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Identification of two-pore domain potassium channels as potent modulators of osmotic volume regulation in human T lymphocytes

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ABSTRACT

Many functions of T lymphocytes are closely related to cell volume homeostasis and regulation, which utilize a complex network of membrane channels for anions and cations. Among the various potassium channels, the voltage-gated $K_V1.3$ is well known to contribute greatly to the osmoregulation and particularly to the potassium release during the regulatory volume decrease (RVD) of T cells faced with hypotonic environment. Here we address a putative role of the newly identified two-pore domain (K_{2P}) channels in the RVD of human $CD4^+$ T lymphocytes, using a series of potent well known channel blockers. In the present study, the pharmacological profiles of RVD inhibition revealed $K_{2P}5.1$ and $K_{2P}18.1$ as the most important K_{2P} channels involved in the RVD of both naïve and stimulated T cells. The impact of chemical inhibition of $K_{2P}5.1$ and $K_{2P}18.1$ on the RVD was comparable to that of $K_V1.3$. $K_{2P}9.1$ also notably contributed to the RVD of T cells but the extent of this contribution and its dependence on the activation status could not be unambiguously resolved. In summary, our data provide first evidence that the RVD-related potassium efflux from human T lymphocytes relies on K_{2P} channels.

1. Introduction

Volume regulation is a basic property of mammalian cell types and tissues, including T cells of the immune system [1–3]. Volume regulatory mechanisms are challenged by a variety of factors, such as changes in extracellular tonicity, membrane channel activity and cellular metabolism, as well as during cell proliferation, mitogen stimulation and apoptosis [4,5]. Numerous studies have also demonstrated the importance of volume regulation in various physiological and pathophysiological conditions, e.g. inflammation, etc. (for review see Ref. [6]).

A large body of literature now exists on the osmotic volume regulation in T lymphocytes and other subtypes of lymphoid cells [7,8]. Even under continuous hypotonic conditions, T lymphocytes can re-adjust their normal isotonic volume after transient swelling by a mechanism known as regulatory volume decrease (RVD). During RVD, the cell swelling causes co-activation of volume-sensitive chloride and various potassium channels. This leads to the net potassium chloride efflux, osmotic water loss and to the restoration of normal cell volume [6,9].

Voltage-gated potassium channels ($K_V1.3$) have been found to be essential for the volume regulation in T lymphocytes [10,11]. Additionally, several alternative pathways have been suggested to contribute to the RVD in lymphocytes, including calcium activated potassium channels ($K_{Ca}3.1$) [12], TRPM7 channels [13], swelling-activated channels for small organic osmolytes [14] and two-pore domain potassium channels, K_{2P} [3].

The family of K_{2P} channels is the most recently described potassium channel family expressed on T lymphocytes [15,16]. In a previous study involving K_{2P} channel knockouts and pharmacological inhibitors [3], we have identified the leading role of $K_{2P}5.1$ in the RVD of murine T cells, with a smaller contribution of $K_{2P}3.1$, $K_{2P}9.1$ and $K_{2P}18.1$. Unfortunately, differences in the K^+ channel expression profiles between murine and human cells, along with the broad variations in expression levels during T cell differentiation and activation do not allow a direct transfer of our previous murine results to the human system [15–18].

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The above considerations prompted us to explore the contribution of various K_{2P} channels to the hypotonic volume regulation of human $CD4^+$ T lymphocytes, using a pharmacological approach. To this end, we analyzed the sensitivity of the RVD in human T cells (including both naïve and stimulated cells) to different well known potassium channel blockers by video microscopy.

2. Materials and methods

2.1. Cell isolation and incubation

Human T lymphocytes were isolated from the peripheral blood of healthy donors, which was provided from the institute of transfusion medicine and hemotherapy of the university clinic in Wuerzburg. Peripheral blood mononuclear cells (PBMCs) were collected by density gradient centrifugation using a lymphocyte separation medium (PAA Laboratories, Pasching, Austria). $CD4^+$ T lymphocytes were separated by magnetic cell sorting (MACS), using human CD4 isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Isolated $CD4^+$ T cells (~90% purity by FACS) were incubated in T cell medium (RPMI1640, 10% human AB serum, 1% penicillin/streptomycin, 1% glutamine) for 48 h with or without stimulation. T cells were stimulated for 48 h with CD3/CD28 human T activator dynabeads in a cell to bead ratio of 4:1 (Invitrogen, Darmstadt, Germany), at 37 °C, 5% CO_2. The research protocol was approved by the local ethics committee (AZ.: 2011-150-f-S).

2.2. Western blot

T lymphocytes were isolated and incubated as described above. 2×10^6 cells (either naïve or stimulated) were lysed in 30 μl lysis buffer (1% NP-40, 10% n-dodecyl-β-D-maltoside, 1 mM sodium monovanadate, 1 mM PMSF, 50 μM TRIS, 10 mM NaF, 10 mM EDTA and 165 mM NaCl) 20 min on ice. After 10 min centrifugation at 18000 rcf and 4 °C the supernatant was mixed with 7.5 μl sample buffer (20 mM TRIS, 10% glycerol, 0.05% bromphenolblue and 1% SDS) and heated 5 min at 99 °C. The proteins were separated on a 10% SDS–PAGE and transferred to a nitrocellulose membrane. Membranes were blocked with 5% dry milk and probed with the following antibodies: anti $K_{2P}3.1$ (1:500, Santa Cruz, sc-32067, 46 kDa), anti $K_{2P}5.1$ (1:1000, Sigma Aldrich, P 1106, 70 kDa), anti $K_{2P}9.1$ (1:500, Santa Cruz, sc-11320, 75 kDa), anti $K_{2P}18.1$ (1:1000, Santa Cruz, sc-51237, 43 kDa) and $K_V1.3$ (1:1000, Sigma Aldrich, P4497, 110 kDa). All antibodies were diluted in 5% BSA and incubated over night at 4 °C. The corresponding horseradish-peroxidase conjugated secondary antibody (Santa Cruz) was diluted 1:3000 in 5% dry milk and incubated 1 h at room temperature. The antibody reaction was detected by enhanced chemiluminescence reaction (ECL, Amersham Biosciences). The analysis was done using image J software.

2.3. Electrophysiological measurements

Human $CD4^+$ T lymphocytes were isolated from buffy coats using the RosettSep® "human $CD4^+$ T cell enrichment cocktail" according to the manufacturer's instructions (Stemcell technologies, Köln, Germany). Isolated $CD4^+$ T cells were either left unstimulated or cultured in well plates coated with 2 μg/ml anti-human CD3 antibodies (clone 2C11, BD Pharmingen) and 1 μg/ml soluble anti-human CD28 antibodies (clone 28.2, BD Pharmingen) for T cell receptor stimulation. All patch-clamp measurements were conducted in the whole-cell configuration 24 to 48 h after T cell isolation. Recording pipettes were fabricated from borosilicate glass (GT150TF-10, Clark Electromedical Instruments, Pangbourne, UK; typical resistance was 6–7 MΩ) and filled with an intracellular solution containing (in mM): K-gluconate, 88; K_3-citrate, 20; phosphocreatine, 15; NaCl, 10; HEPES, 10; $MgCl_2$, 1; $CaCl_2$, 0.5; BAPTA, 3; Mg-ATP, 3; Na_3-GTP, 0.5. The internal solution was set to a pH of 7.25 with KOH and an osmolality of 295 mosm/kg. Since the whole-cell patch-clamp configuration is accompanied by a loss of cytosolic compounds, the internal solution includes energy sources (phosphocreatine, Mg-ATP, Na_3-GTP), Ca^{2+} buffers (citrate, BAPTA), a pH buffer (HEPES), and different salts for physiological ionic conditions. Extracellular solution contained (in mM): NaCl, 125; KCl, 2.5; NaH_2PO_4, 1.25; HEPES, 30; $MgSO_4$, 2; $CaCl_2$, 2; glucose, 10; pH 7.35 and osmolality was set to 305 mosm/kg. Membrane currents were recorded using a EPC-10 amplifier, and digital analysis was done using Fitmaster software (HEKA Elektronik, Lamprecht, Germany [18]). From a holding potential of −80 mV outward currents were elicited by 0.5 s rectangular pulses to +40 mV, applied at 30 s intervals. Outward currents under control conditions and drug application were recorded successively from identical cells.

2.4. Channel blockers

Channel blockers were purchased from Sigma (Germany), except ShK, which was from Bachem AG (Switzerland), and A293 kindly provided by Sanofi Aventis (Frankfurt, Germany, [19]). The blockers were used at the following concentrations: anandamide 3, 30 and 100 μM (targeting primarily $K_{2P}3.1$, and at higher concentrations also $K_{2P}9.1$); A293 1, 3 and 10 μM ($K_{2P}3.1$, $K_{2P}9.1$); quinidine 2, 20, 100 μM ($K_{2P}5.1$, $K_{2P}18.1$); bupivacaine 3, 30, 300 μM ($K_{2P}18.1$, $K_{2P}3.1/5.1/9.1$), spermine 100, 500 and 1000 μM ($K_{2P}9.1$), ruthenium red 10, 100, 1000 μM ($K_{2P}9.1$), ShK 1, 10, 100 nM ($K_V1.3$). For a particular channel blocker, the intermediate concentration corresponded to that reported in the literature as a concentration of maximum inhibition.

2.5. Cell volumetry

Cell volume changes were quantified by video microscopy using the experimental setup described previously [3,14]. Before measurement, a channel blocker was added to 1 ml of human $CD4^+$ T lymphocytes suspended in T cell medium at a density of about 2×10^5 cells/ml. The cell suspension was then injected into the polydimethylsiloxane (PDMS) flow chamber. The chamber was sealed with a poly-D-lysine coated coverslip (0.5 mg/ml poly-D-lysine, Sigma, 5–10 min) and placed on a microscope slide. To allow cell adhesion on the coverslip the flow chamber was incubated upside down for about 15 min. The cells were observed with a 20× objective in transmitted light using a microscope (Axiophot, Zeiss, Germany) equipped with a CMOS video camera (UI-1440-C, UEye, Obersulm, Germany). Images were taken at a time interval of 10 s containing typically 10–20 cells. Thirty seconds after start of the measurement the cell culture medium was exchanged by hypotonic (200 mOsm), isotonic (300 mOsm) or hypertonic (400 mOsm) PBS using a syringe pump (KDS100, KD Scientific, Holliston, USA) with a flow rate of about 1 ml/min. Hypo- and hypertonic PBS solutions were prepared by dilution of 10× Dulbecco's PBS (without Ca^{2+} or Mg^{2+}; PAA, Linz, Austria) with appropriate amounts of deionized water from a Milli-Q purification system (Millipore, Schwalbach, Germany). The solution osmolality (mosmol/kg) was verified using a cryoscopic osmometer (Osmomat 010, Gonotec, Berlin, Germany).

The cell volume (V) of a single cell at each time interval was calculated from its cross section area determined with the aid of the image analysis software ImageJ (Wayne Rasband, NIH, Maryland, USA), by assuming spherical geometry. The cell volume was normalized to the isotonic cell volume (V_0) as: $\nu = V/V_0$. The mean ν values with corresponding standard errors from a sequence of about 120 images were plotted against time.

To estimate the osmotically inactive fraction in untreated naïve and stimulated $CD4^+$ T lymphocytes, the experimental normalized cell volume 2 min after measurement start (ν_2) was plotted against

the reciprocal normalized osmolality in a range between 200 and 400 mOsm and fitted by the Boyle van't Hoff equation:

$$v_2 = \frac{C_{iso}}{C}(1-b) + b \quad (1)$$

where C is the PBS osmolality and $C_{iso} = 300$ mOsm is the isotonic osmolality. The term $b = \frac{Vb}{V_0}$ represents the osmotically inactive volume fraction at 300 mOsm. The linear regression of Eq. (1) to the volumetric data yielded very similar estimates of the osmotically inactive fraction for naive and stimulated CD4$^+$ T lymphocytes.

2.6. Analysis of volumetric data

The effects of potassium channel blockers on hypotonic volume regulation of human CD4$^+$ T lymphocytes were analyzed by the following equation for the RVD inhibition index (IC_{RVD}):

$$IC_{RVD} = \left(1 - \frac{v_2 - v_{end}}{v_2 - v_{end}^{cotr}}\right) * 100\% \quad (2)$$

Symbol v_2 stands for the normalized cell volume at the maximum swelling level achieved ~2–3 min after hypotonic exposure, as illustrated in Fig. 4 below. Symbol v_{end} denotes the normalized cell volume measured 17–20 min after hypotonic shock, for cells pretreated with a channel blocker. The v_{end} values of untreated controls are given by v_{end}^{cotr}. In treated cell samples, no RVD inhibition ($IC_{RVD} = 0$) was observed if their volume restoration was similar to that of untreated control ($v_{end} = v_{end}^{cotr}$). In contrast, cells showed IC_{RVD} of 100% if they were incapable of volume restoration and remained swollen in hypotonic medium ($v_{end} = v_2$). Standard errors of v_2, v_{end} and IC_{RVD} were calculated using Gaussian error propagation.

3. Results and discussion

3.1. Isotonic cell volume changes

T cell receptor stimulation is well known to alter significantly the expression pattern of ion channels in human T lymphocytes, which in turn can markedly affect their electrophysiological, osmotic and other biophysical properties [17,20]. Therefore, this study involved experiments with naïve (unstimulated) and T cell receptor stimulated T cells. As seen in Fig. 1A, the T cell radius increased markedly (i.e. by ~60%) from 3.6 ± 0.2 μm (mean ± SD) to 5.6 ± 1.1 μm upon stimulation with CD3/CD28 coated beads. This change of the cell radius corresponds to a nearly 4-fold increase in the absolute cell volume. Moreover, judging by the coefficients of variation, CV (calculated as CV = SD/mean), T cell receptor stimulation also yielded a much broader population distribution of cell radii (CV = 20%), as compared to naïve T cells (~6%).

Prior to exposure of T cells to anisotonic stress, we analyzed the effects of potassium channel blockers on the cell volume under isotonic conditions. As seen in Fig. 1B, all tested channel blockers slightly increased the radius of naïve T cells by up to ~6%, which corresponded to a cell volume increase by ~18%. The observed isotonic cell swelling might have resulted from an elevated potassium concentration in the cytosol (and subsequent water influx) caused by the chemical blockade of potassium channels in the plasma membrane that are open under control conditions. In case of stimulated cells (see supplementary Fig. S1), the channel blockers caused less significant changes in isotonic cell radii, apparently because of a much greater size variation. Whatever the explanation, the data in Fig. 1B provide clear evidence for the importance of potassium channels in maintaining the T cell volume under physiological conditions.

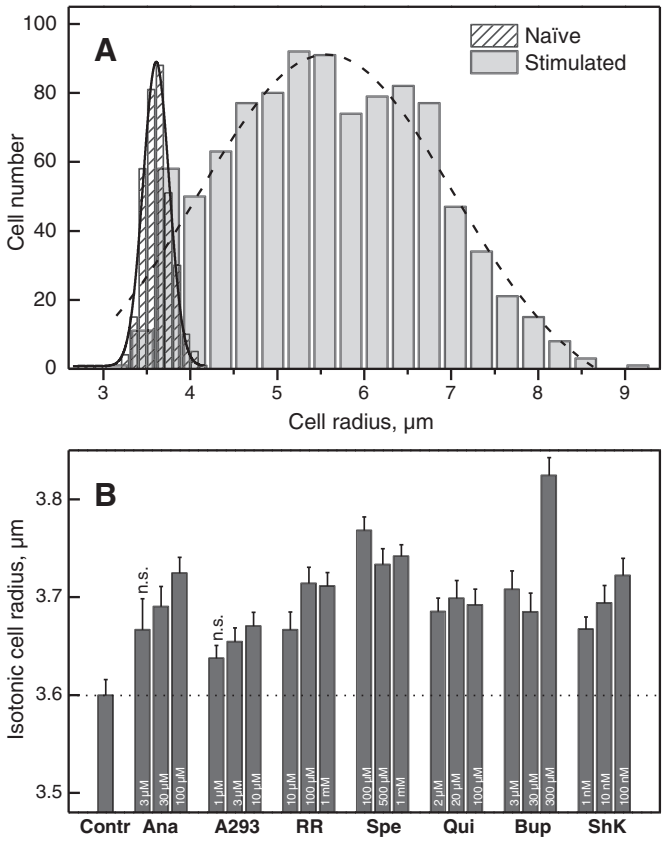

Fig. 1. Changes in the isotonic radius of human CD4$^+$ T lymphocytes induced by T cell receptor stimulation using CD3/CD28 dynabeads (A) and by various concentrations of potassium channel blockers (B). The histograms in A show cell size distributions in naïve and stimulated cell samples (dashed and gray bars, respectively) analyzed by video microscopy. Naïve and stimulated cells exhibit mean radii (±SD) of 3.6 ± 0.2 μm ($N = 350$ cells) and 5.6 ± 1.1 μm ($N = 950$ cells), respectively. Curves are best Gaussian fits to the data. In B: isotonic incubation of naïve T lymphocytes with the potassium channel blockers anandamide (Ana), A293, ruthenium red (RR), spermine (Spe), quinidine (Qui), bupivacaine (Bup) and ShK led to a volume increase. All samples were bathed in isotonic CGM (~300 mOsm) supplemented with the indicated blocker concentrations for ~15 min at room temperature. The mean radii (±SE) of the cells were calculated from the cross-section areas of 40–140 individual cells measured in 3–16 independent experiments. The dotted line shows the mean isotonic radius of the untreated control. Except the lowest concentrations of Ana and A293 (indicated by n.s. = not significant), all treatments gave rise to statistically significant increases ($P < 0.05$) of cell radius. The corresponding data of stimulated cells are given in the Supplement (Fig. S1).

3.2. Cell volume changes and regulation in anisotonic media

Irrespective of the activation status, a sudden exposure of T cells to hypotonic or hypertonic solutions caused T cells to either swell or shrink rapidly within 2–3 min due to the water movement driven by the imposed osmotic gradients (Fig. 2A and B). Despite the great difference in their isotonic radii (Fig. 1A), naïve and stimulated lymphocytes exhibited very similar relative volume changes in response to anisotonic media. In both cell samples, the hypotonic PBS (200 mOsm) gave rise to a rapid transient increase of the cell volume to closely similar maximum v_2 values of ~1.2. After the fast initial swelling, both cell types slowly underwent regulatory volume decrease (RVD), but without reaching the original isotonic cell volume within 20 min. The long-term v values (v_{end}) measured after ~20 min incubation were also similar, i.e. ~1.05 and ~1.07 in naive and stimulated T cells, respectively.

In contrast to the hypotonic volume regulation, both cell types were incapable of undergoing regulatory volume increase (RVI) in hypertonic 400 mOsm PBS (filled symbols in Fig. 2A and B). Moreover, stimulated T cells shrank continuously to $v_{end} = 0.72$ over the

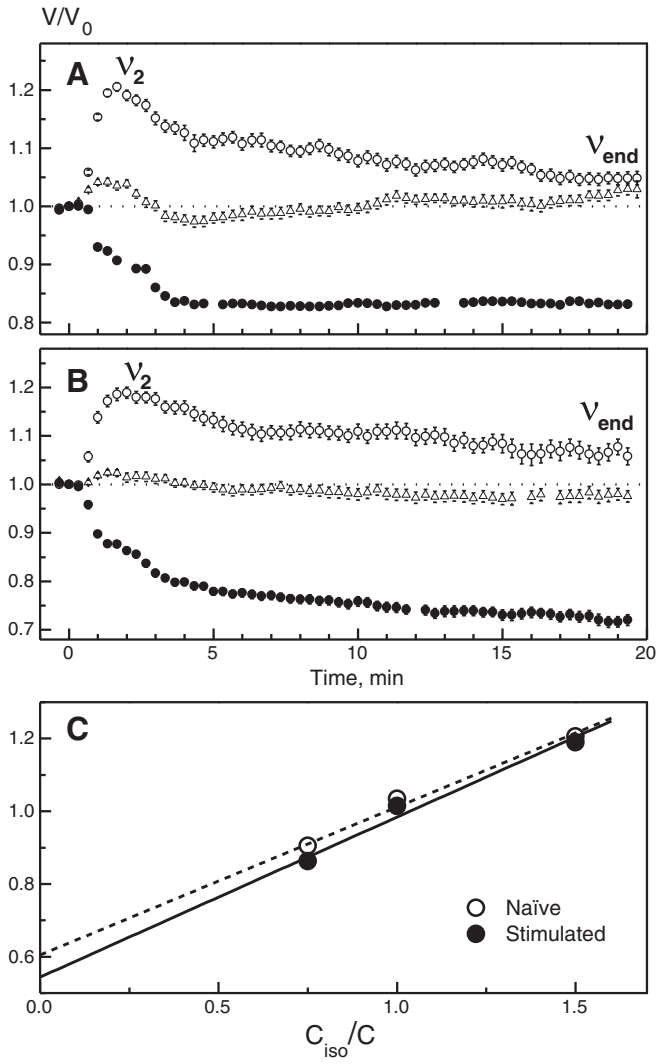

Fig. 2. Temporal changes of the normalized cell volume V/V_0 in naïve and stimulated human T cells (A and B, respectively) in response to PBS of varying osmolalities. All cell samples were bathed initially in isotonic CGM (~300 mOsm) and then exposed (at t = 30 s) to PBS having osmolalities of 200 (open circles), 300 (triangles) or 400 mOsm (filled circles). Under hypotonic conditions (200 mOsm PBS, gray circles), both naïve and stimulated cells first swelled rapidly within 2–3 min and then slowly underwent RVD. Isotonic PBS caused only little volume changes. In response to hypertonic solutions with the tonicity of 400 mOsm the cells shrank rapidly during the first 2–3 min. After that, the cell volume either remained unchanged (A) or slowly decreased (B). The initial cell volume is given by $\nu_0 = 1$. Symbols ν_2 stands for the normalized cell volume observed ~2 min after medium exchange. Each data point represents the mean $\nu \pm$ SE of 40–120 individual cells measured in 4–16 independent experiments. In C: the mean ν_2 values of the initial volume changes are plotted against the reciprocal normalized osmolality (C_{iso}/C, where $C_{iso} = 300$ mOsm), for naïve (open circles) and stimulated T lymphocytes (filled circles). The lines are best least-square fits of the van't Hoff equation (Eq. (1)) to the data. The osmotically inactive volume fractions (b), defined by the Y-intercepts, were found to be 0.61 ± 0.04 for quiescent and 0.54 ± 0.07 stimulated cells.

entire observation time. The lack of RVI in human T lymphocytes reported here corroborates previous findings of RVI incapability in lymphoid cells, e.g. human blood lymphocytes, mouse T lymphocytes, etc. [3,11,21].

To evaluate the impact of T cell receptor stimulation on the osmotically inactive volume fraction, we analyzed the initial volume responses of naïve and stimulated T cells with the Boyle van't Hoff equation (Eq. (1)). Fig. 2C shows the experimental ν_2 values of both cell types plotted versus the reciprocal normalized osmolality C_{iso}/C (symbols in Fig. 2C) over the osmolality range between 200 and 400 mOsm. The linear regression of Eq. (1) to the data yielded slightly different estimates of the osmotically inactive fraction for naïve ($b = 0.61 \pm 0.04$) and stimulated T cells ($b = 0.54 \pm 0.07$).

3.3. Influence of K_{2P} channel inhibition on membrane currents and RVD

In the following electrophysiological experiments, we first proved the presence of functional K_{2P} channels in the plasma membrane of naïve and stimulated T cells. Fig. 3A–H show representative patch-clamp recordings (whole-cell configuration) in the presence of various potassium channel blockers. All tested channel blockers caused a detectable current reduction in both naïve and stimulated T lymphocytes, suggesting that relevant potassium channels were functionally expressed on T cells.

Additionally, potassium channel expression in naïve and stimulated T lymphocytes was quantitatively evaluated by western blot. As seen in Fig. 3I and J, T cell receptor stimulation caused upregulation of $K_{2P}3.1$, $K_{2P}5.1$ and $K_V1.3$. In contrast, the expression levels of $K_{2P}9.1$ and $K_{2P}18.1$ were similar in naïve and stimulated T lymphocytes.

Given that neither naive nor stimulated human T cells were capable of volume regulation in hypertonic PBS media (filled symbols in Fig. 2), the following experiments were restricted to mild hypotonic conditions (200 mOsm PBS). To analyze the effects of channel blockers on RVD we calculated the RVD inhibition indexes (IC_{RVD}, Eq. (2)) using the ν_2 and ν_{end} values derived from the volumetric data, such as illustrated in Fig. 4.

3.4. Inhibition of $K_{2P}3.1$ and $K_{2P}9.1$

The endocannabinoid anandamide has been reported as a potent inhibitor of several K_{2P} channels [22,23]. Within the concentration range of 3–30 μM anandamide is known to preferentially target $K_{2P}3.1$, whereas at 100 μM it additionally inhibits $K_{2P}9.1$ in COS-7 cells [24].

In the present study, anandamide (3, 30 and 100 μM) exerted similar effects on the volume regulation in T cells, independently of the stimulation status. This result suggests that the relative contribution of $K_{2P}3.1$ channels to RVD had been unaffected by stimulation with CD3/CD28 beads, despite an ~threefold upregulation of the channel revealed by western blot (see Fig. 3I and J). 30 μM anandamide inhibited the hypotonic volume regulation in both cell types by ~40% (Fig. 4), which implies a notable contribution of $K_{2P}3.1$ to the RVD process. Furthermore, increasing the anandamide concentration to 100 μM strongly enhanced the RVD inhibition in human T cells (~110 and 80%, in naïve and stimulated cells, respectively), apparently, due to the simultaneous blockade of $K_{2P}3.1$ and $K_{2P}9.1$ (see also Supplementary Table 1). These data indicate a major contribution of $K_{2P}3.1$ and $K_{2P}9.1$ channels in the orchestration of RVD.

The following experiments involving A293, a more selective blocker of $K_{2P}3.1$, further supported the importance of this K_{2P} channel for the RVD in human T cells. A293 has been reported to block human $K_{2P}3.1$ by 50% at a concentration of 222 nM [25]. This substance is therefore the first $K_{2P}3.1$ channel inhibitor acting at submicromolar concentrations. At higher concentrations, A293 additionally inhibits $K_{2P}9.1$ [25].

As seen in Fig. 5, naïve and stimulated T cells exhibited a comparable RVD inhibition (by ~30–40%) upon treatment with A293 over the entire concentration range (1–10 μM). In view of the partial RVD inhibition, this finding corroborates the results with anandamide (Fig. 4), thus confirming a significant (but not sole) contribution of $K_{2P}3.1$ to the RVD in human T cells. Although unspecific channel inhibition at higher concentrations of anandamide and A293 cannot be strictly excluded, our results point to $K_{2P}9.1$ as an additional channel involved in the RVD of human T cells.

To address directly the role of $K_{2P}9.1$ in RVD, we first analyzed the RVD responses of T cells in the presence of ruthenium red (RR) which is well known for its ability to block $K_{2P}9.1$ channels [26,27]. In sharp contrast to the effects of anandamide and A293, the inhibitory effect

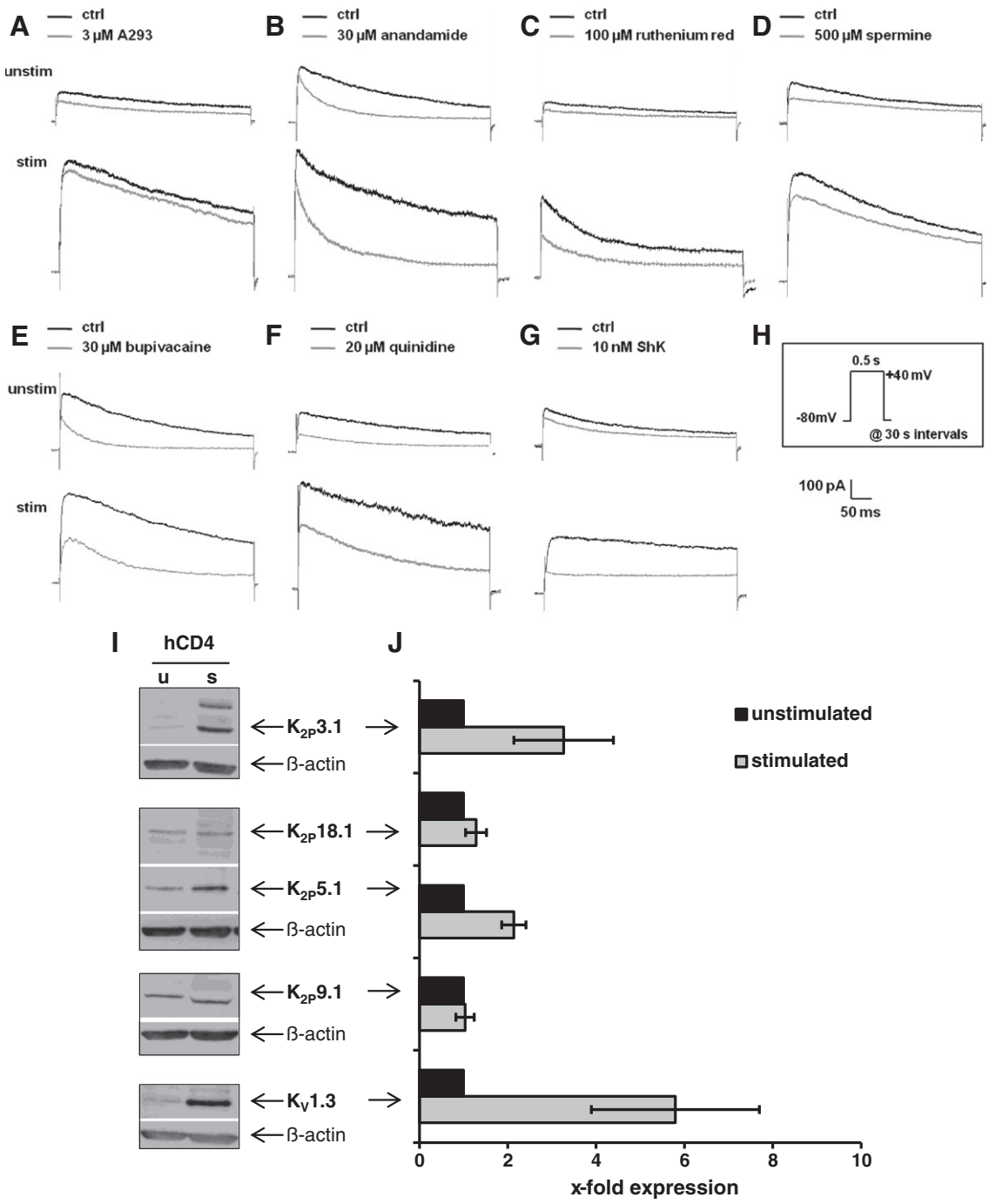

Fig. 3. Functional expression of $K_{2P}3.1$, $K_{2P}5.1$, $K_{2P}9.1$, $K_{2P}18.1$ and $K_V1.3$ on naïve and stimulated human T lymphocytes, probed by patch clamp (A–H) and western blot (I and J). Electrophysiological recordings with naïve and stimulated T lymphocytes show the outward currents in control (black lines) and drug-treated cells (gray lines): (A) A293, (B) anandamide, (C) ruthenium red, (D) spermine, (E) bupivacaine, (F) quinidine and (G) ShK. All blockers caused a noticeable current reduction pointing to the functional expression of the targeted potassium channel ($n = 3$, one representative example is shown for each condition). (H) Patch clamp protocol and legend. Parts (I) and (J) show the expression levels of various potassium channels in naïve (black bars) and stimulated (gray bars) T lymphocytes, analyzed by western blot.

of RR on RVD depended largely on the activation status of T cells (Fig. 5). In naïve T cells, RR caused a near complete RVD inhibition (by ~75–85%) over the entire concentration range (Fig. 5A). T cell receptor stimulation decreased markedly the RVD inhibition capacity of RR to 25–45% (Fig. 5B). In naive cells, the inhibitory effect on RVD was found to be quite independent of the RR concentration (probably due to saturation of the target channel), at least within the range (10 µM–1 mM) used here. Although interactions of RR with other targets, e.g. ryanodine receptors, [28], cannot be fully ruled out, the observed differences in the RR-mediated RVD inhibition, despite comparable expression levels (Fig. 3J), might have been resulted from a greater relative contribution of $K_{2P}9.1$, probed with RR, to volume regulation in naïve cells.

To further prove the importance of $K_{2P}9.1$ in RVD, we treated T cells with spermine, a naturally occurring organic polyamine targeting specifically $K_{2P}9.1$. In earlier electrophysiological experiments performed on thalamocortical relay neurons [26], 100 µM spermine has been

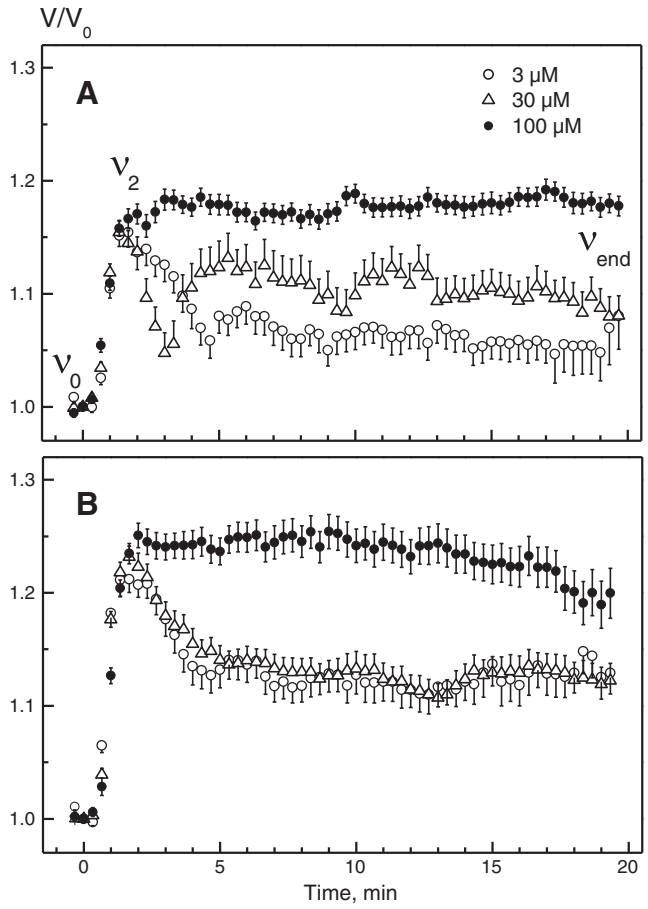

Fig. 4. Effects of various anandamide concentrations on RVD in naïve (A) and stimulated T cells (B), exposed to hypotonic 200 mOsm PBS. Each data point represents the mean $V/V_0 \pm$ SE of 15–140 individual cells measured in 3–16 independent experiments. Symbols ν_0, ν_2 and ν_{end} denote, respectively, the initial isotonic volume ($t=0$), cell volume at the maximum swelling level ($t=2$ min), and the final volume averaged over the time interval of 15–20 min after medium exchange. Substituting the ν_2 and ν_{end} data measured in the volumetric experiments into Eq. (2) yielded the values of the RVD inhibition index for anandamide (and other channel blockers, see supplement Table S1).

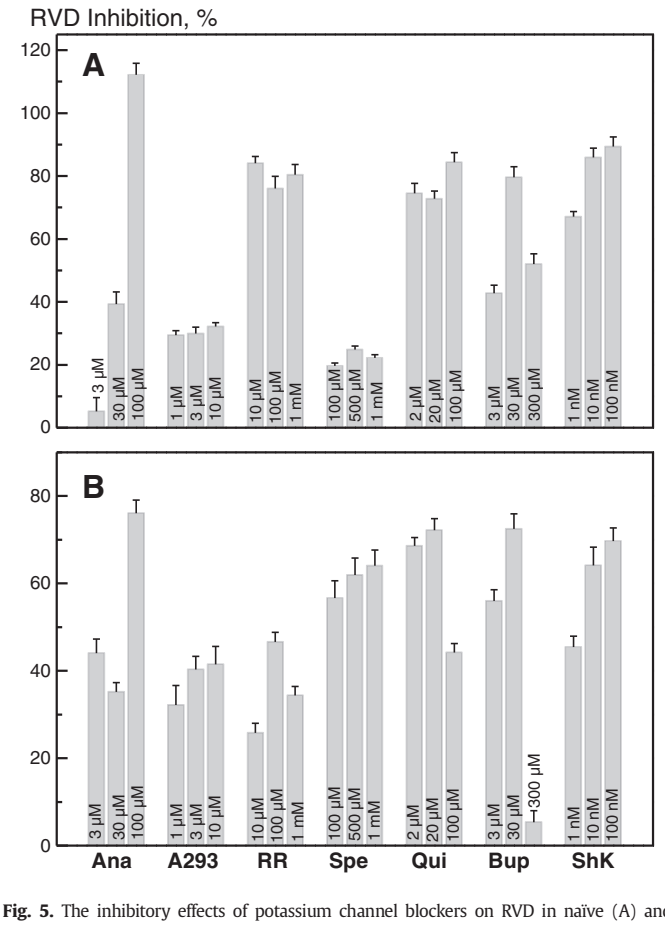

Fig. 5. The inhibitory effects of potassium channel blockers on RVD in naïve (A) and stimulated human T lymphocytes (B). All treated samples and untreated controls were subjected to the same hypotonic stress in 200 mOsm PBS and analyzed by volumetry as illustrated in Fig. 4. For each blocker concentration, the extent of RVD inhibition was calculated from the corresponding ν_2 and ν_{end} data (summarized in Suppl. Table S1) by applying Eq. (2). Note that naïve T cells treated with the highest anandamide concentration (100 μM) were incapable of volume regulation (i.e. remained swollen, see also Fig. 4A) and exhibited a RVD inhibition of ~100%. In contrast, 3 μM anandamide did not impair significantly RVD in naïve cells (RVD inhibition = 0).

shown to reduce by half the K_{2P}9.1-mediated current. In the present study, spermine led to a modest RVD inhibition (~25%) in naïve cells, independent of concentration (0.1–1 mM). In stimulated cells, the RVD inhibition enhanced slightly from 55 to 65%, as the spermine concentration was increased for 100 μM to 1 mM.

The above experiments with a combination of four blockers (anandamide, RR, A293, and spermine) demonstrated a significant role of K_{2P}3.1 and K_{2P}9.1 in the RVD of human T cells. The role of K_{2P}3.1 was also almost independent of the activation status. In case of K_{2P}9.1 probed with RR and spermine, our volumetric data led to controversial results. Thus, RR was a more efficient inhibitor of RVD than spermine in naïve cells, whereas spermine inhibited RVD in stimulated lymphocytes to a higher degree than did RR (Fig. 5). This discrepancy seems to point to non-specific side effects of individual blockers (i.e. RR and spermine), which complicate the analysis of K_{2P}9.1 contribution to RVD. Nevertheless, the strong RVD inhibition in both cell types by the highest anandamide concentration (100 μM) indicate an important role of K_{2P}9.1 in the RVD of human T lymphocytes.

3.5. Targeting K_{2P}5.1

As already shown in numerous studies, e.g. [16,29], K_{2P}5.1 is upregulated in human T cells upon T cell receptor stimulation with CD3/CD28 beads. Moreover, K_{2P}5.1 affects several T cell effector functions, including cytokine production and proliferation [16]. Knock out studies with K_{2P}5.1 deficient mice have demonstrated the importance of this channel for volume regulatory mechanisms in murine T cells [3]. The latter finding is in line with earlier investigations on the role of K_{2P}5.1 in volume regulation of kidney cells and spermatozoa [30–32]. In the present study, we used the antiarrhythmic drug quinidine as a blocker of K_{2P}5.1 [16]. At a concentration of 100 μM, however, quinidine has also been reported to block K_{2P}18.1 [33].

Treatment of naïve T cells with quinidine at concentrations of 2–20 μM caused an about 75% inhibition of RVD (Fig. 5A). Increasing quinidine concentration to 100 μM further enhanced the RVD inhibition to ~85%. These high inhibition rates prove K_{2P}5.1 as one of the major players in the RVD of naïve T cells. In stimulated T cells, the inhibitory capacity of 2–20 μM quinidine (~70%) was similar to that in naïve cells. But in sharp contrast to the concentration dependence found in naïve cells, treatment of stimulated cells with the highest quinidine concentration of 100 μM reduced the extent of RVD inhibition to ~45%. Possible reasons for the inverse concentration dependence may include a limited blocker solubility and/or its cytotoxic effects at high quinidine concentrations. Unfortunately, the lack of more specific blockers for K_{2P}5.1 complicates studies on this channel. Nevertheless, the strong inhibition of RVD by lower quinidine concentrations proves the importance of K_{2P}5.1 for the RVD of human T lymphocytes.

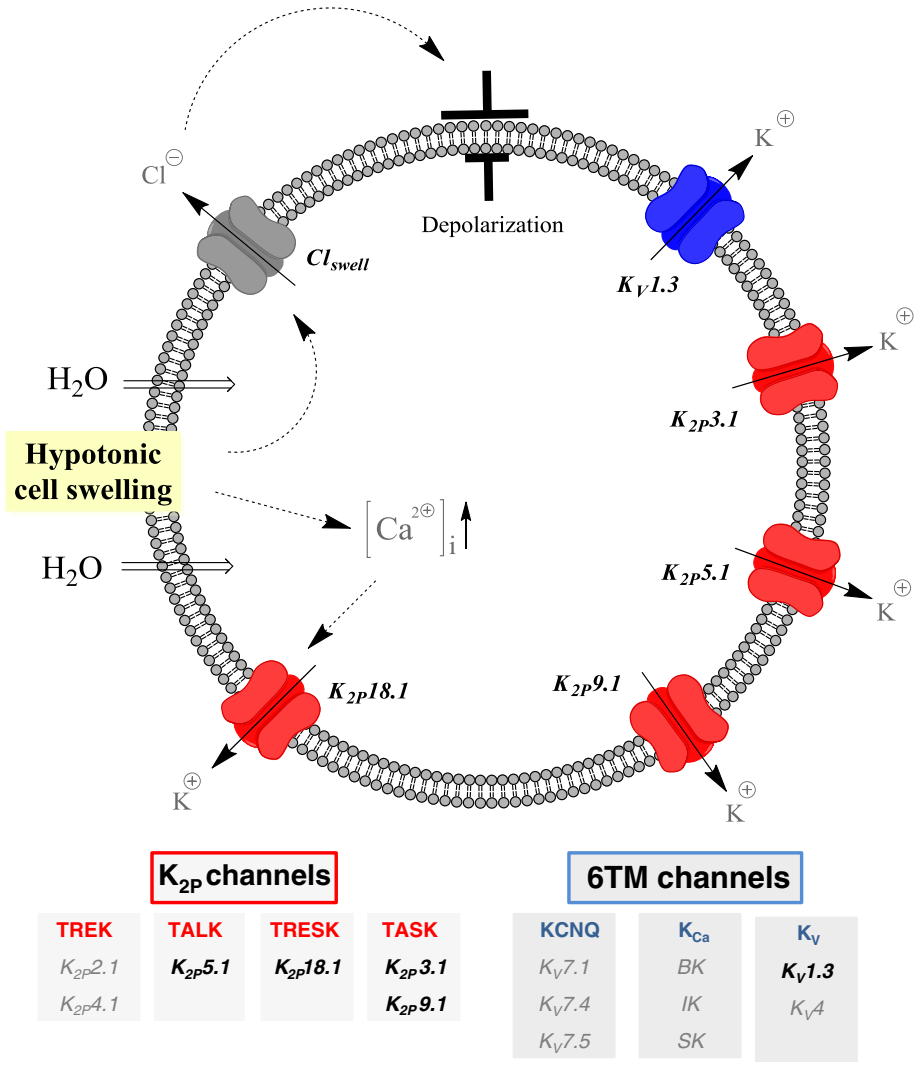

Fig. 6. A network of ion channels is involved in the RVD in human $CD4^+$ T lymphocytes. The pathways of electrolyte efflux following hypotonic volume increase are displayed. Osmotic cell swelling causes activation of different potassium channels either mediated by cell membrane potential changes or intracellular Ca^{2+} level increase (see also Ref. [3]). $K_V1.3$ and $K_{2P}3.1$, 5.1 and 9.1 are activated by depolarization of the cell membrane due to swelling activated chloride efflux (Cl_{swell}). In contrast, $K_{2P}18.1$ channels have been demonstrated to be activated by rising intracellular calcium concentrations. Our results indicate a predominant role of members of the K_{2P} channel (red) and 6 transmembrane domain (6TM) channel families (blue) in RVD of human T cells. The lists below include all potassium channel families and their members known to be involved in volume regulation. Channels expressed on human lymphocytes are highlighted in black.

3.6. Inhibition of $K_{2P}18.1$

The local anesthetic drug bupivacaine has been found to target several K_2P channels in a concentration-dependent manner. Bupivacaine blocks $K_{2P}18.1$ channels in mice and human cells at micromolar concentrations (5–80 μM), as shown elsewhere [34]. Bupivacaine has also been used to block $K_{2P}5.1$, $K_{2P}3.1$ and $K_{2P}9.1$/$K_{2P}18.1$ channels at concentrations higher than 17, 40, and 100 μM, respectively [35–37].

In the present study, the intermediate bupivacaine concentration of 30 μM gave the strongest RVD inhibition of ~75–80% in both cell types. Either decreasing or increasing the concentration to 3 or 300 μM considerably reduced the RVD inhibitory capacity of bupivacaine. The highest concentration of bupivacaine (300 μM) did not cause any RVD inhibition in stimulated T cells, which might have been due to a poor blocker solubility in phosphate buffer, denoted by the manufacturer. The strong inhibitory effect of bupivacaine suggests a significant role of $K_{2P}18.1$ in the RVD of both naïve and stimulated human T cells. In agreement with the protein expression data (Fig. 3I–J), the contribution of $K_{2P}18.1$ to the RVD process is independent of the stimulation status.

3.7. Blocking $K_V1.3$ with Strichodactyla helianthus toxin (ShK)

To compare the relative contributions of different potassium channel families to RVD, we addressed additionally the voltage dependent potassium channel $K_V1.3$, which is well known to play a key role in a variety of T cell functions, including RVD and T cell effector functions, etc. [38,39]. In the present study, we used the blocking agent *S. helianthus* toxin (ShK), a toxic peptide isolated from the sea anemone. ShK is a highly potent $K_V1.3$ blocker, capable of inactivating $K_V1.3$ currents, even in the sub-micromolar range [40,41].

As with murine T lymphocytes studied elsewhere [3], nanomolar concentrations of ShK (1–100 nM) strongly inhibited RVD in human T cells (Fig. 5). Despite a nearly 6-fold increased expression of $K_V1.3$ in stimulated cells (Fig. 3J), the blocking effect of ShK was stronger in naive cells. This finding suggests a greater relative contribution of

$K_V1.3$ to the RVD of the small-sized unstimulated cells, as compared to the large stimulated T lymphocytes. Increasing ShK concentration from 1 to 10 nM enhanced the RVD inhibition from ~65 to ~85% in naïve cells and from ~45 to 65% in stimulated cells. Treatment with 100 nM ShK caused only little further increase in the inhibitory effect. The concentration dependence of RVD inhibition observed here corroborates the data of previous studies on the blocking $K_V1.3$ currents with ShK [42].

3.8. Concluding remarks

Extending our previous findings on murine cells, the pharmacological profile of RVD inhibition observed here points to an essential role of K_{2P} channels in the osmoregulation of human T lymphocytes. Among the examined K_{2P} channels, $K_{2P}5.1$ and $K_{2P}18.1$ seem the most important for the RVD in T cells, with a smaller contribution of $K_{2P}3.1$ and $K_{2P}9.1$. The impact of $K_{2P}5.1$ and $K_{2P}18.1$ on RVD was comparable to that of the voltage-dependent channel $K_V1.3$, whose significance for human T cells was shown previously [11] and confirmed here (Fig. 5). The pharmacological profile and thus the impact of potassium channels on the RVD were found to vary moderately depending on the activation status of T cells. The variations in general correlated with the stimulation-related channel expression patterns reported elsewhere [17]. Our results also underline the heterogeneity of potassium channels involved in the volume regulation of T cells. As a basic physiological process essential for cell survival and function, volume regulation in T cells apparently relies not on a sole potassium channel but rather on an interaction of multiple cation channels, including partially redundant pathways (see Fig. 6).

Conflict of interest statement

The authors declare no conflict of interest.

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Appendix A. Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2012.09.028.

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