operator/repressor. By using these two model systems, this study was aimed at exploring the relationship between BCL-2 and cell volume regulation. The results demonstrated a novel function of BCL-2 overexpression in RVD.

MATERIALS AND METHODS

Cell Culture—Wide-type MDCK cells and two human cervical cancer cell lines (HT-3 and SiHa) were obtained from the American Type Culture Collection (Manassas, VA). Another series of MDCK clones, successfully developed in our laboratory, were included in the study as follows: (i) stable transfection of \( \text{bcl-2} \) gene (B4 and B6 cell lines) or \( \text{bcl-2} \) empty vector (C1 cell) (9); (ii) inducible expression of BCL-2 by the \( \text{lac} \) operator/repressor system in MDCK cells (8). In this system, MDCK

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1 The abbreviations used are: RVD, regulatory volume decrease; IPTG, isopropyl-\( \beta \)-thiogalactoside; MDCK, Madin-Darby canine kidney; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; \( [\text{Ca}^{2+}]_{i} \), intracellular \( \text{Ca}^{2+} \); \([\text{Ca}^{2+}]_{o}\), extracellular \( \text{Ca}^{2+} \); CCE, capacitative \( \text{Ca}^{2+} \) entry; TG, thapsigargin; ORCC, outwardly rectified CI\(^{-} \) channel.
cells were cotransfected with the lac repressor gene and the human bcl-2 gene that had been inserted downstream of a simian virus 40 (SV40) promoter containing the lac operator sequence (11). The induction of BCL-2 expression is dependent on the incubation time as well as the concentration of the lactose analog isopropyl-ß-D-thiogalactoside (IPTG). Cells were incubated at (1024 ± 5 mol liter\(^{-1}\), pH 7.4). After wash of the lipid bilayer with a 1:1 voltage step, applied every 15 s from a holding potential of −20 mV to test currents to potentials from −80 to +80 mV with an increment of 20 mV. Currents were sampled at 1-ms intervals. Current densities were determined by normalizing the whole-cell current to the membrane capacitance. The cell volume was calculated from the shift of the reversal potential in anion substitution experiments. In this case, an agar bridge was used to minimize junction potential, and permeability ratios were calculated from a modified Goldman-Hodgkin-Katz Equation 1,

\[
P_i = \frac{[Cl^-]}{[X^-]} \exp\left(\frac{-\Delta E_{rev} - FRT}{[Cl^-]}\right) - [Cl^-],
\]

where [Cl\(^-\)], and [X\(^-\)], are the Cl\(^-\) concentrations in the normal and substituted external solutions; [X\(^-\)], is the concentration of the substituting anion; F is the Faraday constant; R is the gas constant, and T is absolute temperature.

Fluorescence Measurements of \([Ca^{2+}]_i\) and \([Ca^{2+}]_o\), was measured with the fura-2 fluorescence ratio method on an a fluorimeter (F-2000, spectrofluoromter, Hitachi, Tokyo, Japan) as described previously (18). In brief, cells were attached on coverslips were loaded with 2 μM fura-2/AM (fura-2/AM) in Dulbecco's modified Eagle's culture medium at room temperature for 40 min and then at 37°C for 20 min. After loading, the coverslip was washed three times with phosphate-buffered saline. After washing, the cover slip was mounted in a custom-made holder and placed in a 5-ml quartz cuvette. Fluorescence emission was collected from a group of \(10^6\) cells located in the excitation path. Excitation wavelength was alternated between 340 (I\(_{340}\)) and 380 nm (I\(_{380}\)), and fluorescence intensity was monitored at 510 nm. \([Ca^{2+}]_i\), was calculated from the I\(_{340}/I_{380}\) ratio using Equation 2 proposed by Grynkiewicz et al. (19).

\[
\frac{[Ca^{2+}]_i}{K_d} = \left(\frac{F_{340}}{F_{380}}\right) \times \left(\frac{[R - R_{	ext{min}}]}{R - R_{	ext{max}} - R}\right)
\]

where \(K_d\) is the dissociation constant for fura-2 in the cytosol (250 mM), \(F_{340/380}\) are the 380 nm fluorescence intensity and \(F_{340/380}\) ratio at low \([Ca^{2+}]_i\); \(R_{	ext{max}}\) and \(R_{	ext{min}}\) are the 380 nm fluorescence intensity and \(F_{340/380}\) ratio at \([Ca^{2+}]_i\); and \(F_{340/380}\) recorded during experiments. Calibration measurements of \(R_{	ext{max}}\) and \(R_{	ext{min}}\) were performed after incubating cells for 10 min in nominally \(Ca^{2+}\)-free isosmotic solution containing 3 mM EGTA. Cells were then superfused with isosmotic solution containing 1 μM thapsigargin, 5 μM iberiotoxin, and 10 mM Ca\(^{2+}\) to evaluate \(F_{340/380}\) and \(R_{	ext{max}}\).

Data Recording and Analysis—Data from electrophysiological experiments were digitized and analyzed using pCLAMP software (version 6.0.3, Axon Co., Foster City, CA). All values in the present study were reported as mean ± S.E. Student’s paired or unpaired t test was used for statistical analyses. Differences between values were considered significant when \(p < 0.05\).

RESULTS
Enhanced Capability of RVD by BCL-2 Overexpression—As shown in Fig. 1A, BCL-2 was differentially expressed in wild-type, plasmid control (C1), and BCL-2-transfected (B4 and B6) MDCK clones. The typical volume changes induced by hypotonic stress in wild-type MDCK and C1 cells could be divided into three phases as follows: 1) an initial and rapid osmotic swelling, reaching a peak cell volume (1.27 ± 0.08 of original cell size, \(n = 60\); Fig. 1B) at 2.8 min; 2) a rapid shrinkage in the following 2 min; and 3) a more gradual decrease of cell volume that finally reached a plateau that was 19% above the original cell size at 7–10 min (Fig. 1B). Hypotonic stress rapidly triggered B4 cells to reach a peak volume of 1.19 ± 0.05 (\(n = 60\)) of initial cell volume at about 2 min. A rapid decrease of cell volume subsequently appeared in the following time course, and cell volume returned to the original size at about 7–10 min (Fig. 1B). Moreover, osmotic swelling B6 cells reached a peak

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**Fig. 1.** Response of cell volume to hypotonic stress in MDCK cells with different BCL-2 expression. A, different BCL-2 levels in MDCK cells with stable BCL-2 transfection. Western blotting analysis demonstrates the expression of BCL-2 in homogenates of wild-type, plasmid control (C1), and BCL-2 transfected (B4 and B6) MDCK clones. B, time course of volume changes in C1, B4, B6, and wild-type MDCK cells following superfusion with hypotonic bath solution (230 mosmol liter⁻¹). **, p < 0.01, compared with the volume ratio with wild-type MDCK cells at 10 min, unpaired t test. The y axis (V/Vₒ) depicts the cell volume at the indicated times divided by the cell volume at zero time. Each point represents mean ± S.E. (n = 60 cells). C, effect of BCL-2 antibodies on the time course of volume changes in response to hypotonicity for C1 and B6 cells. Antibodies were delivered by microinjection. Each point represents mean ± S.E. (n = 25 cells). **, p < 0.01, compared with the volume ratio of B6 cells without or with antibody treatment at 20 min. D, inducible BCL-2 expression by the lac operator/repressor system in MDCK cells. Time course induction of BCL-2 by 1 mM IPTG for the indicated time (upper panel). The levels of BCL-2 were determined by Western blotting. P, positive control, taken from the stable BCL-2 transfectants. Lower panel, results (n = 3) of densitometric analysis of the Western blotting. E, progressively enhancing capability of RVD by inducible BCL-2 expression. *, p < 0.05; **, p < 0.01, compared with the volume ratio with control groups (Day 0) at 10 min, unpaired t test. The y axis (V/Vₒ) depicts the cell volume at the indicated time divided by the cell volume at zero time after superfusion with hypotonic bath solution (230 mosmol liter⁻¹). Each point represents mean ± S.E. (n = 60 cells).

The volume of 1.14 ± 0.07 (n = 60) of initial cell volume at 1.5 min and then returned to the original cell size in about 5 min (Fig. 1B). These results indicate the sequence of RVD capability is B6 > B4 > C1 = wild-type MDCK cells, which is well correlated with BCL-2 levels in these clones.

To investigate whether the advantage of B6 cells in RVD is a specific effect, BCL-2 antibody was delivered into cells by microinjection. Compared with control group, the treatment of BCL-2 antibody increased initial osmotic swelling, attenuated the shrinkage phase, and inhibited the gradual decrease of volume regulation in B6 cells (Fig. 1C). In addition, B6 cells treated with BCL-2 antibody showed a similar responsive curve with the swelling C1 cells and wild-type MDCK cells (Fig. 1B). However, BCL-2 antibody has no effect on the volume regulation of C1 cells (Fig. 1C). This indicates that the advantage of B6 cells in volume regulation results from BCL-2 overexpression.

We also studied the RVD response in a clone of MDCK cells that differentially expressed BCL-2 induced by the lactose analog IPTG. Compared with the control group (Day 0), the RVD process was not significantly changed after 1-day induction of 1 mM IPTG, in spite of the fact that a certain amount of BCL-2 was expressed (Fig. 1, D and E). The progress of capability for RVD became significant after 2 and 3 days of IPTG induction, suggesting that BCL-2 overexpression enhances the RVD capability in a dose-dependent manner.

To ensure the changing ability of volume regulation did not result from the drug effect of IPTG, we also investigated the effect of IPTG on RVD process. IPTG (1 mM) did not affect the RVD response of wild-type and other clones of MDCK cells (data not shown).

**Fig. 2.** Up-regulation of swelling-activated Cl⁻ channel by BCL-2 overexpression. Representative recordings of Cl⁻ current traces (step protocol) in isotonic (300 mosmol liter⁻¹) and hypotonic (230 mosmol liter⁻¹) solution for C1 cells (A) and B6 cells (B). Horizontal lines represent zero current levels. Current-voltage relationships were obtained from traces in isotonic and hypotonic solutions. Closed and open circles are hypotonic and isotonic currents, respectively. The step protocol for (A and B) consisted of a 1-s voltage step, applied every 15 s from a holding potential of −20 mV to test potentials from −80 to +80 mV with an increment of 20 mV. C, normalized swelling-activated Cl⁻ currents measured at +80 mV in wild-type MDCK cells and MDCK cells transfected with plasmid control (C1) and bcl-2 (B4 and B6) gene. Each column represents mean ± S.E. (n = 50). *, p < 0.05; **, p < 0.01 by unpaired t test, compared with wild-type MDCK cells.

**BCL-2 Overexpression Up-regulates the Swelling-activated Cl⁻ Channel**—The swelling-activated Cl⁻ channel plays a critical role in RVD (3). We subsequently investigated whether the changing RVD capability of MDCK cells is because of up-regulating the swelling-activated Cl⁻ channel.

In Fig. 2, A and B, shows the representative recordings of swelling-activated Cl⁻ currents, obtained from MDCK cells with plasmid control (C1 cell) and stable BCL-2 transfected (B6 cell). C1 cells had a small isotonic background current, averaging 8.0 ± 0.8 pA pF⁻¹ at +80 mV and −5.5 ± 0.8 pA pF⁻¹ at −80 mV and with a slope conductance of 0.084 ± 0.009 nS pF⁻¹ (n = 50). Application of a hypotonic solution induced cell swelling, which was accompanied by an activation of large outwardly rectifying currents. At potentials more positive than +40 mV, the currents showed time-dependent inactivation, which became more pronounced at higher membrane potentials. The swelling-activated current was reversed at a potential close to the theoretical equilibrium potential for Cl⁻ (ECl⁻ ≈ −25 mV), indicating that the swelling-activated current is mainly carried by Cl⁻ (Fig. 2A). The sequence of anion permeability, calculated from the shifts in reversal potential, was I⁻ > Br⁻ > Cl⁻ (1.53 ± 0.20: 1.26 ± 0.10: 1, n = 5).

As depicted in Fig. 2B, B6 cells also presented a small isotonic background current of 10 ± 0.5 pA pF⁻¹ at +80 mV and −8.0 ± 0.4 pA pF⁻¹ at −80 mV, with a slope conductance of 0.10 ± 0.01 nS pF⁻¹ (n = 50), which was not significantly different from those of C1 cells. Hypotonicity induced a remarkable...
able outwardly rectified current (Fig. 2B) with the anion permeability of I– > Br– > Cl– (1.58 ± 0.18:1.30 ± 0.15:1, n = 5). To compare the activities of swelling-activated Cl– channel among different MDCK clones, we normalized the swelling-activated Cl– current, which was defined as the differences of current densities between isotonic and hypotonic solutions and was expressed as per unit membrane capacitance. For C1 cells, the normalized swelling-activated Cl– current was 56.2 ± 8.4 pA pF–1 (n = 50) at +80 mV which was similar to that of wild-type MDCK cells (Fig. 2C). For B4 and B6 cells, the normalized swelling-activated Cl– current significantly increased to 80 ± 3.0 pA pF–1 (n = 50, p < 0.05, unpaired t test) and 105 ± 5.0 pA pF–1 (n = 50, p < 0.01), respectively.

In addition to altering the current amplitude, the activation rate of swelling-activated Cl– channel was also significantly increased by BCL-2 overexpression (Fig. 3). In wide-type MDCK and C1 cells, exposure to hypotonicity induced an outward rectifying current with an activation rate of 0.30 ± 0.03 (n = 50) and 0.28 ± 0.03 (n = 50) pA pF–1 s–1 at +80 mV, respectively. B4 and B6 cells expressed a faster current activation (B4 cells, 0.55 ± 0.05 pA pF–1 s–1, n = 50; B6 cells, 0.74 ± 0.02 pA pF–1 s–1, n = 50). These results clearly demonstrate that BCL-2 overexpression up-regulates the activation of swelling-activated Cl– channel. Other fundamental characteristics of this channel were similar among these different MDCK clones, such as sensitivity to Cl– channel inhibitor NPPB (Fig. 3A), time-dependent inactivation at more positive potentials, and anion permeability.

We also studied the activities of swelling-activated Cl– channel in MDCK cells with inducible BCL-2 expression (Fig. 4). In the absence of IPTG (Day 0, control), the normalized swelling-activated Cl– current was 55 ± 2.7 pA pF–1 s–1 at +80 mV (n = 50). In the presence of IPTG for 1 day, the normalized swelling-activated Cl– current was 55 ± 2.7 pA pF–1 s–1 at +80 mV (n = 50), which was similar to that of the control group. However, after 2 days of IPTG induction, the normalized swelling-activated Cl– current was significantly increased to 75 ± 2.3 pA pF–1 (n = 50) at +80 mV (p < 0.05, unpaired t test; Fig. 4B). After 3 days of IPTG induction, the normalized swelling-activated Cl– current further increased to 85 ± 3.0 pA pF–1 (n = 50) at +80 mV (p < 0.05, unpaired t test; Fig. 4B). IPTG itself showed no effect on the activity of swelling-activated Cl– current. These results confirm that the increasing activity of swelling-activated Cl– channel is a specific effect of BCL-2 overexpression.

Normal RVD Requires Extracellular Ca2+ ([Ca2+]o)—In addition to Cl– channels, Ca2+ signaling is deemed responsible for the normal RVD in some cell types (5). To ascertain the role of Ca2+ signaling in volume regulation of MDCK cells, we
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The swelling-activated [Ca\textsuperscript{2+}] transient is enhanced by BCL-2 overexpression, as shown by the summary of the changes of [Ca\textsuperscript{2+}] in swollen C1 and B6 cells. In the presence of [Ca\textsuperscript{2+}]\textsubscript{i}, the [Ca\textsuperscript{2+}] transient was observed to be blocked effectively by trivalent metal cations (12). Gadolinium (Gd\textsuperscript{3+}) inhibited the swelling-activated [Ca\textsuperscript{2+}] transient of B6 cells in a dose-dependent manner. The swelling-activated [Ca\textsuperscript{2+}] transient in C1 cells is also sensitive to Gd\textsuperscript{3+} (data not shown).

It has been reported that BCL-2 overexpression results in an up-regulation of capacitative Ca\textsuperscript{2+} entry (CCE) in human promyeloid leukemia cell line and human B-cell lymphoma cell line (20). CCE is the specific gating of Ca\textsuperscript{2+} entry across the plasma membrane in response to depletion of intracellular stores during Ca\textsuperscript{2+} signaling and can be triggered by thapsigargin (TG), an irreversible inhibitor of the endoplasmic reticulum Ca\textsuperscript{2+}-ATPase (21). A prompt question arises: is swelling-activated [Ca\textsuperscript{2+}] transient different from CCE in BCL-2 overexpressed cells?

Therefore, C1 and B6 cells were analyzed to determine the level of CCE after stimulation with thapsigargin. [Ca\textsuperscript{2+}] was measured in fura-2/AM-loaded cells in the absence of [Ca\textsuperscript{2+}]\textsubscript{i} (Fig. 7A). TG, a well established inducer of CCE, was added at 100 nM to trigger the opening of the plasma membrane calcium release-activated calcium channels. Generally, after the addition of TG, Ca\textsuperscript{2+} is released immediately from intracellular stores, resulting in an elevation of [Ca\textsuperscript{2+}]\textsubscript{i}. However, in these experiments, cells have been incubated in Ca\textsuperscript{2+}-free media plus 1.5 mM EGTA for 30 min before [Ca\textsuperscript{2+}] measurement, which is long enough to deplete intracellular Ca\textsuperscript{2+} stores; therefore, no immediate Ca\textsuperscript{2+} release was detected. Only after [Ca\textsuperscript{2+}]\textsubscript{i} is replenished 500 s later does [Ca\textsuperscript{2+}] rise as the ion crosses the plasma membrane. B6 cells had significantly higher levels of [Ca\textsuperscript{2+}]\textsubscript{i} than C1 cells, indicating that CCE is up-regulated in BCL-2-overexpressing cells (Fig. 7A). This result is consistent with the findings in human promyeloid leukemia cells and human B-cell lymphoma cells (20).

However, swelling-activated [Ca\textsuperscript{2+}] transient could be distinguished from the CCE. In TG-treated B6 cells, [Ca\textsuperscript{2+}] rose to a peak at ~150 s with an activation rate of 1.5 ± 0.1 nM s\textsuperscript{-1} (n = 10), after [Ca\textsuperscript{2+}]\textsubscript{i} is replenished in isotonic solution. In contrast, [Ca\textsuperscript{2+}]\textsubscript{i} rose rapidly to a peak at ~80 s with an activation rate of 2.0 ± 0.1 nM s\textsuperscript{-1} (n = 10), after [Ca\textsuperscript{2+}]\textsubscript{i} is replenished in hypotonic solution. In TG-treated C1 cells, swelling-activated [Ca\textsuperscript{2+}] transient could also be distinguished from the CCE (Fig. 7, A–C). In addition, in B6 cells, Gd\textsuperscript{3+} blocked Ca\textsuperscript{2+} entry during CCE activation more potently than Ca\textsuperscript{2+} influx during swelling-activated [Ca\textsuperscript{2+}] transient, consistent with an IC\textsubscript{50} of 6 and 65 μM, respectively (Fig. 7D). Moreover, the swelling-activated [Ca\textsuperscript{2+}] transient could be elicited after 10 μM Gd\textsuperscript{3+} completely inhibited the CCE activation (Fig. 7E). These results indicate that swelling-activated [Ca\textsuperscript{2+}] transient and CCE represent separate pathways for Ca\textsuperscript{2+} entry in MDCK cells.

BCL-2 Antibody Affects the RVD Response of Cells Expressing Endogenous BCL-2—We further investigated the volume regulation of cells with endogenous BCL-2 expression. As shown in Fig. 8A, human cervical cancer HT-3 cells expressed the endogenous BCL-2, whereas cervical cancer SiHa cells did not. These two cell lines need hypotonicity-induced Ca\textsuperscript{2+} entry for the normal RVD response and the activation of swelling-
activated Cl\(^{-}\) channel (15, 22). Delivered by microinjection, BCL-2 antibody showed a significantly inhibitory effect on RVD response of HT-3 cells but had no effect on the volume regulation of SiHa cells (Fig. 8, B and C). Moreover, in patch clamp recordings, intracellular dialysis of BCL-2 antibody significantly blocked the activation rate and amplitude of swelling-activated Cl\(^{-}\) channel in HT-3 cells (Fig. 8D, n = 10). In contrast, the activation of swelling-activated Cl\(^{-}\) channel was not affected by BCL-2 antibody in SiHa cells (Fig. 8E, n = 10).

**DISCUSSION**

Here we show the novel function of BCL-2 overexpression in the regulation of cell volume and ion flux. In this study, various clones of MDCK cells were initially seeded in a similar cell density and were used for experiments in the logarithmic growth phase. Moreover, neutralization of endogenous BCL-2 by antibody blocks the normal RVD response in human cervical cancer cells, supporting that BCL-2 can play an important role in volume regulation. Therefore, the presented observation is a critical signal for normal volume regulation of MDCK cells. BCL-2 overexpression results in enhanced swelling-activated Ca\(^{2+}\) entry and has a better buffering capacity for this Ca\(^{2+}\) entry than that of MDCK cells.

In addition to up-regulation of swelling-activated Ca\(^{2+}\) transient, BCL-2 overexpression in MDCK cells enhances the capacitative Ca\(^{2+}\) entry which is thought to be essential for maintaining Ca\(^{2+}\) homeostasis and may therefore be an important regulator of apoptosis during both the induction and execution phase, because both phases contain Ca\(^{2+}\)-dependent components (20). In human promyeloid leukemia cells and B-cell lymphoma cells, BCL-2 overexpression results in up-regulation of capacitative Ca\(^{2+}\) entry and resistance to apoptosis induced by the inhibitor of capacitative Ca\(^{2+}\) entry (20). However, the swelling-activated Ca\(^{2+}\) transient apparently does not share the same pathway with capacitative Ca\(^{2+}\) entry and has an unknown role in the ant apoptotic effect of BCL-2.

Osmotic swelling of MDCK cells led to a transient hyperpolarization followed by a sustained depolarization of cell mem-

**FIG. 7.** BCL-2 overexpression enhances CCE which can be differentiated from swelling-activated [Ca\(^{2+}\)] transient. A and B, representative recordings from 10 different experiments to show [Ca\(^{2+}\)] transient, induced by 2 \(\mu\)M TG in extracellular Ca\(^{2+}\) (Ca\(^{2+}\))-free media followed by replenishment of [Ca\(^{2+}\)], in the isotonic (A) or hypotonic (B) solution. In these experiments, cells were incubated in Ca\(^{2+}\)-free media plus 1.5 mM EGTA for 30 min before [Ca\(^{2+}\)] transient, measurement. C, comparison of the activation rate of [Ca\(^{2+}\)] transient in replenishment of [Ca\(^{2+}\)] in the isotonic or hypotonic solution. Each column represents mean ± S.E. (n = 10). *p < 0.05, unpaired t test. ISO, isotonic solution, 300 mosmol liter\(^{-1}\); HYPO, hypotonic solution, 230 mosmol liter\(^{-1}\); M, TG, 10 \(\mu\)M Gd\(^{3+}\), and hypotonic solution (B). Arrowheads indicate that B6 cells were treated with 2 \(\mu\)M TG, 10 \(\mu\)M Gd\(^{3+}\), and hypotonic solution (HYPO, 230 mosmol liter\(^{-1}\)).

In contrast, the activation of swelling-activated Cl\(^{-}\) channel was not affected by BCL-2 antibody in SiHa cells (Fig. 8E, n = 10).

**DISCUSSION**

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Figure 8. Neutralization of endogenous BCL-2 by antibody blocks the normal RVD response. A, Western blotting analysis demonstrates that human cervical cancer HT-3 cells express the endogenous BCL-2, but cervical cancer SiHa cells do not. P, positive control for BCL-2 expression, taken from the stable BCL-2 transfectants (B6 cells). B and C, effects of BCL-2 antibody (0.5 μg/ml) on the time course of volume changes in response to hypotonicity for HT-3 and SiHa cells. Antibody was delivered by microinjection. The y axis (V/V0) depicts the cell volume at the indicated time divided by the cell volume at zero time. Each point represents mean ± S.E. (n = 20 cells). ***, p < 0.01, compared with the volume ratio between groups without or with antibody treatment at 20 min. D and E, time courses of membrane currents activated at +80 mV or −80 mV for HT-3 and SiHa cells with or without intracellular dialysis of BCL-2 antibody. Data points were obtained from the voltage ramp protocol that was applied every 15 s. Horizontal bars indicate the application of hypotonic solution (HYPO, 230 mosmol liter−1) with or without treatment at 20 min.

Results

The malignant transformation of human cervical epithelial cells is accompanied by the significant up-regulation of swelling-activated Cl− channel and a sustained activation of K+ channel (27, 28). The swelling-activated Cl− channel has also been suggested to be involved in pH-regulatory steps, and its inhibition may induce cell alkalinization and arrest cell proliferation (1). Accordingly, increasing capability of RVD by up-regulation of swelling-activated Cl− channels may give cells an advantage on growth and metabolism and a better ability to handle stress.

Although there is evidence that changes in cellular ionic concentrations are important early events in apoptosis, the regulation of ion fluxes across the plasma membrane during this process is poorly understood. Little information is available on the role of ion channels in apoptosis. Lang and coworkers (14, 29) have proposed a model of lymphocytes for the relationship among ion channels, cell volume, and apoptotic cell death. Stimulation of the CD95 receptors leads to a rapid activation of outwardly rectified Cl− channel (ORCC), which shares some similar characteristics with volume-sensitive Cl− channel. Ceramide, a lipid metabolite synthesized upon CD95 receptor triggering, also induces the activation of ORCC in cell-attached patch clamp experiments. The activation of this type of Cl− channel is mediated by Src-like tyrosine kinases, because it is abolished by the tyrosine kinase inhibitor or by genetic deficiency of p56lck (14). These results suggest that tyrosine kinase-mediated activation of ORCC may play a role in CD95-induced cell death in T lymphocytes. Another study demonstrated that apoptotic volume decrease was an early prerequisite to apoptotic cell death, and this apoptotic volume decrease process could be prevented by blocking the volume-regulatory Cl− or K+ channel (30). However, the role of cell volume-regulatory mechanisms in programmed cell death is still ill-defined, and the functional importance remains a matter of speculation.

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