Compensatory Anion Currents in Kv1.3 Channel-deficient Thymocytes*

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Kv1.3 is a voltage-gated potassium channel with roles in human T cell activation/proliferation, cell-mediated cytotoxicity, and voltage regulation and is thus a target for therapeutic control of T cell responses. Kv1.3 is also present in some mouse thymocyte subsets and splenocytes, but its role in the mouse is less well understood. We report the generation and characterization of Kv1.3-deficient (Kv1.3−/−) mice. In contrast to wild-type cells, the majority of Kv1.3−/− thymocytes had no detectable voltage-dependent potassium current, although RNA and protein for several potassium channel subunits were found in the thymocyte population. Surprisingly, the level of chloride current in the Kv1.3−/− thymocytes was increased approximately 50-fold over that in wild-type cells. There were no abnormalities in lymphocyte types or absolute numbers in thymus, spleen, and lymph nodes and no obvious defect in thymocyte apoptosis or T cell proliferation in the Kv1.3−/− animals. The compensatory effects of the enhanced chloride current may account for the apparent lack of immune system defects in Kv1.3−/− mice.

The voltage-gated K+ channel, Kv1.3, is expressed in B and T cells, natural killer cells, macrophages (for review, see Ref. 1), and microglia (2, 3). Large currents that resemble those of Kv1.3 in heterologous expression systems have been described in human thymocytes (4) and in resting peripheral human T cells (for review, see Ref. 1), and the currents increase somewhat after cell activation (5, 6). Likewise, Kv1.3-like currents, termed n-type currents, increase after mitogen stimulation of splenic and lymph node T cells from several mouse strains (7–9). Kv1.3-like (n-type) currents are differentially expressed in mouse thymocyte subsets and splenocytes, but there are some discrepancies in the literature. Very small n-type currents were reported for CD4+ CD8− thymocytes in some studies (7, 8, 10), but others observed large currents in these cells and in CD4+ CD8− thymocytes (11). Moderate currents were seen in CD4+ CD8− thymocytes (7, 8, 10), whereas CD4− CD8− thymocytes had either moderate or no Kv1.3 current (10, 11).

Studies with selective drug inhibitors have suggested Kv1.3 to be involved in naive T cell activation and lymphoblast proliferation (5, 6, 12–14), cell-mediated cytotoxicity (15, 16), T cell volume regulation (6, 17), and thymocyte development (14, 18). A proposed common role for Kv1.3 in these functions is to maintain the conditions necessary for a sustained rise in intracellular Ca2+. Following stimulation of the T cell receptor, production of inositol 1,4,5-triphosphate causes a transient release of Ca2+ from intracellular stores followed by a sustained Ca2+ influx that is required for full activation (19, 20). Kv1.3 channels help to maintain a negative membrane potential (Nernst potential for K+ is about −85 mV) and a large driving force for Ca2+ entry through Ca2+ -release-activated Ca2+ channels (21).

We have now generated Kv1.3-deficient (Kv1.3−/−) mice to determine whether they have compromised thymocyte development or peripheral T cell activation. Despite an increase in levels of transcripts and protein for certain other potassium channel subunits (Kv1.1 and Kv1.4) in the total Kv1.3−/− thymocyte population, deletion of the Kv1.3 gene results in the complete loss of voltage-dependent potassium current in the majority of thymocytes. Surprisingly, this is accompanied by an approximately 50-fold increase in chloride conductance. Because chloride channels would be expected to maintain negative membrane potentials in lymphocytes, this compensatory change in membrane permeability may account for the apparent absence of abnormalities in the immune responses of Kv1.3−/− animals.

MATERIALS AND METHODS

Generation of Kv1.3-deficient Mice—The Kv1.3 gene was isolated from a lambda Fix II 128S/J library (Stratagene) using a 5′ region of the rat homologue (GenBankTM number m30441) as a probe. The identity of the clone was confirmed by restriction mapping and sequencing. The rat Kv1.3-coding sequence (GenBankTM number m30441) possesses particular PouII and SacI sites that are unique among members of the Kv1 family, as well as ScaI (used in the targeting strategy), HincII, PsI, and Smal sites.

The targeted region spans an 8.2-kbp region between an upstream BamHI site and the 3′-end of the lambda Fix II genomic clone (see Fig. 1). The BamHI site was subsequently eliminated by Klenow polymerase end-filling and re-ligation. The construct was then linearized with XhoI by partial digestion, and a herpes simplex virus thymidine kinase gene

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1 The abbreviations used are: FCS, fetal calf serum; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; PE, phycoerythrin; 7-AAD, 7-amino actinomycin D; DiOC6, 3,3’-dihexyloxacarbocyanine iodide; NPPB, 4-nitro-2-(3-phenylpropylamino)benzoic acid; DIDS, 4,4’-diisothiocyanatostilbene-2,2’-disulfonic acid; TEA, tetraethylammonium; AgTx-2, agitoxin-2; BSA, bovine serum albumin; RT, reverse transcription; SK, small-conductance k channel; ConA, concanavalin A.

mented with 5 µg/ml FcBlock (BD Pharmingen). Samples were left on ice for 30 min before primary antibodies were added and left on ice in the dark for a further 1 h. Samples were washed in 1.2 ml of PBS/1% FCS before centrifugation at 1000 rpm for 4 °C for 5 min. Secondary antibody incubation and washing were performed as above. Four-color fluorocytometry employed a FACSCalibur with argon and helium-neon lasers (BD Biosciences). Data were analyzed with CellQuest software by first gating on lymphocytes/lymphoblasts, based on forward and side scatter, BD Pharmingen antibodies and other reagents used included anti-IgD (clone 11-26c.2a)-FITC, anti-αβ T cell receptor (clone H57-597)-biotin, anti-CD3e (clone 2C11)-FITC, anti-CD4 (clone RM4-5)-allophtocyanin, anti-CD8a (clone 53-6.7)-allophycocyanin, anti-CD24 (clone M1/69)-biotin, anti-CD44 (clone IMT)-Cy-Chrome, anti-CD45R (B220, clone RA3-6B2)-Cy-Chrome, anti-CD45R (B220, clone RA3-6B2)-phycoerythrin (PE), anti-CD62L (clone Mel-14)-PE, streptavidin-Cy-Chrome, streptavidin-PE, and annexin V-PE. Anti-mouse IgM (donkey polyclonal)-Cy5 was from Jackson Immunoreagents.

**Apoptosis Assays**—Thymocytes were prepared in Bruf’s/5% FCS as above and used in two types of apoptosis assays. In one assay, aliquots of 10^6 cells/ml were treated with 1 µM dexamethasone (Sigma), 2 µM staurosporine (Clontech), or medium alone in 24-well plates in a 37 °C, 5% CO_2 incubator for 6 h. Alternatively, aliquots were treated with 10 µg of immobilized anti-CD3 antibody (clone 2C11; grown in-house), 10 µg of immobilized anti-Fas antibody (clone Jo2; BD Pharmingen), or medium alone in 24-well plates for 24 h as above. Ultraviolet light treatment was performed on a standard transilluminator followed by culturing for 24 h.

Thymocytes were then centrifuged at 1000 rpm for 5 min and resuspended in 0.1 ml of annexin V-staining buffer (BD Pharmingen) containing 1 µg/ml 7-amino actinomycin D (7-AAD, Calbiochem), 20 µg/ml annexin V-PE (BD Pharmingen), and 20 µM 3,3’-dihexyloxacarbocyanine iodide (DiOCP, Molecular Probes). Samples were then left at room temperature in the dark for 20 min before being made up to 0.5 ml with annexin V-staining buffer for fluorocytometry, as above. Percentages of thymocytes that were "live" (7-AAD-low), annexin-V (Ann) positive or negative and DiOCP positive or negative were then determined from the fluorocytometry data and represented as mean percentage of thymocytes ± S.D. from three mice per group. 7-AAD is a fluorescent DNA stain that allows discrimination of live cells from necrotic and late-apoptotic cells that have lost cell membrane integrity (23). Annexin V binds phosphatidylserine and as such is useful in fluorescent-conju-
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A. Kv1.3+/−

Control

5 nM AgTx-2

B. Kv1.3−/−

Control

100 μM NPPB

5 nM AgTx-2

100 μM NPPB

50 ms 60 pA 50 ms

C.

-120 -80 0 100 200 Voltage (mV)

NaCl NaAsp

Current (pA/pF)

Fig. 2. Whole-cell patch clamping of thymocytes. A, the predominant current in wild-type thymocytes resembles Kv1.3. Representative whole-cell patch-clamp current was recorded from a 9-week-old wild-type mouse thymocyte using 145 mM NaCl bath and 140 mM K+ aspartate pipette solution. Current traces are responses to a series of 200-ms-long voltage steps from a holding potential of −80 mV, at intervals of 60 s (to allow recovery from inactivation), to −50, −30, −10, +10, +30, +50, +70, and +90 mV. Upper panel, control recordings were performed by adding 0.1% BSA and 0.2% Me2SO to the bath. Middle panel, 5 nM AgTx-2 was prepared in the bath solution with 0.1% BSA and 0.2% Me2SO and added to the bath during recording. Lower panel, 100 μM NPPB was prepared in the bath solution with 0.1% BSA and 0.2% Me2SO to the bath. B, no Kv1.3 current but increased chloride current in Kv1.3−/− thymocytes. Representative whole-cell patch-clamp current was recorded from a 6-week-old Kv1.3 knockout mouse thymocyte using 145 mM NaCl bath and 140 mM K+ aspartate pipette solution. Current traces are responses to a series of 200-ms-long voltage steps from a holding potential of −80 mV, at intervals of 15 s, to −110, −70, −30, +10, and +50 mV. Upper panel, control recordings were performed by adding 0.1% BSA and 0.2% Me2SO to the bath. Middle panel, 5 nM AgTx-2 was prepared in the bath solution with 0.1% BSA and 0.2% Me2SO and added to the bath during recording. Lower panel, 100 μM NPPB was prepared in the bath solution with 0.1% BSA and 0.2% Me2SO and added to the bath containing 100 μM NPPB. C, current-versus-voltage (I-V) relations for Kv1.3−/− thymocytes with standard NaCl saline in the bath (filled triangles) and after the bath was exchanged with sodium aspartate saline (open circles). Values represent mean ± S.E., n = 4.
4-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) and 4,4'-diisothiocyanato-stilbene-2,2'-disulfonic acid (DIDS), and the K+ channel blocker tetraethylammonium (TEA). Agatoxin-2 (AgTx-2) was from Alomone Laboratories. Stock solutions of NPPB (500 μM) and DIDS (500 μM) were dissolved in Me2SO and stored at −20 °C. When Me2SO was used as the solvent, the maximal final concentration in the bathing solution was about 0.2%. Thus, control recordings included 0.2% Me2SO in the bath solution. Stock solutions of AgTx-2 (1 μM) and charybdotoxin (50 μM) were made in standard bath medium (see below) supplemented with 0.1% bovine serum albumin (BSA, Sigma) to prevent adhesion to surfaces, and stored at −20 °C. Control recordings were done by adding 0.1% BSA to the bath solution.

Patch-clamp Electrophysiology—Thymocytes were prepared as above and then washed five times in RPMI 1640% glutamine supplemented with 10% fetal calf serum (both from In Vitrogen). Whole-cell currents were measured using an Axopatch 200 amplifier and PC-LAMP version 6.04 software (both from Axon Instruments). Patch electrodes of resistance 8–12 MΩ were pulled from thick-walled borosilicate glass (World Precision Instruments). During data acquisition, capacitive currents were canceled by analog subtraction, and all currents were filtered at 2 kHz via the amplifier. Data analysis was performed using pCLAMP and Origin (version 5, Microcal Software), and curve fitting used the iterative Levenberg-Marquardt algorithm of non-linear regression. All recordings were made at room temperature (18–21 °C). All data were corrected for junction potentials between bath and pipette solutions.

The standard bathing solution contained (mM): 145 NaCl, 5 KCl, 1 MgCl2, 1 CaCl2, 5 HEPES, adjusted with NaOH to pH 7.4. The standard pipette solution contained (mM): 145 K aspartate, 1 K 4BAPTA, 5 MgCl2, 1 CaCl2, 5 HEPES, adjusted with NaOH to pH 7.2 with KOH. The resulting low Ca2+ (10 mM) pipette solution allowed Cl− currents and voltage-gated K+ currents to be monitored without contaminating Ca2+-activated currents. Fresh K2ATP (Sigma) was added to pipette solutions just before use to help maintain channel activity during whole-cell recording. Occasionally, to isolate K+ currents, Cl− currents were reduced by substituting aspartate for most of the Cl− in the bath.

RESULTS

Generation of Kv1.3−/− Mice—Mice deficient in Kv1.3 (Kv1.3−/− mice) were generated by homologous recombination in embryonic stem cells using conventional techniques (Fig. 1). Our strategy deletes a 1.8-kbp region of Kv1.3 that includes the promoter/transcriptional start site and a 5′-region of the coding sequence (see “Materials and Methods”). Kv1.3−/− mice were initially obtained by interbreeding Kv1.3+/− mice. Kv1.3−/− mice were born at a normal Mendelian ratio, and subsequent interbreeding of Kv1.3+/− mice did not reveal any obvious lack of breeding or reduced litter sizes.

Kv1.3 is expressed in certain regions of mouse brain (29) and in CD4+ CD8− thymocytes (see the introduction). To verify the loss of Kv1.3 transcription, RT-PCR was conducted with RNA from thymus and brain cortex of 3- to 6-week-old mice (Fig. 1C). Although a Kv1.3 RT-PCR product was easily demonstrable with RNA from Kv1.3+/+ thymus and brain cortex, this product could not be generated with RNA from Kv1.3−/− tissues (Fig. 1C). Western blot analysis (Fig. 1D) and patch-clamping of Kv1.3−/− thymocytes (see below) also confirmed the complete absence of Kv1.3.

Whole-cell Currents in Kv1.3+/+ and Kv1.3−/− Thymocytes—Thymocyte currents were recorded in the whole-cell patch clamp configuration. Most recordings were expected to be from CD4+ CD8+ thymocytes, because they represent ~85% of thymocytes in both Kv1.3+/+ mice and Kv1.3−/− mice (see later and Table III below). Consistent with a previous report that CD4+ CD8+ thymocytes in Kv1.3+/+ mice have a large n-type current (11), the predominant current in Kv1.3+/+ thymocytes strongly resembled Kv1.3 (Fig. 2A). Biophysical features in common with Kv1.3 (1, 30–32) include time-dependent activation with depolarization at about −30 mV, inactivation during prolonged depolarizing steps, and use-dependent inactivation. Although we did not examine details of kinetics and voltage dependence of the Kv current, about 30% inactivation was seen (e.g. Fig. 2A) with voltage pulses of 200-ms duration (which are relatively short). Faster inactivation has been observed for the Kv1.3 current (formerly called “n”-type); e.g. a time constant of ~100 ms (at +40 mV) in mouse thymocytes and ~180 ms in human T cells (8, 9). However, Kv1.3 inactivation is affected by many factors, including temperature, internal and external divalent ions, and the type of internal anion. In particular, intracellular fluoride was previously used (8, 9), and this accelerates inactivation (33) compared with aspartate, which we used in the present study. Also consistent with Kv1.3, there was complete block by AgTx-2, a scorpion toxin that blocks Kv1.3 at very low concentrations (Kd = 4 pm (34)). We found that 2 mM (not shown) and 5 mM AgTx-2 (Fig. 2A, middle panel) blocked the time-dependent current completely. The current remaining after AgTx-2 treatment of Kv1.3+/+ cells was only about 20% of control levels and was insensitive to the anion channel blocker, NPPB (0.1 mM) (Fig. 2A, lower panel). For Kv1.3+/− thymocytes, only two channels among those whose transcripts were found (see below) are apparently inhibited by AgTx-2 (34): Kv1.3 (Kd = 4 pm) and Kv1.6 (Kd ~40 pm). Although Kv1.1 is also blocked (Kd ~40 pm), it was not detected in Kv1.3+/− thymocytes. Because we used 2 or 5 mM AgTx-2, in principle, Kv1.6 expression could also contribute to the current in Kv1.3+/+ thymocytes. One possibility is that Kv1.6 may not form functional channels on the cell surface of thymocytes, because Kv1.6 transcripts were also present in Kv1.3−/− cells, but there was no Kv current (see below). Although we cannot rule out a difference in Kv1.6 activity in the two mice strains, the simplest interpretation is that Kv1.3 accounts for the AgTx-2-sensitive current in thymocytes from wild-type mice.

In contrast to Kv1.3+/+ cells, no AgTx-2-sensitive current could be detected in cells from Kv1.3−/− mice. Instead, a substantial time-independent current appeared (Fig. 2B), the properties of which closely matched those of the small conductance chloride current in human T cells (35). The current was blocked by 0.1 mM NPPB (Fig. 2B, middle panel), a well known blocker of whole-cell Cl− current in normal human T lymphocytes (35) and microglia cells (36), but only weakly by the Cl− channel blocker, DIDS (0.1 mM, not shown), also as previously seen in normal human T lymphocytes (35). NPPB also blocks some other Cl− currents, including cloned CIC-2 channels (37). It is not known to block voltage-gated K+ channels, and because the only current observed in the Kv1.3-deficient thymo-
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### Table I

**List of primers used for RT-PCR amplification**

| Gene   | GenBank™ accession no. | Primer sequence (5' to 3') (FP = forward primer; RP = reverse primer) Expected product size |
|--------|------------------------|--------------------------------------------------------------------------------------------------|-----------------------------------------------|
| Kv1.1  | M30439                 | AAGAATCGGTATGTTGATCC (FP) AACGGCTTAAACACCGGTCAG (RP) 270 bp |                                          |
| Kv1.3  | M30312                 | AAGAATCGGTATGTTGATCC (FP) AACGGCTTAAACACCGGTCAG (RP) 270 bp |                                          |
| Kv1.4  | M32867                 | CCAATGGAAGGAATTGAGCAGCCCG (FP) GCCATATCTCTCCTCCGAGC (RP) 790 bp |                                          |
| Kv1.5  | M27158 or 17621        | AAATTTGACGAGTTGATGTTGCTA (FP) TGGCGATCTCGTGATAGACC (RP) 1000 bp |                                          |
| Kv1.6  | U45979                 | AAAGATCAGCAATGCTGTTG (FP) AGAGGCTTACAGGGACCTT (RP) 552 bp |                                          |
| Kv2.1  | AF029056               | AAAGATCAGCAATGCTGTTG (FP) AGAGGCTTACAGGGACCTT (RP) 552 bp |                                          |
| Kv3.1  | Y07521                 | AAAGATCAGCAATGCTGTTG (FP) AGAGGCTTACAGGGACCTT (RP) 552 bp |                                          |
| SK2    | U68982                 | GAGATCGATGATGCTGTTG (FP) AGAGGCTTACAGGGACCTT (RP) 1320 bp |                                          |
| SK3    | U68984                 | GAGATCGATGATGCTGTTG (FP) AGAGGCTTACAGGGACCTT (RP) 1320 bp |                                          |
| SK4    | AF000972               | GAGATCGATGATGCTGTTG (FP) AGAGGCTTACAGGGACCTT (RP) 1320 bp |                                          |
| CIC-2  | X64139                 | CTGGCTAAGCAGCTAGCTAGG (FP) TGGGACTTACGTTGACCTTT (RP) 857 bp |                                          |
| CIC-3  | AF029347               | CTGGCTAAGCAGCTAGCTAGG (FP) TGGGACTTACGTTGACCTTT (RP) 857 bp |                                          |
| CIC-5  | Z56277                 | GTTATCCGCTCTCTCTTCTT (FP) GGATATCGAGAGAGAGAGAG (RP) 362 bp |                                          |
| CIC-6  | AF030101               | GTTATCCGCTCTCTCTTCTT (FP) GGATATCGAGAGAGAGAGAG (RP) 362 bp |                                          |
| CIC-7  | AF063101               | GTTATCCGCTCTCTCTTCTT (FP) GGATATCGAGAGAGAGAGAG (RP) 362 bp |                                          |
| pCln   | U17899                 | AAAGGCTTCCGAGCGGCTG (FP) AAGGCTTCCGAGCGGCTG (RP) 271 bp |                                          |
| β-Act  | M12481                 | AAGGCTTCCGAGCGGCTG (FP) AAGGCTTCCGAGCGGCTG (RP) 271 bp |                                          |

### Table II

**Expression of messenger RNAs of several ion channels in the thymus of wild-type and homozygous Kv1.3-deficient mice**

Relative mRNA levels were determined by RT-PCR with gene-specific primers (n = 3 animals; at least four independent PCRs per sample). Analyses were done using total thymocyte populations. CD4+CD8+ cells in both Kv1.3+/− mice and Kv1.3−/− mice represented about 85% of the total thymocyte population, with a relatively normal distribution of other subsets (see Table III). Band densities were quantified using the Quantity One software program (GelDoc system, Bio-Rad).

| Gene  | WT† | KO† |
|-------|-----|-----|
| Kv1.1 | −   | +   |
| Kv1.3 | +   | −   |
| Kv1.4 | +   | −   |
| Kv1.5 | +   | −   |
| Kv1.6 | +   | −   |
| Kv2.1 | +   | −   |
| Kv3.1 | −   | +   |
| SK2   | −   | +   |
| SK3   | −   | +   |
| SK4   | −   | +   |
| CIC-2 | −   | +   |
| CIC-3 | −   | +   |
| CIC-5 | −   | +   |
| CIC-6 | −   | +   |
| pCln  | −   | +   |
| β-Act | −   | +   |

†, undetectable; +, detected

Cyto3 was a time- and voltage-independent Cl− current, NPPB appears to be selective in this context. After block by NPPB, the remaining current reversed at about 0 mV (as expected for a non-selective leak current) and was comparable in amplitude to the leak current (AgTx-2-insensitive component) in Kv1.3−/− thymocytes. The whole-cell current and conductance, which is the slope of the current-versus-voltage (I-V) relation, of Kv1.3−/− thymocytes were outwardly rectifying (Fig. 2C). With the standard NaCl bath and K+ aspartate pipette solutions the I-V relation reversed at −50 mV, from which the calculated aspartate permeability is about 15% that of Cl−; the same value calculated for the Cl− current in human T cells (35). To further corroborate the anion selectivity, NaCl in the bath was replaced with aspartate. The resulting reversal potential shifted to 0 mV (Fig. 2C), as predicted for an anion channel.

Despite the presence of alpha subunits for other voltage-dependent potassium channels (Kv1.4 and Kv4.2, see below) in both Kv1.3+/− and Kv1.3−/− thymocytes, in only 3 of 16 cells (1 Kv1.3+/−, 2 Kv1.3−/−) was any non-Kv1.3 (i.e. AgTx-2-insensitive) voltage-dependent potassium current detected. Conceivably, this lack of current could be due to failure to express at the cell surface (2) or to Kv1.4 and Kv4.2 being expressed only in a minority of thymocytes and not in the 85% or so of thymocytes that are CD4+ CD8−. When present, the unidentified voltage-dependent potassium current was also insensitive to charybdotoxin, which blocks Kv1.2 and Kv1.3 (34) but was fully inhibited by 2 mM tetraethylammonium (TEA). This moderately large current (110 pA/pF in the Kv1.3+/− cell; 27 and 39 pA/pF in the Kv1.3−/− cells) was most likely the l-type current (Kv3.1), which is found in CD4− CD8− thymocytes (11), or the Kv1.1-like current reported in CD4− CD8− thymocytes (18), both of which are very sensitive to TEA block.

Fig. 3 summarizes the currents seen in Kv1.3+/− and Kv1.3−/− thymocytes. Values represent the mean peak current amplitude measured at +50 mV. In Kv1.3−/− thymocytes, the
AgTx-2-sensitive Kv1.3 current was 505 ± 208 pA/pF (n = 7); similar in amplitude to that previously reported (11). Although ~85% of the cells are CD4+ CD8+, other cells may have been included in the small sample sizes. This would contribute to the variability, because different thymocyte subsets express different amounts of Kv1.3 current. None of the Kv1.3\(^{-/-}\) thymocytes had detectable NPPB-sensitive anion currents (n = 7). In contrast, none of the Kv1.3\(^{-/-}\) thymocytes (n = 9) had detectable AgTx-2-sensitive Kv1.3 current, but all had a substantial anion current (289 ± 72 pA/pF at +50 mV, n = 9) that was almost as large as the Kv1.3 current in Kv1.3\(^{+/+}\) thymocytes. All cells tested had comparably small leak currents (the AgTx-2-insensitive and NPPB-insensitive portion): 62 ± 15 pA/pF (n = 7) in Kv1.3\(^{+/+}\) thymocytes and 54 ± 15 pA/pF (n = 9) in Kv1.3\(^{-/-}\) thymocytes. Further corroboration that this was simply the leak current was that the remaining current was not inhibited by 100 μM gadolinium, a blocker of non-selective cation currents (see Ref. 38) or by 5 nM apamin, a blocker of the SK2 Ca\(^{2+}\)/calmodulin-activated K\(^{+}\) channel (39), and the only SK channel found by using RT-PCR in these cells.

Expression of Potassium and Chloride Channel Genes in Kv1.3\(^{+/+}\) and Kv1.3\(^{-/-}\) Thymocytes—To characterize further the effects of eliminating the Kv1.3 gene, we examined the expression of transcripts and proteins for several channel subunits in Kv1.3\(^{+/+}\) and Kv1.3\(^{-/-}\) thymocytes. Total thymocyte populations were used. CD4+ CD8+ cells in both Kv1.3\(^{+/+}\) and Kv1.3\(^{-/-}\) mice represented ~85% of the total thymocyte population with a relatively normal distribution of other subsets (see later and Table III). Potassium channel subunits were selected for study if they had previously been found in immune cells or tissues (32, 40). In addition, several previously cloned Cl\(^{-}\) channels were assessed as possibly underlying the lymphocyte Cl\(^{-}\) current (1, 35). mRNAs, determined by RT-PCR with gene-specific primers (Table I), were compared with a positive control; i.e. β-actin mRNA levels in the same samples (n = 3 animals; >4 independent tests per sample) and summarized in Table II as present or undetectable.

Kv1.3\(^{+/+}\) thymocytes expressed detectable levels of mRNA for several Kv channels (Kv1.3, Kv1.4, Kv1.5, Kv1.6, and Kv2.1) and Cl\(^{-}\) channels (CIC-2, CIC-3, CIC-6, and CIC-7). No mRNA was detected for the Ca\(^{2+}\)/calmodulin-activated channels, SK1–4. In thymocytes from Kv1.3\(^{-/-}\) mice, mRNA was detected for several voltage-dependent K\(^{+}\) channels (Kv1.1, Kv1.4, Kv1.6, Kv2.1, and Kv3.1), several Cl\(^{-}\) channels (CIC-2, CIC-3, CIC-6, and CIC-7), one Ca\(^{2+}\)/calmodulin-activated channel (SK2) and pICln. pICln was examined because it is thought to be either a Cl\(^{-}\) channel (41) or an important modulator of some Cl\(^{-}\) channels (42). We were interested in the possibility that pICln could up-regulate functional expression of the Cl\(^{-}\) current. As expected, Kv1.3 was undetectable in Kv1.3\(^{-/-}\) thymocytes, confirming the knockout at the genetic level (Fig. 1C). Kv1.5 was also undetectable in Kv1.3\(^{-/-}\) thymocytes. Kv1.1 is normally expressed at low levels by wild-type CD4+ CD8+ thymocytes (18) but was not detected in RT-PCR of Kv1.3\(^{+/+}\) thymocytes, most likely because CD4+ CD8+ thymocytes represent only about 2% of the total population used to harvest mRNA. Most striking was that limiting dilution of mRNA (with normalization for β-actin) showed at least 20- to 40-fold more Kv1.1 mRNA in Kv1.3\(^{+/+}\) thymocyte mRNA than in Kv1.3\(^{-/-}\) thymocyte mRNA, and at least 2-fold more Kv3.1 mRNA (n = 3 animals; >4 independent tests per sample).

Although we observed a small Kv current in two Kv1.3\(^{+/+}\) thymocytes, we did not exhaustively analyze the protein expression for all channels that were expressed at the mRNA level in Kv1.3\(^{+/+}\) cells. Some of the antibodies were not reliable; e.g. we found that the SK2 antibody produced nonspecific bands and cross-reacted with all SK channel types (43). As shown in Fig. 4, thymocytes from Kv1.3\(^{+/+}\) mice had bands of correct size for Kv1.3 (68 kDa), Kv1.4 (90 kDa), Kv4.2 (80 kDa), Kv4.3 (70 kDa), and CIC-3 (88 kDa). Kv1.3\(^{-/-}\) thymocytes had no Kv1.3 protein but expressed Kv1.1 (68 kDa), Kv1.4, Kv4.2, CIC-2, and CIC-3 proteins. Of these, Kv1.4 protein was significantly higher on average in Kv1.3\(^{-/-}\) thymocytes compared with Kv1.3\(^{+/+}\) thymocytes (Fig. 4); however, this may not be a significant finding, because it was highly elevated in only one of the four samples. CIC-3 appeared to be elevated in Kv1.3\(^{-/-}\) thymocytes, but this increase was not statistically significant. Finally, in agreement with the mRNA results, total Kv1.3\(^{-/-}\) thymocytes had much more Kv1.1 protein than Kv1.3\(^{+/+}\) thymocytes (Fig. 4). The nearly undetectable Kv1.1 reactivity seen in Kv1.3\(^{+/+}\) thymocytes and the low level in Kv1.3\(^{-/-}\) thymocytes are consistent with Kv1.1 being normally expressed only...
interleukin-converting enzyme (47).

Blockade of Kv1.3 activity might conceivably be a pre-

requisite to K\textsuperscript{+} depletion from cells, which occurs early in

apoptosis and stimulates a molecule in the apoptosis pathway,

Fas stimulation caused tyrosine phosphorylation of Fas-associated

dehaptoxin, whereas Fas-induced apo-

ptosis in thymocytes (48) in a manner attributable to early ceramide generation caused by the activation of an acidic sphingomyelinase (49). Inhibition of ceramide generation inhibited caspase activation and thymocyte death (49). Like dexamethasone, anti-CD3 antibody treatment causes thymocyte apoptosis via caspase 9 activation, whereas Fas-induced apo-

Thus, we next challenged thymocytes in vitro with various apoptosis-inducing agents to examine whether or not Kv1.3 is involved in one or other pathway of thymocyte apoptosis. Dexamethasone, a synthetic glucocorticoid hormone, causes apoptosis in thymocytes (48) in a manner attributable to early ceramide generation caused by the activation of an acidic sphingomyelinase (49). Inhibition of ceramide generation inhibited caspase activation and thymocyte death (49). Like dexamethasone, anti-CD3 antibody treatment causes thymocyte apoptosis via caspase 9 activation, whereas Fas-induced apo-

TABLE III

Normal lymphocyte numbers and types in Kv1.3\textsuperscript{+/−} mice

|                     | Kv1.3\textsuperscript{+/−} mice | Kv1.3\textsuperscript{−−} mice |
|---------------------|---------------------------------|-------------------------------|
| Total thymocytes    | 173.3 ± 17.9                    | 165.0 ± 44.5                  |
| CD4\textsuperscript{+} thymocytes (% of total) | 6.6 ± 0.3                      | 7.7 ± 1.0                     |
| CD8\textsuperscript{−} thymocytes (% of total) | 2.8 ± 0.2                      | 3.0 ± 0.6                     |
| CD4\textsuperscript{+}/CD8\textsuperscript{−} thymocytes (% of total) | 2.3 ± 0.5                      | 2.0 ± 0.3                     |
| Total splenocytes   | 96.7 ± 25.7                     | 96.0 ± 14.0                   |
| CD4\textsuperscript{+} splenocytes (% of total) | 20.5 ± 2.0                     | 21.3 ± 2.2                    |
| CD8\textsuperscript{−} splenocytes (% of total) | 12.5 ± 1.1                     | 12.4 ± 2.1                    |
| CD4\textsuperscript{+} CD44\textsuperscript{hi} CD62L\textsuperscript{hi} memory T cells (% of CD4\textsuperscript{+}) | 13.9 ± 3.4                     | 10.6 ± 3.5                    |

Lymphocyte data are from a representative experiment with three mice per group ± S.D. Subsets were analyzed by flow cytometry (see “Materials and Methods”). The vast majority of the remaining thymocytes and splenocytes not listed by percentage of total were CD4\textsuperscript{+}CD8\textsuperscript{−} thymocytes and B220\textsuperscript{−} B cells, respectively.

![Fig. 5. Normal proliferative response of Kv1.3\textsuperscript{−−} splenocytes to T cell polyclonal stimulation.](image)

Splenocytes were then employed to test the significance of Kv1.3 deficiency on T cell proliferation capacity in response to the polyclonal T cell mitogen, concanavalin A (ConA). Fig. 5 shows that splenocytes from 6- to 8-week-old Kv1.3\textsuperscript{−−} mice with several antibodies failed to reveal any significant differences in relative or absolute numbers of thymocytes or splenic B cells and T cells (Table III). This included the presence of a relatively normal frequency of splenic CD62L-low/CD44-high memory CD4 T cells. The small difference in absolute numbers of thymocytes was not significant, nor was there a significant difference in absolute numbers of any thymocyte subset (data not shown, but see Table III).

Splenocytes were then employed to test the significance of Kv1.3 deficiency on T cell proliferation capacity in response to the polyclonal T cell mitogen, concanavalin A (ConA). Fig. 5 shows that splenocytes from 6- to 8-week-old Kv1.3\textsuperscript{−−} mice responded normally to ConA. Equivalent results were obtained with the polyclonal T cell mitogen anti-CD3 (data not shown).

Although the role of ion channels in apoptosis is largely unexplored, Kv1.3 current is reduced during CD95/Fas-induced apoptosis in the human T cell line, Jurkat (44, 45). Moreover, Fas stimulation caused tyrosine phosphorylation of Kv1.3 (44, 45), a modification that inhibits Kv1.3 activity (3, 44, 46). Blockade of Kv1.3 activity might conceivably be a pre-

requisite to K\textsuperscript{+} depletion from cells, which occurs early in

apoptosis and stimulates a molecule in the apoptosis pathway, interleukin-converting enzyme (47).

![Fig. 6. Normal apoptosis in Kv1.3\textsuperscript{−−} thymocytes.](image)

Thymocytes were treated with various apoptosis-inducing agents for either 6 h (A) or 24 h (B) as described under “Materials and Methods.” Dead cells were excluded on the basis of high fluorescence with 7-aminoactinomycin D (7-AAD\textsuperscript{−} cells). Annexin V staining (Ann\textsuperscript{+}) increases significantly upon loss of cytoplasmic membrane integrity, whereas loss of DiOC\textsubscript{6} fluorescence (DiOC\textsubscript{6}\textsuperscript{−}) is due to loss of normal mitochondrial membrane potential, both of which are indicators of apoptosis. A representative experiment is shown as mean ± S.D. from triplicate experiments.
apotosis employs a caspase 8 pathway (50). Staurosporine is a protein kinase inhibitor that induces apoptosis with cytoplasmic features that appear to be independent of caspase activation (such as externalization of phosphatidylserine and loss of mitochondrial membrane integrity), although nuclear features of staurosporine-induced apoptosis are caspase activation-dependent (51). Finally, UV irradiation causes, among other things, DNA damage leading to the expression of Fas ligand and subsequent apoptosis (52).

Thymocyte apoptosis was assessed using annexin V and DiOC₆ (see “Materials and Methods”). These assays did not reveal any significant difference between Kv1.3⁺/++ and Kv1.3⁻/− thymocytes in anti-CD8-, anti-Fas-, UV-, or drug-induced apoptosis (Fig. 6).

DISCUSSION

We have generated Kv1.3⁻/− mice by deleting a large promoter region and the N-terminal third of the Kv1.3 coding sequence. This resulted in complete loss of Kv1.3 as demonstrated by RT-PCR, Western blot analysis, and whole-cell current recordings. Despite the loss of Kv1.3, Kv1.3⁻/− mice had a normal distribution of lymphocytes in the thymus and spleen (Table III) as well as mesenteric lymph nodes (data not shown). There were also no apparent abnormalities in thymocyte development or apoptosis, or in the ability of peripheral T cells to proliferate. These findings might at first seem to contradict studies suggesting that Kv1.3 has a critical role in the immune system. For example, margatoxin, which blocks both Kv1.3 and Kv1.1 (53, 54), caused thymic atrophy in mature mini-swine (14). In addition, there are aspects of Kv1.3 function that we have not yet addressed. For example, selective blockade of Kv1.3 ameliorated CD4⁺ T cell-mediated experimental allergic encephalomyelitis in a rat model (55).

One possible explanation for the apparent lack of abnormality in Kv1.3⁻/− mice is that the loss of all detectable voltage-dependent potassium current in most Kv1.3⁻/− thymocytes was accompanied by the emergence of a major component of chloride current whose amplitude matched that of the Kv1.3 current in wild-type cells. In addition, Kv1.3⁻/− thymocytes also appear to have up-regulated mRNA for Kv1.1, Kv3.1, and SK2, suggesting that there was a concerted compensatory increase in expression of these genes as a result of the loss of Kv1.3 currents. One role of Kv1.3 channels in T cells is to maintain a negative membrane potential necessary for Ca²⁺ influx into thymocytes (21). Because the Nernst potential for chloride is also negative (−30 to −40 mV (56)), in principle, Cl⁻ channels could play a similar role. Although it would be desirable to compare the membrane potential of individual thymocytes from Kv1.3⁺/++ and Kv1.3⁻/− mice, this is problematic. Thymocytes have very small maximal currents and even smaller currents at the resting potential; i.e. they have a much higher membrane resistance than the electrode seal resistance. Thus, the leak around the patch pipette will depolarize the cells from their true membrane potential value.

We also observed greatly increased Kv1.1 protein in Kv1.3⁻/− total thymocytes, without detecting a corresponding current. The presence of mRNA and even protein for an ion channel does not guarantee a corresponding membrane current for several reasons. (i) There are many examples of Kv channels not being present on the plasma membrane; e.g. because of trapping in the endoplasmic reticulum or other internal compartments or mis-processing. Thus, even if protein is detected on a Western blot, it may not be on the cell surface. For instance, we found that Kv1.5 protein in rat microglia was restricted to an intracellular location (2). (ii) Even if the channel is on the cell surface, channel modifications may inhibit the current; e.g. the inhibition of Kv1.3 and 1.5 by tyrosine phosphorylation (46, 57). (iii) CD4⁺ CD8⁺ thymocytes, which represent about 85% of all thymocytes, may lack these currents, and Kv1.1 in total Kv1.3⁻/− thymocyte preparations might have been restricted to CD4⁻ CD8⁻ thymocytes, where Kv1.1 is normally expressed (18).

The prevalent chloride current in Kv1.3⁻/− thymocytes had some properties that are the same as the small conductance chloride current in normal human T cells (35). Both lacked time- or voltage-dependent gating, were outwardly rectifying, had a calculated aspartate permeability of about 15% of Cl⁻, and were fully blocked by 0.1 mM NPPB but only weakly blocked by 0.1 mM DIDS. The molecular identity of the chloride channel has not been determined, but it bears several similarities to the cloned CIC-3 channel, which produces a voltage-insensitive outwardly rectifying current with an iodide > chloride >> aspartate selectivity sequence. However, other properties diverge from CIC-3, which is strongly inhibited by 0.1 mM DIDS (100 μM) and inactivates at potentials above +80 mV (58). The lymphocyte current is less similar to CIC-2, which has a chloride > bromide > iodide selectivity sequence and is closed unless activated by a strong hyperpolarization (59) or a hypo-osmotic shock (60). One intriguing possibility is that these two proteins, like other CIC family members, form heteromultimers with novel properties (61).

In conclusion, deletion of the Kv1.3 gene results in the complete loss of voltage-dependent potassium current in the majority of thymocytes. This is accompanied by an increase of approximately 50-fold in chloride conductance. Because chloride channels would be expected to maintain negative membrane potentials in lymphocytes, this compensatory change in membrane permeability may account for the apparent absence of abnormalities in the immune responses of Kv1.3⁻/− animals. Our findings also highlight the need for caution when interpreting studies on channel-deficient mice because of the possibility of compensatory channel up-regulation.

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