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Heterologous expression of specific K^+ channels in T lymphocytes: Functional consequences for volume regulation

CAROL DEUTSCH AND LI-QIONG CHEN

Department of Physiology and Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, PA 19104-6085

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ABSTRACT **It has been postulated that the K^+ channel isoform Kv1.3 plays a role in regulatory volume decrease (RVD) in response to hypotonic shock. We show that a mouse cytotoxic T-lymphocyte line, CTLL-2, is devoid of voltage-dependent K^+ channels and is unable to volume regulate. Transient transfection of these cells with Kv1.3 reconstitutes their ability to volume regulate. As predicted by our model, this ability depends critically on volume-induced changes in membrane potential and the isoform of the K^+ channel used. When the cells were transfected with Kv3.1, an isoform believed to be expressed in a specific subclass of mouse thymocytes, the CTLL-2 cells did not show RVD. The difference in the ability of the two isoforms to confer the capacity for RVD is expected from differences in the voltage dependence of activation of the channels, according to our proposed model for RVD. The experimental approach that we use, transient transfection and panning to select positive transfectants, is highly effective; it has a >95% efficiency. This method, and this cell line, may be important tools in studying lymphocyte K^+ channels and their function *in situ*.**

The cloning and heterologous expression of voltage-dependent potassium (K^+) channels has produced a plethora of data about the molecular determinants of K^+ currents (see ref. 1 for review). Many questions remain, however, about the physiological roles of these ubiquitous, diverse proteins, especially in nonexcitable cells. For example, it has been proposed, but not proven, that the ability of a cell to regulate volume in response to a hypotonic shock (regulatory volume decrease; RVD) depends on voltage-dependent K^+ conductance (2–5). Two lines of indirect evidence support this hypothesis. First, K^+ channel antagonists block RVD (3, 6–8). Second, mitogen stimulation of quiescent mouse T cells leads to up-regulation of the voltage-gated K^+ channel concomitant with acquisition of the ability of the cells to undergo RVD (3). In this paper, we present direct evidence that K^+ channels are required for the volume regulatory response in T lymphocytes. Moreover, we show, as predicted by our previously proposed model (2, 3), that this volume regulatory response depends critically on the isoform of K^+ channel present.

Our experimental protocol involved transfecting CTLL-2 cells, a mouse T-lymphocyte cell line devoid of voltage-gated K^+ currents, with two K^+ channel genes, Kv1.3 (the *n*-type channel) and Kv3.1 (a putative *l*-type channel) believed to be present in lymphocytes (9–13). Expression of Kv1.3 and Kv3.1 in *Xenopus* oocytes gives currents that resemble, but are not identical with, endogenous lymphocyte K^+ currents (11, 12). Our goals were (*i*) to examine whether expression of Kv1.3 and Kv3.1 genes in CTLL-2 cells produces currents indistinguishable from the *n*-type and *l*-type currents in native lymphocytes, and (*ii*) to test whether the *n*-type channel mediates RVD.

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MATERIALS AND METHODS

Cytotoxic murine T cells (CTLL-2) were transiently cotransfected with a plasmid using a cytomegalovirus (CMV) promoter (pRc/CMV; 5.4 kb) and a Kv1.3 insert (1.8 kb) or a Kv3.1 insert (1.8 kb; gift from Teresa Perney, Yale University, New Haven, CT) along with a Ccd4neo plasmid (5.0 kb; pUC-based plasmid with a CMV promoter driving CD4; gift from Ray Sweet, SmithKline Beecham) containing human surface membrane CD4 (1.7 kb).

Transfection. Using sterile techniques, cells were collected and suspended in Opti-MEM plus recombinant interleukin 2 (rIL-2) (8.3 ng/ml; Cetus) at 2×10^7 cells per ml with Ccd4neo (10 μg/ml) and pCMV/Kv1.3 or pCMV/Kv3.1 (80 μg/ml). Control cells were transfected only with Ccd4neo at 10 ng/ml. These suspensions were incubated on ice for 10 min in 0.4-cm electrode gap cuvettes (400 μl) and then electroporated using a BTX electroporator (San Diego) with settings determined previously to give ≈50% viability (voltage, 220–320 V; capacitance, 800 μF; resistance, 72 Ω). The resultant time constants were 24–30 ms. Cells were incubated for 10 min more on ice and then transferred back to culture medium (14) in the incubator and grown for 48 hr.

Harvesting of Cells. Cells were collected from culture and incubated with monoclonal anti-human CD4 antibody (AMAC, Westbrook, ME; 0.5 μg/10^6 cells) and adhered to Petri dishes (35 mm) coated with goat anti-mouse IgG as described in ref. 15. Dishes were washed gently five times with 1 ml of phosphate-buffered saline (PBS) containing 5% fetal bovine serum and rIL-2 (8.3 ng/ml) for the RVD experiments or with 1 ml of normal extracellular medium plus rIL-2 (8.3 ng/ml) for the patch-clamp experiments. All experiments were carried out at room temperature.

Electrophysiology. Standard patch-clamp techniques were used, as described in ref. 2. Pipettes were made from 0010 glass, coated with Sylgard, and fire-polished to give electrodes of ≈3 MΩ. The bath solutions were 147 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 5.5 mM glucose, 10 mM Hepes, and rIL-2 (8.3 ng/ml) (pH 7.35, 305 milliosmolar). The pipette solution was 130 mM KF, 11 mM K_2EGTA, 1 mM $CaCl_2$, 2 mM $MgCl_2$, and 10 mM Hepes (pH 7.20, 283 milliosmolar). Because of the large amplitude of K^+ currents in transfected cells, we restricted our kinetic analysis to experiments in which we used 60–70% compensation of the series resistance, reducing voltage errors to <6 mV.

Cloning of pCMV/Kv1.3. The kanamycin gene (1.2 kb) was ligated into *Spe* I/*Not* I-digested pGEM-A that contained the full-length (1.8 kb) open reading frame of Kv1.3 plus 0.18 kb of 5′ untranslated sequence. An *Eco*RI blunt-ended/*Not* I fragment was isolated from this clone and ligated into *Hin*dIII blunt-ended/*Not* I-digested pRc/CMV to generate pCMV/Kv1.3/Kanamycin. Kanamycin was deleted by *Hin*dIII/*Not* I digestion, the clone was filled in using the Klenow reaction,

Abbreviations: rIL-2, recombinant interleukin 2; ChTX, charybdotoxin; RVD, regulatory volume decrease; CMV, cytomegalovirus.

and the blunt ends were religated to give pRc/CMV/Kv1.3. This plasmid was used in all the experiments.

Volume Determinations. Photographs of CTLL-2 cells in isotonic PBS or hypotonic medium (25% PBS containing rIL-2 at 8.3 ng/ml) were taken at the appropriate times using TMAX 100 film and a Zeiss/Ikon camera (×600) attached to an inverted microscope (×10 objective). The photographs were enlarged to allow accurate measurement of cell diameters, and the volumes were calculated according to the formulae $\frac{4}{3}\pi r^3$ for spherical cells (>95%) or $\frac{4}{3}\pi ab^2$, where a = smaller axis and b = larger axis for elliptical cells (<5%). To prevent bias in the measurements, the identities of the photographed cells were not known to the person measuring them.

RESULTS AND DISCUSSION

Since CTLL-2 cells are transfected with poor efficiency (≈1.7%), we used a cotransfection strategy to select for positively expressing cells. K^+ channel-encoding plasmids were cotransfected with a plasmid encoding an abundantly expressed cell surface molecule to which an antibody was available. Transfected cells were then selectively adhered for our experiments (15, 16). Human CD4 was chosen as the selection surface marker because T cells normally process and express large quantities of homologous CD4 on their surface, and this isoform of CD4 is absent in CTLL-2 cells. Furthermore, this panning procedure has no effect on conductance (15).

The CTLL-2 line is an ideal expression system because these T cells contain no voltage-gated K^+ currents, as shown in Fig. 1*A*. Moreover, in our laboratory we have used a mouse T-cell-derived clone (MTK1) for the *n*-type voltage-gated K^+ channel to probe Northern blots of both quiescent and IL-2-stimulated CTLL-2 cells. There was no detectable mRNA for this channel (17).

As shown in Fig. 1 *B* and *C*, both channel types express well in CTLL-2 cells. The experimental conditions (see legend to Fig. 1) yield a panned population of CD4-adhered cells that are >95% positive for K^+ currents. Cells transfected with CD4 alone had no current when they were selectively adhered and studied in whole-cell patch-clamp (data not shown). However, cells cotransfected with CD4 and K^+ channel genes and then selectively adhered with CD4 antibody expressed currents at high levels (typically >1 nA per cell at voltages more positive than +20 mV) for both K^+ channel genes. Whole-cell recordings are shown in Fig. 1 *B* and *C* for Kv1.3 and Kv3.1, respectively. Chord conductance versus voltage curves (Fig. 2*A*) for Kv1.3 and Kv3.1 were similar to curves obtained for the native *n*- and *l*-type K^+ currents studied in human T cells and in mouse thymocytes, respectively. Threshold for activation is −45 to −60 mV for Kv1.3 and approximately −20 mV for Kv3.1. The reversal potential is approximately −75 to −80 mV (E_K for our solutions is −87 mV). The inactivation time constant of Kv1.3-transfected cells is of similar magnitude (150–300 ms for voltages greater than +20 mV) and has the same voltage dependence as that observed for the native K^+ currents in human T lymphocytes (18, 19). The half-time for activation of the expressed Kv1.3 is voltage-dependent and is 3.8 ± 0.5 ms (mean ± SD; n = 3) at +20 mV, similar to the activation characteristics of the native channel (18, 19). Heterologously expressed Kv1.3 current is blocked by charybdotoxin (ChTX) at nanomolar concentrations (data not shown) as is the native current (20, 21). Thus, the heterologous Kv1.3 currents expressed in CTLL-2 cells are virtually identical to the native *n*-type currents with respect to gating, selectivity, kinetics, and pharmacology. We have not yet determined the single-channel conductance of the heterologously expressed channel.

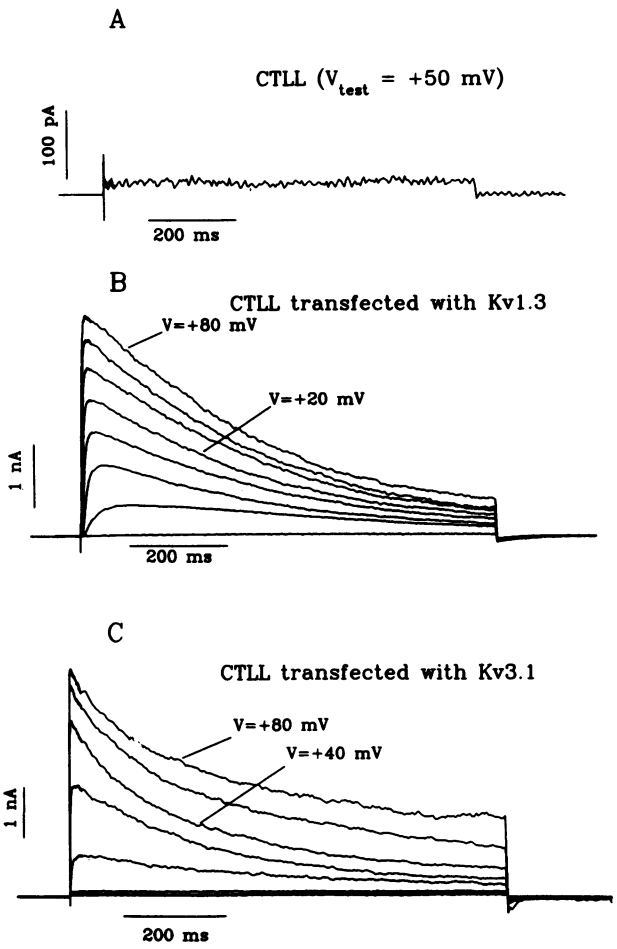

FIG. 1. CTLL-2 cell current in whole-cell mode. (*A*) Nontransfected CTLL-2 cell. Cell capacitance was 2.88 pF. Current was elicited by a step from the holding potential of −70 mV to +50 mV. The absence of endogenous K^+ current was seen in each of 17 cells. (*B*) Whole-cell current in CTLL-2 cells transfected with Kv1.3. Cell capacitance was 7.3 pF; series resistance was 9 MΩ. Current was elicited by a step from the holding potential of −100 mV to voltages of −60, −40, −20, 0, +20, +40, +60, and +80 mV at intervals of 30 s. Data were filtered at 3 kHz. (*C*) Whole-cell current in CTLL-2 cells transfected with Kv3.1. Cell capacitance was 8.04 pF; series resistance was 6.2 MΩ (60% series resistance compensation). Current was elicited as described for *A*, except that the pulses were delivered at 10-s intervals.

Kv3.1 expressed in CTLL-2 cells (Fig. 1*C*) also exhibits functional properties that correspond well with native *l*-type currents found in $CD4^-CD8^+$ thymocytes (12, 22). Specifically, the transfected current displays slower single-pulse inactivation compared to Kv1.3 (≈3-fold; Fig. 1*C*), half-time for activation of 0.9 ± 0.08 ms (n = 3) at +50 mV, and faster deactivation kinetics (note tail currents in Fig. 1*C* compared to 1*B*).

Yet another compelling difference between the two native currents is faithfully manifested in the expressed currents and is shown in Fig. 2 *B* and *C*. The transfected Kv3.1 current displays rapid recovery from inactivation (τ < 1 s) vis-a-vis the slow recovery (τ > 10 s) exhibited by Kv1.3.

Having expressed these K^+ channels at high levels per cell, we were now able to evaluate the physiological consequences of introducing K^+ channels into cells lacking voltage-activated K^+ channels. As shown in Fig. 3, CTLL-2 cells transfected with CD4 alone swell (Fig. 3*B*) in response to hypotonic shock, reaching a maximum volume in 2–5 min, but do not volume regulate (Fig. 3*C*). However, CTLL-2 cells transfected with both CD4 and Kv1.3 and then exposed to

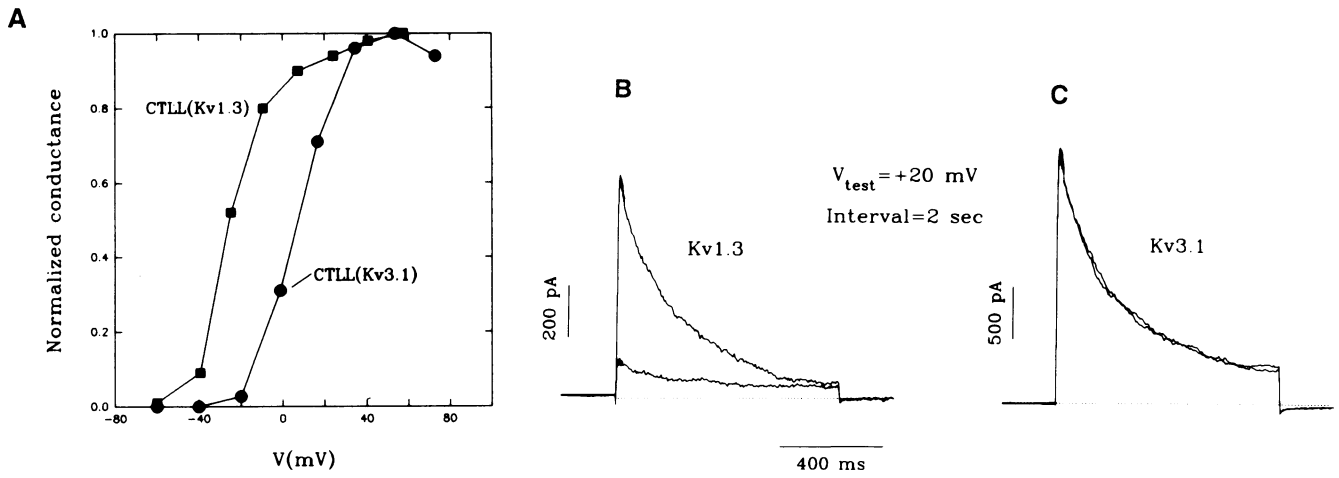

FIG. 2. (*A*) Voltage dependence of normalized conductance for Kv1.3 and Kv3.1 for the data in Fig. 1 *B* and *C*, respectively. (*B*) Recovery from inactivation for whole-cell current in CTLL-2 cells transfected with Kv1.3. Cell capacitance was 6.1 pF; series resistance was 6.8 MΩ (50% series resistance compensation). Current was elicited by a step from a holding potential of −100 mV to +20 mV. The interval between each pulse was 1 sec. (*C*) Recovery from inactivation for whole-cell current in CTLL-2 cells transfected with Kv3.1. This cell was the same as that used in Fig. 1*C*. Current was elicited by a step from −100 mV to +20 mV. The interval between each pulse was 1 s.

hypotonic shock first swell to the same maximum volume within the same time and then return toward isotonic volume (RVD) as shown in *D–F*, respectively. In these experiments, the cells transfected with Kv1.3 were tested in patch-clamp experiments to determine the efficiency and level of functional expression. Of 10 cells tested, all had *n*-type K^+ currents: only 2 had currents <1.5 nA at +50 mV; all the rest had currents between 1.5 and 6 nA. Regardless of transfection, all cells under isotonic conditions had similar median volumes. The median (± SD) volume was 1741 ± 169 μm^3, which agrees quite well with median values, ≈1600 μm^3, obtained from Coulter Counter/Channelyzer measurements of the volume distribution of nontransfected CTLL-2 cells in a Hepes-buffered Hanks' medium (GIBCO) containing rIL-2 (8.3 ng/ml; Cetus). Antibody binding to T-cell surface antigens had no effect on the volume response to hypotonic shock in nontransfected primary human T cells (data not shown). These results show that transfection of Kv1.3 into CTLL-2 cells has conferred upon these cells the ability to volume regulate.

Sufficient K^+ permeability is requisite for RVD. In either the absence of adequate endogenous, activatible K^+ permeability (3) or a pharmacologically blocked K^+ pathway (6–8), RVD will not occur. We (2, 3, 6) and others (4, 8) have proposed a model for RVD. The model states that hypotonic shock induces volume expansion, which results in increased

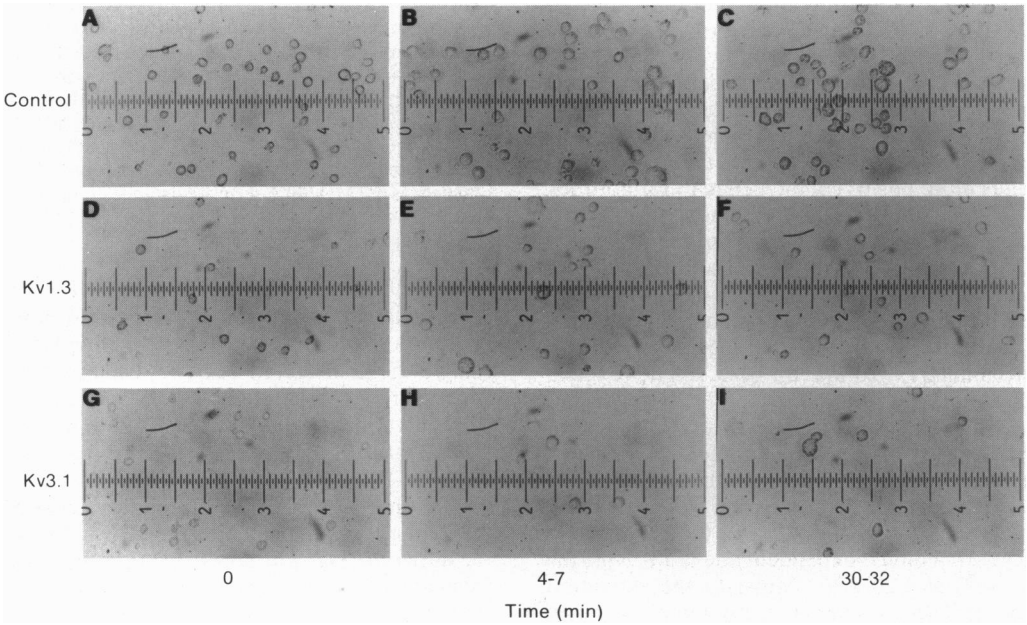

FIG. 3. Hypotonically induced changes in cell volume. Cells were transfected with either CD4 alone (*A–C*), CD4 plus Kv1.3 (*D–F*), or CD4 plus Kv3.1 (*G–I*); selected for CD4 expression; and washed in isotonic PBS plus rIL-2 as described in Fig. 1. All cells adhered are CD4-transfected since five washes are sufficient to remove any nonspecifically attached cells (data not shown). *A*, *D*, and *G* show cells in isotonic medium prior to swelling (t = 0). Medium was decanted and replaced with 25% PBS containing rIL-2 (8.3 ng/ml). Photographs were taken at the indicated times using TMAX 100 film and a Zeiss/Ikon camera (×600) attached to an inverted microscope (×10 objective). *B*, *E*, and *H* show cells after 4–7 min in hypotonic medium. *G*, *F*, and *I* show cells after 30–32 min in hypotonic medium. The calibration scale is 92 μm per unit marking. The mean cell volumes in *A–I* are 1589, 3383, 2998, 1404, 3780, 2185, 1450, 4150, and 2833 μm^3, respectively. The specific fields presented were chosen for clarity in the outlines of the cells. Complete data are quantitated in Fig. 4.

opening of Cl^- channels, thus depolarizing the cell toward the chloride equilibrium potential [estimates of E_{Cl} range from −10 to −35 mV (5)]. Depolarization activates voltage-gated K^+ channels, thereby providing an efflux pathway for K^+, accompanied by efflux of water. Grinstein and coworkers (7, 23) have shown that RVD in human T cells involves activation of both a Cl^- and a K^+ conductance. T cells possess osmotically gated Cl^- channels (24), and swelling leads to a Cl^--mediated depolarization of at least 15 mV (8). Depolarization would activate Kv1.3, which has a threshold of approximately −60 to −45 mV (Fig. 2). The results of our experiments are consistent with this model. Furthermore, this model predicts that if the cells undergoing hypotonic shock contain K^+ channels with thresholds for activation that are substantially more positive than the volume-induced depolarization, these cells will not exhibit RVD. Indeed, this is the case, as shown in Figs. 3 and 4 for cells transfected with Kv3.1. CTLL-2 cells transiently transfected with CD4 and Kv3.1 swell in response to hypotonicity, but they do not exhibit RVD (Fig. 3 *G–I*). In these experiments, cells transfected with Kv3.1 were tested using patch-clamp to determine the efficiency and level of functional expression. Of seven cells tested, all had *l*-type K^+ currents: only two had currents <1 nA at +20 mV; all the rest had currents between 1 and 5 nA. The level of functional expression was similar for both isoforms as the maximum chord conductance after correcting for series resistance was 3.0 ± 1.6 nS/pF (n = 4) and 3.8 ± 1.3 nS/pF (n = 5) for Kv1.3 and Kv3.1, respectively, for the experiments described in Fig. 3. Taken together our findings imply that the mechanism for RVD includes an initial swelling-induced depolarization to voltages more negative than −20 mV and that the CTLL-2 cells have a significantly high chloride permeability but a limiting cation permeability.

Fig. 4 shows detailed time courses for the volume changes following hypotonic shock at time zero. The time course of the volume response was fit as a weighted sum of two exponentials. Fig. 4*A* includes data for cells transfected with CD4 alone or CD4 plus Kv1.3 and exposed to hypotonicity in the absence or presence of 50 nM recombinant ChTX, a toxin that blocks Kv1.3 (see above; refs. 20 and 21) but not Kv3.1 (12, 20). Regardless of the transfection plasmid, all cells swelled with similar time constants of 1.40 ± 0.5 (mean ± SD) min (n = 11 experiments). In contrast, the time constant for the shrinking depended on the type of K^+ channel expressed and whether or not ChTX was present. Cells transfected with CD4 alone or CD4 plus Kv3.1 exhibited time constants of 188 ± 22 (mean ± SD) min (n = 8). Cells expressing Kv1.3 had significantly faster ($P < 0.001$) RVD than control cells in each of three transfections, with a mean decrease of a factor of 4.6 in the time constant. ChTX increased the time constant for RVD in Kv1.3 cells to a level not significantly different from cells transfected with CD4 alone (Fig. 4*A*) and had no effect on the time constant for Kv3.1 cells (data not shown). These results demonstrate that RVD requires conductance through K^+ channels and that the different responses of cells transfected with two different isoforms are likewise mediated by the biophysical properties of the functionally expressed K^+ channel.

RVD proceeds over many minutes, a time course significantly slower than that of the inactivation of K^+ currents at positive voltages. Despite this apparent paradox, several considerations indicate that the biophysical properties of Kv1.3 are compatible with it playing a role in RVD. First, Grinstein and Smith (8) have shown that hypotonic shock leads to a depolarization between −50 and −60 mV that is manifested over 15 min. Second, inactivation is 3 times slower at −50 mV than it is at, for example, +50 mV. Therefore, although hypotonic shock depolarizes the lymphocyte to a voltage where the probability of activating Kv1.3 is low, the inactivation time constant is quite large compared to more depolarized voltages. Thus, during RVD a prolonged K^+ current is active and is capable of mediating RVD. Third, preliminary data from perforated patch recordings indicate that inactivation is ≈5-fold slower at +30 mV than it is in whole-cell recordings (D. Levy and C.D., unpublished data). Therefore, an even greater amount of current may be activated for longer times in the intact cell than can be inferred from standard whole-cell recordings.

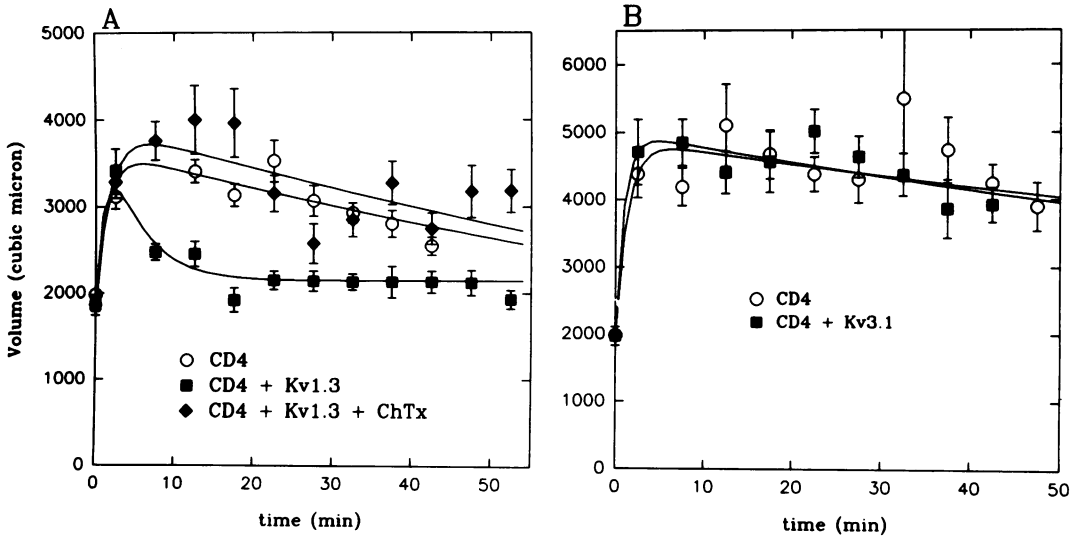

FIG. 4. Time course of volume changes in response to hypotonicity. The hypotonic solution was 25% PBS plus rIL-2 at 8.3 ng/ml. (*A*) CTLL-2 cells transfected with CD4 alone or CD4 and Kv1.3 and swollen in the absence or presence of 50 nM ChTX. (*B*) CTLL-2 cells transfected with CD4 alone or CD4 and Kv3.1. For both *A* and *B* data as shown in Fig. 3 were collected at ≈1- to 2-min intervals for 50 min. Calculated volumes were binned into 5-min intervals, and means ± SE are plotted. The average number of cells in each bin is 93. The solid lines drawn through the symbols are best fits determined by weighted nonlinear least-squares regression. The estimated time constants of RVD for *A* are 152 ± 33 min, 4.2 ± 0.3 min, and 162 ± 34 min for CD4 alone, CD4 plus Kv1.3, and CD4 plus Kv1.3 plus ChTX, respectively. Those for *B* are 184 ± 40 min and 189 ± 31 min for CD4 alone and CD4 plus Kv3.1, respectively. The amount of swelling initially induced by hypotonic shock was variable and ranged from 1.7- to 2.7-fold for nine experiments.

Quiescent, nongrowing primary lymphocytes demonstrate volume homeostasis under isotonic conditions; the standard deviation of the volume distribution is <15% of the mean volume (3, 6). Under anisotonic conditions, lymphocytes regulate their volume toward isotonic volume. Although vertebrate cells are usually bathed in solutions of highly regulated tonicity, lymphocytes may experience local, transient external anisotonicities (e.g., in sites of inflammation or passage through the renal medulla), or they may generate transient internal anisotonicities (e.g., during growth and proliferation) (3, 6). In either case, lymphocyte volume regulation may be necessary for lymphoproliferation and an effective immune response and may even serve as a feedback signal for production of specific transport systems, receptors, or soluble factors.

Though the K^+ channel encoded by the Kv1.3 gene serves to regulate cell volume, it also serves at least one other purpose: it is a major determinant of the resting membrane potential (−70 mV) in quiescent T cells (8, 25). One importance of a negative resting potential is that it is required for specific growth factor production during mitogenesis (14). It is not yet clear what roles are served by other lymphocyte K^+ channel isoforms (e.g., those described in ref. 26 and those reviewed in ref. 27); however, we suggest that different functional roles may be conferred by different biophysical properties (e.g., see above) of the channel isoforms.

In summary, our results provide a direct demonstration of the necessity for functional K^+ channels in RVD. Moreover, to our knowledge this is the first report of the heterologous expression of an ion channel in a lymphocyte and confirms that Kv1.3 does in fact encode the T lymphocyte *n*-type K^+ channel. The method employed, transient expression and panning to select positive transfectants, is highly effective; it has >95% efficiency. This method, and this cell line, may be important tools for studying lymphocyte function. Finally, the difference between K^+ channel isoforms shows that RVD requires the voltage-dependent activation of K^+ channels.

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