

CHANGES IN THE EXPRESSION OF POTASSIUM CHANNELS DURING MOUSE T CELL DEVELOPMENT

BY DAVID MCKINNON AND RHODRI CEREDIG

From the Departments of Physiology and Experimental Pathology, John Curtin School of Medical Research, Australian National University, Canberra A. C. T. 2601, Australia

Voltage-dependent, potassium-selective ion channels have an important functional role in nerve cells, muscle cells, and some hormone-releasing cells (1). In these cell types, a K^+ selective ion channel, known as the delayed rectifier K^+ channel, is opened, or activated, after depolarization of the cell membrane to potentials more positive than approximately -50 mV. Opening of the ion channel increases the permeability of the cell membrane to potassium ions which, in turn, acts to restore the cell membrane potential towards the equilibrium potential for potassium ions, typically, in the range of -80 to -100 mV. A voltage-dependent K^+ conductance with similar characteristics to the delayed rectifier K^+ conductance of nerve axons (2) has recently been described in human peripheral blood T lymphocytes (3, 4), human T cell clones (5), mouse T cell clones (6), mouse peripheral lymph node T lymphocytes of unknown surface phenotype (5), and mouse peritoneal macrophages (7). Using electrophysiological recording techniques, it is possible to measure the number of functional K^+ channels in the plasma membrane and quantitate the differences in expression of this particular membrane protein among different T cell subpopulations.

While it has been recognized for many years that the thymus is the primary site for differentiation of T lymphocytes (8), attempts to elucidate the pathways of T cell maturation within the thymus have proved difficult and inconclusive. Using mAbs to the cell surface determinants Lyt-2 and L3T4 and flow microfluorometry (FMF),¹ four major subpopulations of thymocytes can be identified. A putative precursor subpopulation, the Lyt-2⁻/L3T4⁻ cells, represents ~3-5% of the total thymocyte population. This population of cells can be further subdivided, using a variety of markers, including the group of mAbs designated B2A2 (9), MI-69 (10), and J11d (11). The other subpopulations are: the Lyt-2⁺/L3T4⁺ cells that make up the majority (~80%) of all cells within the adult thymus, and the two "mature" phenotype subpopulations, the Lyt-2⁺/L3T4⁻ and Lyt-2⁻/L3T4⁺ cells representing 5 and 10% of the total thymocyte population, respectively. The lineage relationships between these subpopulations remains uncertain, although data obtained from thymic reconstitution experiments suggest that the Lyt-2⁻/L3T4⁻ subpopulation contains cells that can give rise to all other thymocyte subsets.

Address correspondence to David McKinnon, Department of Physiology, John Curtin School of Medical Research, Australian National University, G. P. O. Box 334, Canberra City, A. C. T. 2601, Australia.

¹ *Abbreviations used in this paper:* CRT, cortisone-resistant thymocytes; FLS, forward light scatter; FMF, flow microfluorometry; PI, propidium iodide; S/N, supernatant.

In this study we have, for the first time, combined the whole-cell electrophysiological recording technique with FMF to isolate phenotypically defined thymocytes and T lymphocytes. Results obtained showed that J11d⁻/Lyt-2⁻/L3T4⁻ thymocytes express none or very few K⁺ channels, whereas most other Lyt-2⁻/L3T4⁻ cells, as well as typical cortical thymocytes (Lyt-2⁺/L3T4⁺), do express K⁺ channels. Mature (Lyt-2⁺/L3T4⁻ or Lyt-2⁻/L3T4⁺) thymocytes, which are heterogeneous for J11d expression, were also found to be heterogeneous for K⁺ channel expression. Consistent with this finding was the observation that the cortisone-resistant subpopulation of thymocytes, which express low levels of J11d, were enriched for cells expressing low levels of K⁺ channels. Mature phenotype peripheral T lymphocytes expressed very low levels of K⁺ channels, but upon activation with Con A were found to express high levels of K⁺ channels. These results will be discussed in terms of models of thymocyte differentiation.

Materials and Methods

Mice. Female C57BL/6 mice, 6–10 wk old, were bred at the John Curtin School of Medical Research under specific pathogen-free conditions and maintained in a clean animal room for up to 1 wk before use. Fetal mice were obtained from timed matings of C57BL/6 males and BALB/c female mice. Male and female mice were caged together overnight and the following morning plugged females were isolated. The day of finding a vaginal plug was designated day 0 of embryonic development.

Cell Suspensions. Single cell suspensions were prepared in DME containing 10% (vol/vol) FCS. For the preparation of adult thymocyte or lymphocyte suspensions, mice were killed by cervical dislocation. Thymuses were removed free of parathyroid lymph nodes. Lymph node cells were obtained from pooled inguinal and axillary lymph nodes. Pregnant female mice were killed by ether anesthetic and we dissected embryos from the uterus. Fetal thymuses were removed using a dissecting microscope and fine forceps. Cortisone-resistant thymocytes (CRT) were obtained from adult mice 2 d after a single intraperitoneal injection of 4 mg hydrocortisone acetate (Merck, Sharp and Dohme (Australia) Pty. Ltd., Granville, New South Wales).

Antibodies. All mAbs were obtained as culture supernatants from hybridomas grown in vitro. The following mAbs were used: hybridoma GK-1.5 (anti-L3T4, rat IgG2b) (12); hybridoma LICR.LAU.RL172.4 (RL 172) (anti-L3T4, rat IgM) hybridoma PC 61 (anti-IL-2-R, rat IgG1) (13); hybridoma 53-6.7 (anti-Lyt-2, rat IgG2a) (14); hybridoma 31M (anti-Lyt-2, rat IgM) and hybridoma AT 83 (anti-Thy-1.2, rat IgM) (15); hybridoma J11d (recognizing B cells, most thymocytes but not mature T cells, rat IgM) (11). FITC-coupled sheep anti-rat Ig (Silenus Laboratories, Melbourne, Australia) or rabbit anti-rat Ig (Nordic Immunological Laboratories, Tilburg, The Netherlands) sera were used as second stage reagents depending upon the class of the primary antibody. The source of complement (C) was agarose-absorbed rabbit complement.

Negative Selection Cytotoxic Procedures. Adult thymocytes, lymphocytes, or CRT were incubated at cell concentrations $<20 \times 10^6$ cells/ml with a 1:10 dilution of anti-Lyt-2 (31M) or anti-L3T4 (RL172) or with a 1:3 dilution of J11d hybridoma supernatants for 10 min at 37°C. Rabbit C was then added at a 1:10 dilution and the cells were incubated for a further 45 min at 37°C. Viable cells were recovered after centrifugation over Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden). In some instances, and for the preparation of J11d⁻/Lyt-2⁻/L3T4⁻ thymocytes, mAb and C treatment was carried out twice.

Fluorescence Staining and FACS Analysis. Indirect immunofluorescence was used in all instances and all incubations were carried out at 4°C. For analytical purposes, samples of 10^6 cells were incubated for 30 min in 0.1 ml primary antibody hybridoma supernatant. Samples were then centrifuged through FCS and the pellets were resuspended in a 1:10 dilution of the appropriate FITC-coupled anti-Ig sera for a further 30 min. The cells were centrifuged again through FCS and resuspended in 500 μ l of DME. For sorting

purposes, 5×10^6 cells were stained with the same final concentrations of primary and second-step reagents used above. Control samples were incubated with the FITC-coupled reagent only.

We used a FACS IV from Becton Dickinson Immunocytometry Systems, Sunnyvale, CA. Viable cells were gated by narrow-angle forward light scatter (FLS). Laser excitation was with a Spectra-Physics laser (Spectra-Physics Inc., Mountain View, CA) with 800 mW output at 488 nm. FITC fluorescence was measured using a 500–540 nm band pass filter. For each sample, 10,000 gated events were accumulated. Results for fluorescence intensity, number of cells and FLS are expressed in arbitrary linear units (AU). The percentage of positive cells was calculated, for biphasic distributions, by counting cells in the range positive to the inflexion point and then subtracting the percentage of control cells in the same interval of fluorescence to yield a net value for percent positivity.

Analysis of Cellular DNA Content. Samples were processed for cellular DNA content according to the method of Taylor and Milthorpe (16). After treatment with 80 kunitz U/ml RNase (Sigma Chemical Co., St. Louis, MO), cells were stained with the DNA binding dye propidium iodide (PI) (50 $\mu\text{g/ml}$; Sigma Chemical Co.) in the presence of 0.05% NP-40. The samples were then analyzed on the FACS.

FACS Sorting. Sorting was only attempted on cell populations with clearly biphasic fluorescence profiles. Gating parameters were set to include the majority of fluorescence-positive cells. A percentage of cells around the inflexion point were always discarded. Cells were collected in glass tubes whose inside surfaces had been coated with FCS. The flow rate of viable cells being sorted was in the range 0.6 to 1×10^3 gated events per second.

In Vitro Cell Culture. To generate lymphoblasts, peripheral T cells were stimulated with 2 $\mu\text{g/ml}$ of Con A (Pharmacia Fine Chemicals) in the presence of 30–40 U/ml IL-2. The source of IL-2 was the supernatant (S/N) of an EL-4 thymoma subline (17) stimulated for 24 h with 10 ng/ml PMA (Sigma Chemical Co.). This EL4 S/N contained 1,000 U/ml IL-2 as determined using the CTLL-2 cell-proliferation assay (18).

Electrophysiology. The whole-cell, voltage-clamp recording procedure used was similar to that described by Sakmann and Neher (19). Briefly, a glass micropipette was positioned above a cell using a three-dimensional fluid-drive micromanipulator (Narashige Scientific Instrument Laboratory, Tokyo, Japan). During the period while the pipette was maneuvered into position, a positive pressure was maintained to stop debris from blocking the electrode tip. This pressure was released when the electrode tip was within close proximity to the cell surface and the tip was then slowly advanced until it touched the membrane surface. A small negative pressure was then applied to pull the membrane of the cell more tightly against the pipette. This pressure was then released and a high-resistance seal would regularly form spontaneously over the next few minutes. Seal resistances were in the range of 50 to 100 G Ω . Any stray capacitance associated with the electrode and electrode holder was compensated at this point. To break the membrane patch separating the inside of the pipette from the inside of the cell, brief pressure pulses were applied using an electrically controlled hydraulic switch (Clippard Instrument Laboratory, Inc., Cincinnati, OH). Pulses of increasing pressure were used until a sudden increase in capacitive current was seen, indicating entry into the cell.

Ionic currents were recorded using an LS/EPC 7 patch clamp amplifier (List Electronics, Darmstadt, Federal Republic of Germany) and current signals were low-pass filtered through a 4-pole Bessel filter set at a corner frequency of 500 Hz or 1 kHz, depending upon the sampling frequency. Series resistance compensation was used when voltage step errors were ≈ 2 mV. The holding potential for all experiments was -80 mV and voltage steps were applied at a maximum frequency of 1 pulse per 30 seconds to avoid the effects of cumulative inactivation. A PDP 11/23 microcomputer (Digital Equipment Corp., Marlboro, MA) was used to deliver voltage pulses and to record current responses. Analysis of currents was done off-line using a PDP 11/44 minicomputer (Digital Equipment Corp.). All experiments were performed at room temperature (20–22°C).

The maximum peak potassium conductance was calculated by first plotting the peak potassium conductance for each voltage step (which was calculated from the peak current after leakage subtraction and assuming a linear I-V relationship) against membrane

potential. The resulting data points were then fitted by the following equation using least squares minimization (1):

$$g_K(V_m) = g_{K,max} / (1 + e^{(V_m - V_n)/k_n})^4$$

where, $g_{K,max}$ is the maximum peak potassium conductance, $g_K(V_m)$ is the peak potassium conductance for a given membrane potential (V_m), V_n is the mid-point of the curve, and k_n gives the steepness of the voltage dependence. For some cells, instead of fitting the above equation, the maximum peak conductance was calculated by taking the arithmetic mean of peak conductances measured from voltage steps to potentials $>+10$ mV. The cell membrane capacitance was measured by reading the dial settings of the LS/EPC 7 capacitive cancellation network following compensation of the capacitive current response to a 10 mV hyperpolarizing voltage step.

Patch pipettes were prepared from either microhematocrit tubes (Modulohm I/S, Herlev, Denmark) or specialized electrode glass (Kovar 7052; Garner Glass Co., Claremont, CA). Electrodes were coated with Sylgard (Dow Corning Corp., Midland, MI) and then fire-polished to give final resistances in the range of 5 to 8 M Ω . The standard pipette solution contained 130 mM KF, 10 mM KCl, 11 mM K-EGTA, and 5 mM K-Hepes. The standard bath solution contained 150 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, and 5 mM Na-Hepes. No correction was made for the junction potential between the pipette and bath solutions.

Results

Characteristics of the Delayed-Rectifier Potassium Conductance in Mouse Thymocytes and T Lymphocytes. Whole-cell voltage clamp current records from an adult Lyt-2⁻/L3T4⁻ mouse thymocyte are shown in Fig. 1A. The current response to a series of depolarizing steps from a holding potential of -80 mV indicates the presence of a delayed rectifier potassium conductance (G_{K^+}) with similar kinetic and ion permeation properties to that described previously in T cells (3-6). The conductance activates at membrane potentials positive to -50 mV with a sigmoidal time course and saturates in the voltage range of +10 to +20 mV (Fig. 1B). Steady-state inactivation was insignificant at the normal holding potential of -80 mV. Steady-state inactivation curves were well fitted by a Boltzman distribution, with an average midpoint value of -46 ± 1.0 mV, and a slope factor of -5.4 ± 0.9 mV (mean \pm SEM, $n = 7$).

Expression of G_{K^+} in Peripheral Lymph Node T Lymphocytes. To obtain recordings from Lyt-2⁺/L3T4⁻ lymphocytes from peripheral lymphoid tissues, lymphocytes from inguinal and axillary nodes were first depleted of B cells using the mAb J11d (11) to remove anti-Ig staining cells. By FACS, the resultant cell population was 98% Thy-1⁺ with ~34% Lyt-2⁺/L3T4⁻ and 66% Lyt-2⁻/L3T4⁺ phenotypes. These cells were stained for Lyt-2 and sorted to give a population of 98% Lyt-2⁺/L3T4⁻ lymphocytes (Fig. 2A). The sorted cells were in good physical condition and the binding of primary antibody and the secondary fluoresceinated antibody did not appear to inhibit seal formation between the glass micropipette and the cell membrane. Voltage clamp records from these cells were very different to those obtained from a typical thymocyte shown in Fig. 1A. Only a very few functional K⁺ channels were present in the cell membrane, and the sensitivity of the whole cell recording technique as an assay is shown by the fact that currents corresponding to the opening of a single K⁺ channel could easily be seen in the current traces (Fig. 2B). The level of expression of K⁺ channels is obviously quite low. The mean peak conductance of the Lyt-2⁺/L3T4⁻ lymphocytes was 0.12 ± 0.05 nS and the cell membrane

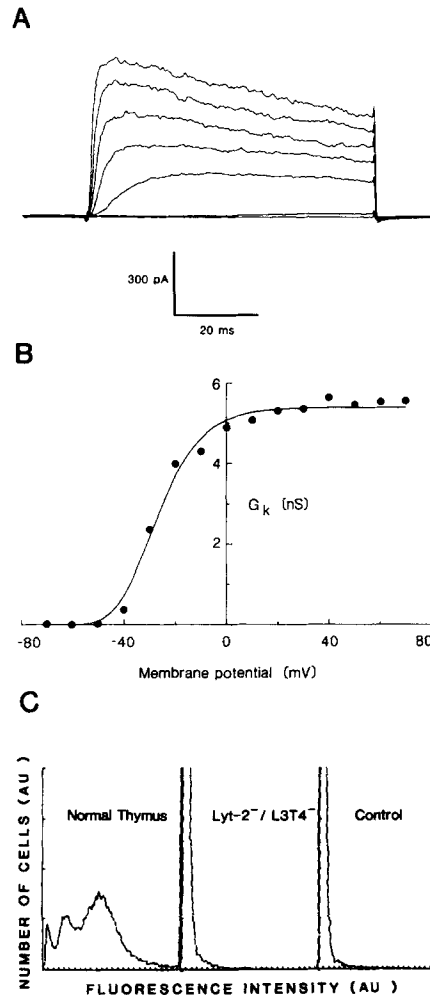


FIGURE 1. Voltage-activated K^+ channel expression by $Lyt-2^-/L3T4^-$ thymocytes. (A) Whole-cell, voltage-clamp current records from an adult $Lyt-2^-/L3T4^-$ mouse thymocyte. Holding potential was -80 mV and voltage steps were applied at 30-s intervals. Voltage was stepped over the range of -70 to $+70$ mV in 10 mV increments. (Only steps to -60 , -40 , -20 , 0 , 20 , 40 , and 60 mV are shown). Sampling interval, $100 \mu s$; filter, 1 kHz. (B) Peak conductance-voltage relationship for the data shown in A. Data were fitted using equation (1) with $V_n = -44.5$ mV, $k_n = -10.7$ mV, and $g_{k,max} = 5.4$ nS. (C) Negative selection of $Lyt-2^-/L3T4^-$ thymocytes. The *left* panel shows the staining pattern of a normal thymus fluorescently labeled with both anti-Lyt-2 and anti-L3T4 mAbs. The *center* panel shows the staining pattern obtained after complement lysis of cells bearing Lyt-2 and/or L3T4 determinants. There is very little fluorescence labeling above the background fluorescence of the negative control shown in the *right* panel.

capacitance was 0.8 ± 0.05 pF (Table I). Cell membrane capacitance is directly proportional to cell membrane surface area and, as such, is an indicator of cell size.

It is possible that selection of J11d⁻ lymphocytes may have biased the resultant cell population against T lymphocytes strongly positive for K^+ channel expression since it is known that this can occur in the thymus (see later sections) and, in this

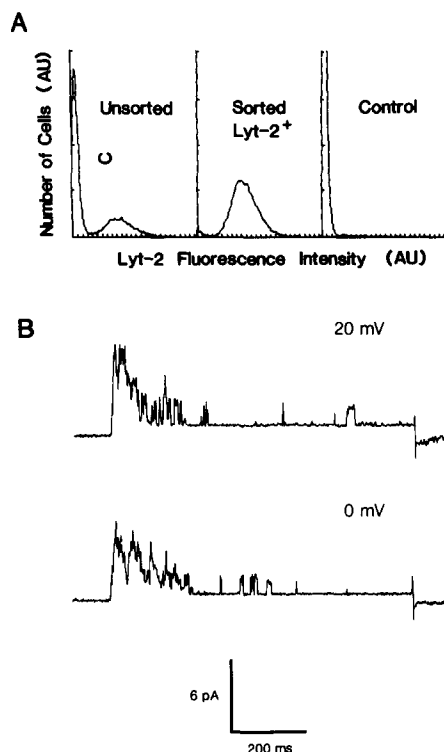


FIGURE 2. Lyt-2⁺/L3T4⁻ peripheral lymph node T cells express very few voltage-activated K⁺ channels. (A) Fluorescence profile of J11d⁻ pooled inguinal and axillary lymph node lymphocytes before and after sorting for Lyt-2⁺/L3T4⁻ lymphocytes. *Left* panel, cells labeled with anti-Lyt-2 mAb were 34% positive. *Center* panel, cells sorted for Lyt-2 fluorescence were 98% Lyt-2⁺. *Right* panel, negative control. (B) Whole-cell voltage-clamp records from a Lyt-2⁺/L3T4⁻ peripheral lymphocyte. Holding potential -80 mV, 800 ms steps to 0 mV and +20 mV reveal single K⁺ channels. Sampling interval, 1 ms; filter, 500 Hz.

TABLE I
Peak Potassium Conductance and Cell Membrane Capacitance of Subpopulations of Mouse Peripheral Resting and Con A-stimulated T Lymphocytes

Cell type	Peak potassium conductance	Cell membrane capacitance	Fraction of cells (G _K ⁺ >0.5 nS)
	nS	pF	
Adult Lyt-2 ⁺ /L3T4 ⁻ lymphocytes	0.12 ± 0.05*	0.8 ± 0.05	0/9
3-d Con A Lyt-2 ⁺ /L3T4 ⁻ blasts	3.5 ± 0.2	3.4 ± 0.5	7/7
3-d Con A Lyt-2 ⁻ /L3T4 ⁺ blasts	3.9 ± 0.4	2.9 ± 0.2	10/10
7-d Con A blasts	2.0 ± 0.3	1.8 ± 0.2	12/12

* Mean ± SEM.

respect, some of our early experiments in which unselected peripheral lymph node lymphocytes were used for electrophysiological experiments are relevant. In these preliminary experiments only 1 cell of 36 expressed a K⁺ conductance >0.5 nS. A value of 0.5 nS was chosen as an arbitrary cut-off point differentiating high from low expression levels of K⁺ channels because this value clearly

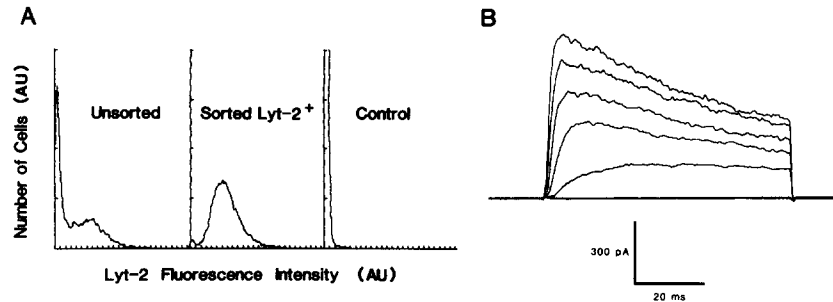


FIGURE 3. Lyt-2⁺/L3T4⁻ thymocytes express high levels of voltage-activated K⁺ channels. (A) Positive selection of Lyt-2⁺/L3T4⁻ thymocytes. The *left* panel shows the fluorescence profile of thymocytes after depletion of L3T4⁺ cells and labeling with an anti-Lyt-2 mAb. ~55% of cells were Lyt-2⁺. The *center* panel shows the profile of positively selected cells, with 97% of cells fluorescence positive compared with the negative control shown in the *right* panel. (B) Whole-cell, voltage-clamp records from a Lyt-2⁺/L3T4⁻ thymocyte. Voltage steps to -40, -20, 0, 20, 40, and 60 mV are shown. Sampling interval, 100 μ s; filter, 1 kHz.

distinguished between peripheral T cells and in vitro-activated T cells (Table I). Although this untreated cell preparation is only ~82–87% positive for Thy-1, by FMF, it is clear that the majority of peripheral T lymphocytes do not express high levels of K⁺ channel.

Expression of G_K⁺ in In Vitro-activated Peripheral T Lymphocytes. Expression of K⁺ channels in mouse peripheral lymphocytes can be up-regulated in vitro by stimulation with the mitogen Con A (5). Peripheral T lymphocytes of either Lyt-2⁺/L3T4⁻ or Lyt-2⁻/L3T4⁺ surface phenotype were stimulated with Con A in the presence of IL-2. 3 days later their surface phenotype was analyzed by FMF and any cells of the inappropriate phenotype were removed by negative selection using mAb and C. Voltage clamp studies of these cells showed that the number of K⁺ channels per cell had increased considerably compared with resting cells of similar phenotype and, as expected from their blast morphology, there was an approximately fourfold increase in membrane capacitance (Table I).

Lymphoblasts cultured for 7 d still expressed K⁺ channels at moderate levels, although the cell membrane capacitance had fallen (Table I). No effective protocol was found in which K⁺ channel expression could be first induced and then seen to fall back to resting levels. Cells from mixed lymphocyte cultures left for up to 11 d still continued to express K⁺ channels at high levels (data not shown).

Expression of G_K⁺ by Subsets of Mouse Thymocytes Defined by the Antigens Lyt-2 and L3T4. To study thymocytes with the "mature" Lyt-2⁺/L3T4⁻ cell surface phenotype, normal thymocyte suspensions were first depleted of L3T4⁺ cells by antibody and complement lysis. FMF analysis of the remaining cells showed that they were either Lyt-2⁺/L3T4⁻ or Lyt-2⁻/L3T4⁻. The L3T4-depleted cells were then stained for Lyt-2 (Fig. 3A) and sorted for positive fluorescence staining. A population, ~97% Lyt-2⁺/L3T4⁻, could be obtained by sorting. Unlike peripheral T cells of similar phenotype (Fig. 2), voltage clamp records from these cells (Fig. 3B) revealed a relatively high average peak K⁺ conductance of 4.8 ± 0.6 nS (Table II), indicating a greater number of K⁺ channels in the cell membrane.

Small Lyt-2⁺/L3T4⁺ cortical thymocytes, which make up ~60% of cells in the normal thymus, can also be isolated for electrophysiological experiments by

TABLE II
Peak Potassium Conductance and Cell Membrane Capacitance of Subpopulations of Mouse Thymocytes

Cell type	Peak potassium conductance	Cell membrane capacitance	Fraction of cells ($G_{K^+} > 0.5$ nS)
	nS	pF	
Adult Lyt-2 ⁺ /L3T4 ⁻ thymocytes	4.8 ± 0.6*	1.2 ± 0.05	12/13
Adult Lyt-2 ⁺ /L3T4 ⁺ thymocytes	2.7 ± 0.6	0.6 ± 0.03	7/7
Adult Lyt-2 ⁻ /L3T4 ⁻ thymocytes	2.7 ± 0.5	1.3 ± 0.1	13/15
14-d fetal Lyt-2 ⁻ /L3T4 ⁻ thymocytes	1.4 ± 0.2	1.9 ± 0.1	17/18

* Mean ± SEM.

positive selection. A total thymus suspension was first stained for Lyt-2, then sorted using a combination of fluorescence intensity and low FLS to select small cells. To ensure that only the small Lyt-2⁺/L3T4⁺ cortical thymocytes were used for recordings, it was also necessary to rely on visual identification of the smallest cells during electrophysiological experiments. Voltage clamp records from all cells examined indicated the presence of a K⁺ conductance, similar to that seen in Lyt-2⁺/L3T4⁻ thymocytes with an average peak conductance of 2.7 ± 0.6 nS (Table II). The low cell membrane capacitance value (0.6 ± 0.03 pF), is consistent with the relatively small size of these cells.

Fig. 1C shows that a homogeneous Lyt-2⁻/L3T4⁻ cell population could be obtained by negative selection using complement lysis. The average maximum peak conductance for adult Lyt-2⁻/L3T4⁻ thymocytes was 2.7 ± 0.5 nS and the average cell capacitance was 1.3 ± 0.1 pF (Table II). The adult Lyt-2⁻/L3T4⁻ thymocyte population is thought to largely comprise cells at very early stages in the pathway committed to T lymphocyte development. Since the majority of these cells express K⁺ channels at relatively high levels it was of some interest to see at what stage during the embryological development of the thymus that K⁺ channels first appear. Thymocytes obtained from 14-d fetal thymuses are all Lyt-2⁻/L3T4⁻ (20). Voltage clamp records of these fetal cells revealed a K⁺ conductance similar to that seen in adult Lyt-2⁻/L3T4⁻ thymocytes, although the average mean peak conductance was somewhat lower (Table II). This result indicates that embryologically, expression of K⁺ channels is an early event in the differentiation pathway of T lymphocytes within the thymus.

Expression of G_{K^+} by J11d⁻ Subsets of Thymocytes. It has recently been shown (21) that ~87% of thymocytes with apparently mature surface phenotypes (Lyt-2⁺/L3T4⁻ or Lyt-2⁻/L3T4⁺), express high levels of the surface antigen recognized by the mAb B2A2. In contrast, apart from recent thymus migrants, peripheral lymph node T cells express very low levels of the antigen and are resistant to B2A2-directed complement lysis (21). Since differential expression of this antigen is one of the few indices, other than K⁺ channel expression, that can differentiate between mature phenotype thymocytes and peripheral T lymphocytes, it was of some interest to examine the distribution of K⁺ channels in B2A2⁻ thymocyte subpopulations.

It has been suggested (21) that the mAbs J11d and B2A2 recognize similar determinants. Since our initial studies showed that, for the preparations of mAbs

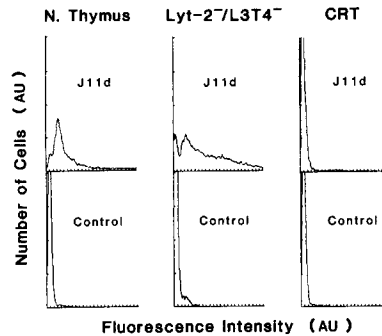


FIGURE 4. J11d staining profile of normal thymocytes, $\text{Lyt-2}^-/\text{L3T4}^-$ and CRT. Normal thymocytes were 86% positive, $\text{Lyt-2}^-/\text{L3T4}^-$ thymocytes were 85% positive, and CRT were ~5% positive.

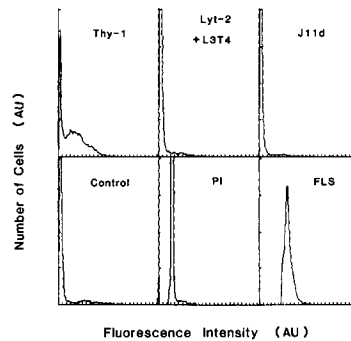


FIGURE 5. Fluorescence profile of $\text{J11d}^-/\text{Lyt-2}^-/\text{L3T4}^-$ thymocytes. Cells were stained with mAbs to Thy-1, Lyt-2 plus L3T4, and J11d. Cells were stained for DNA content with the DNA-binding dye PI. Also shown is the FLS profile indicating a homogeneous population of cells with regard to cell size.

available, J11d was more effective than B2A2 at complement-mediated killing and fluorescence staining, J11d was used for all experiments.

Fig. 4 shows the profile of normal, $\text{Lyt-2}^-/\text{L3T4}^-$, and CRT labeled with J11d. The majority (86%) of normal thymocytes stained positively with J11d. $\text{Lyt-2}^-/\text{L3T4}^-$ cells are clearly heterogeneous for J11d expression with 85% staining above the control. CRT stain very weakly with J11d, with only ~5% of cells staining above control levels. The profiles obtained with FMF agree with data obtained by complement-mediated lysis (data not shown).

Clearly, ~15% of the $\text{Lyt-2}^-/\text{L3T4}^-$ thymocytes are also J11d^- (Fig. 4, *middle*). These $\text{J11d}^-/\text{Lyt-2}^-/\text{L3T4}^-$ cells could be obtained by negative selection, using two rounds of mAb and C treatment. The FMF profile of such cells is shown in Fig. 5. Negative selection for a minor thymic subpopulation carries the danger of enriching for any non-T cell lineage cells found within the thymus. This problem is reduced, though not entirely eliminated, with J11d depletion because this mAb recognizes a number of non-T cell lineages including B cells and neutrophils (11). As can be seen from the negative control in Fig. 5, there is <6% staining with anti-Ig reagents. Compared with this control, 70% of $\text{J11d}^-/\text{Lyt-2}^-/\text{L3T4}^-$ cells were Thy-1^+ with no cells staining for Lyt-2, L3T4, or J11d. Interestingly, as determined by PI staining, most (~95%) of such cells

TABLE III
Peak Potassium Conductance and Cell Membrane Capacitance of J11d⁻ and Cortisone-resistant Subpopulations of Mouse Thymocytes

Cell type	Peak potassium conductance	Cell membrane capacitance	Fraction of cells (G _K ⁺ >0.5 nS)
	nS	pF	
J11d ⁻ /Lyt-2 ⁻ /L3T4 ⁻ thymocytes	0.5 ± 0.3*	1.2 ± 0.2	2/13
J11d ⁻ thymocytes	0.4 ± 0.1	1.0 ± 0.04	2/12
J11d ⁻ /Lyt-2 ⁺ /L3T4 ⁻ thymocytes	1.1 ± 0.5	1.3 ± 0.2	4/8
Lyt-2 ⁺ /L3T4 ⁻ CRT	1.2 ± 0.6	1.1 ± 0.1	3/8
Lyt-2 ⁻ /L3T4 ⁺ CRT	0.4 ± 0.08	1.0 ± 0.2	1/8

* Mean ± SEM.

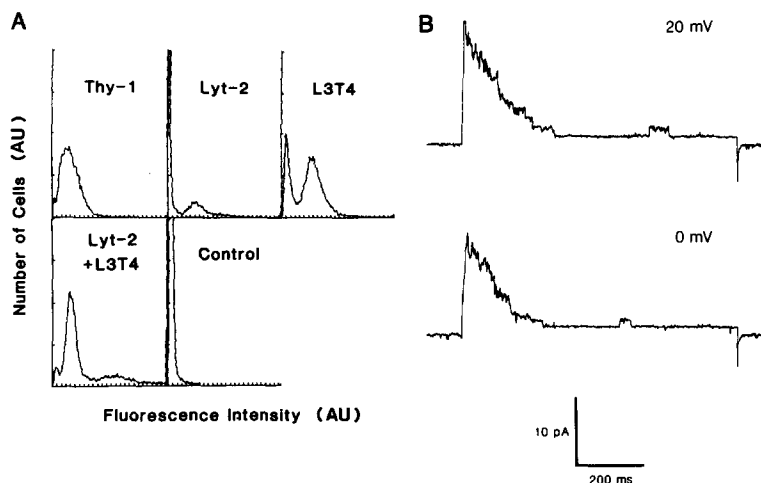


FIGURE 6. J11d⁻ thymocytes express very few voltage-activated K⁺ channels. (A) Surface phenotype of adult C57BL/6 J11d⁻ thymocytes. Negatively selected thymocytes were stained by indirect immunofluorescence with anti-Thy-1, anti-Lyt-2, anti-L3T4, and a mixture of both anti-Lyt-2 plus anti-L3T4 mAbs. (B) Whole-cell, voltage-clamp records from a J11d⁻ thymocyte. J11d⁻ thymocytes were obtained by negative selection using the mAb J11d plus complement. Holding potential, -80 mV; 800 ms steps to 0 mV and +20 mV. Sampling interval, 1 ms; filter, 500 Hz.

were in the G₀/G₁ phases of the cell cycle (Fig. 5). When examined electrophysiologically, 4 of 13 of the J11d⁻/Lyt-2⁻/L3T4⁻ cells tested had no K⁺ channels at all, and overall the mean conductance was low (0.5 ± 0.3 nS; Table III).

A total population of J11d⁻ thymocytes, was also obtained by complement-mediated depletion and analyzed by FMF. As shown in Fig. 6, the majority of cells (~90%) were weakly Thy-1⁺, with ~27% Lyt-2⁺ and 67% L3T4⁺ and when we added both anti-Lyt-2 and anti-L3T4 mAb simultaneously, only 5% of cells did not stain.

Typical voltage clamp recordings from J11d⁻ cells are shown in Fig. 6B. The level of K⁺ channel expression (0.4 ± 0.1 nS) is clearly much reduced compared with the majority of thymocytes (Table II). The mean peak K⁺ conductance is closer to the value found for peripheral T lymphocytes, and only 2 of 12 cells

expressed a conductance >0.5 nS (Table III). It is clear that in most J11d⁻ thymocytes K⁺ channel expression is down regulated.

As shown previously by Scollay and Shortman (21), B2A2 (or J11d) expression among Lyt-2⁺/L3T4⁻ cells is heterogeneous. The results shown in Fig. 6 show the existence of J11d⁻/Lyt-2⁺/L3T4⁻ cells. To examine whether K⁺ channel expression among Lyt-2⁺/L3T4⁻ thymocytes was related to J11d expression, J11d⁻/Lyt-2⁺/L3T4⁻ cells were obtained by positive selection from thymocyte suspensions first depleted of J11d⁺ and L3T4⁺ cells by mAb and C. These cells were found to have a mean peak conductance (1.1 ± 0.5 nS, Table III) significantly (Student's *t* test, $p < 0.001$) lower than that of the total subpopulation of Lyt-2⁺/L3T4⁻ thymocytes (4.8 ± 0.6 nS, Table II).

Expression of G_{K+} in CRT. A distinct subpopulation of thymocytes can be obtained 2 or 3 d after pretreatment of mice with an injection of corticosteroids. The pattern of expression of Lyt-2 and L3T4 on such cells was similar to that previously reported (20), with 33% Lyt-2⁺/L3T4⁻, 61% Lyt-2⁻/L3T4⁺, and only 6% Lyt-2⁻/L3T4⁻. Since only 3–5% of all thymocytes are cortisone resistant (22), and ~15% of the total number of thymocytes are mature phenotype, this implies that cortisone pretreatment results in the recovery of only a subpopulation of the mature phenotype thymocytes. As has already been shown in Fig. 4, expression of J11d is very much reduced on CRTs compared with the normal thymus, suggesting that cortisone treatment specifically spares J11d⁻ mature thymocytes. Voltage clamp studies on CRT show that such cells have reduced K⁺ channel expression compared with the majority of both mature and immature thymocytes (Table III).

Discussion

This is the first report in which an analysis has been made of the expression of voltage-activated K⁺ channels in subpopulations of mouse thymocytes and T lymphocytes. The results suggest that the level of expression of K⁺ channels by T cells is related to their stage of differentiation. As a consequence, analysis of K⁺ channel expression in different T cell subpopulations provides additional information about the differentiation pathways of T cells.

The kinetic and ion permeation properties of the delayed rectifier K⁺ conductance seen in mouse thymocytes and T lymphocytes in this study are very similar to those described previously in human and mouse T lymphocytes and T cell clones (3–6, 23). One notable difference between the human and mouse studies is the relatively high expression of K⁺ channels by resting human peripheral blood T lymphocytes compared with that of mouse lymph node T cells (5, 24). Since there is, at present, no electrophysiological study of either human lymph node lymphocytes or mouse peripheral blood T cells, it is uncertain whether this discrepancy is due to differences between the general level of activation of T cells in the two tissues or to genuine species differences.

Bregestovski et al. recently saw (25) that internal calcium ions can block K⁺ channels in human T lymphocytes. This calcium-dependent channel blockade was, however, seen at [Ca]_i concentrations of the order of 0.1 μM, which are considerably higher than the [Ca]_i of the pipette solutions used in this study. The peak K⁺ conductance values calculated for any given cell in this study should,

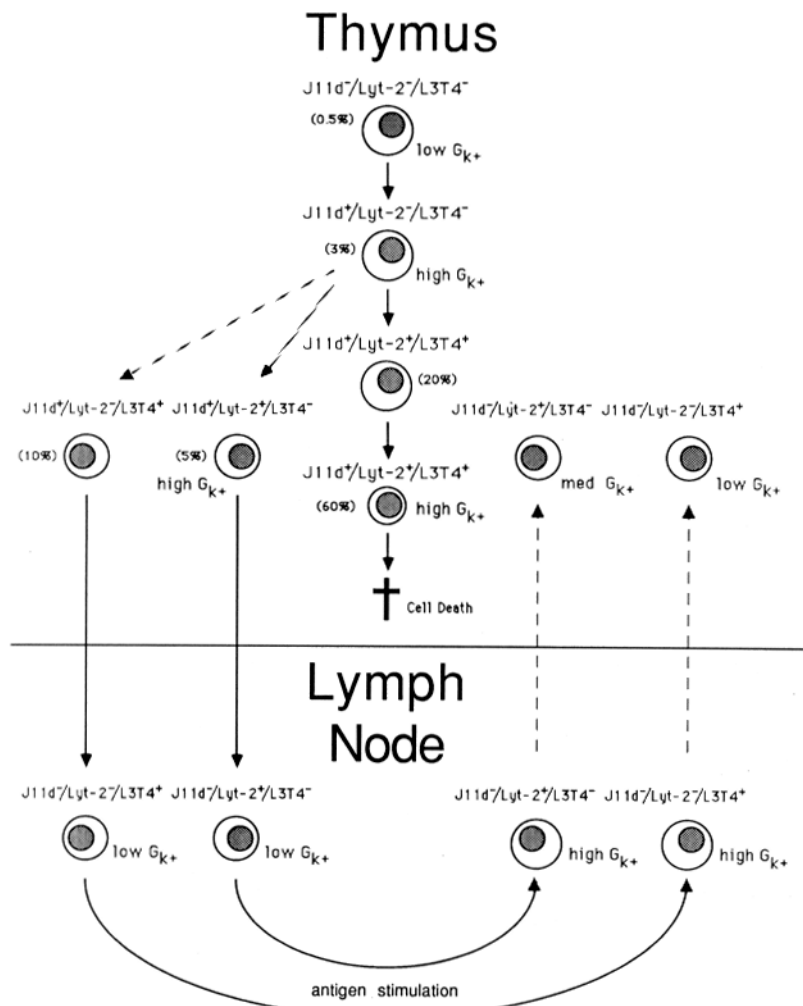


FIGURE 7. Summary of voltage-activated K^+ channel expression (G_{K^+}) during T cell maturation. Subpopulations of T cells are defined by the surface antigens Lyt-2, L3T4, and the antigen recognized by the mAb J11d. Lineage relationships, where they remain uncertain, are shown as dashed lines. The number of thymocytes within each subpopulation (if it has been determined), is shown in brackets as a percentage of the total number of thymocytes.

therefore, accurately reflect the number of K^+ channels available for activation in the cell membrane.

Shown in Fig. 7 is a summary of our data on the expression of K^+ channels among subpopulations of thymocytes and peripheral T cells. The finding that K^+ channel expression in peripheral lymph node T lymphocytes is upregulated after activation by mitogenic lectins suggests that T cells may, as a general response to mitogenic stimulation, increase their level of expression of K^+ channels (see also 5, 24, 26). It has been shown previously (27) that K^+ channels are apparently necessary for mitogenesis, although their exact function remains uncertain.

As for the thymus, the factors that control proliferation of cells therein are

still unknown. It is clear that the $\text{Lyt-2}^-/\text{L3T4}^-$ cells are rapidly proliferating cells (9, 28) and that approximately half of these cells express an IL-2-R (13). The high level of K^+ channel expression by $\text{Lyt-2}^-/\text{L3T4}^-$ cells is consistent with the proposition that K^+ channel expression is high in cells recently stimulated to proliferate. This conclusion is also supported by results obtained from $\text{J11d}^-/\text{Lyt-2}^-/\text{L3T4}^-$ cells, which generally have a low level of K^+ channel expression and are largely noncycling (Fig. 5) and IL-2-R⁻ (data not shown). In further confirmation of this general trend are the results obtained from day 14 fetal thymocytes, a high percentage of which are rapidly proliferating and express both the IL-2-R (13, 29) and the K^+ channel.

The relationship of $\text{J11d}^-/\text{Lyt-2}^-/\text{L3T4}^-$ cells to other subpopulations of thymocytes is, at present, uncertain. One possibility is that these cells are recent immigrants from the bone marrow which have not yet received an appropriate stimulus from the thymic microenvironment (Fig. 7). Consistent with this suggestion is the low rate of mitogenesis within this subpopulation and the lack of expression of the IL-2-R. We cannot at present, however, exclude the possibility that these cells are a quiescent population within the thymus unrelated to the major pathway of T cell maturation.

The very high K^+ channel expression, relative to membrane area, seen in the small cortical $\text{Lyt-2}^+/\text{L3T4}^+$ thymocytes is, in some ways, surprising since these cells have generally reduced rates of protein synthesis and are thought to die within the thymus (30). These small thymocytes are presumably derived from the rapidly cycling $\text{Lyt-2}^+/\text{L3T4}^+$ blast cells, and the K^+ channels that they express are, quite possibly, inherited from these precursor blast cells. This inheritance of K^+ channels may be analogous to the inheritance of the TL antigen by small cortical thymocytes (30). There is, at present, no data available on the rate of turnover of the delayed rectifier K^+ channel in cell membranes. It is probable however, that the channel has a half life in the cell membrane of the order of a few days at least; a proposition that is supported by the relatively slow decline of K^+ channel numbers in 7-d Con A blasts and mixed lymphocyte culture cells.

The $\text{Lyt-2}^+/\text{L3T4}^+$ blasts do not express the IL-2-R, and it therefore seems probable that the mechanisms controlling expression of the K^+ channel and the IL-2-R are independent, even though expression of these two membrane proteins appears to be linked in both immature ($\text{Lyt-2}^-/\text{L3T4}^-$) thymocytes and T lymphocytes. The significance of the failure of proliferating $\text{Lyt-2}^+/\text{L3T4}^+$ cells to express the IL-2-R is still not understood, and it is possible that a similar failure to express the IL-2-R by half of the $\text{Lyt-2}^-/\text{L3T4}^-$ cells indicates that these cells are already committed to becoming $\text{Lyt-2}^+/\text{L3T4}^+$ cells; although, the alternative possibility, that they are precursors of the IL-2-R⁺ cells cannot, at present, be excluded.

The high level of K^+ channel expression by $\text{Lyt-2}^+/\text{L3T4}^-$ thymocytes suggests that these cells may be the direct products of a rapidly proliferating parent cell of either $\text{Lyt-2}^-/\text{L3T4}^-$ or $\text{Lyt-2}^+/\text{L3T4}^+$ phenotype. The lower level of expression of K^+ channels by $\text{J11d}^-/\text{Lyt-2}^+/\text{L3T4}^-$ cells indicates that these cells represent a distinct stage of T cell maturation. There is evidence that peripheral T lymphocytes can recirculate back into the thymus (31). One possible interpretation of our data is that $\text{J11d}^-/\text{Lyt-2}^+/\text{L3T4}^-$ cells isolated from the thymus

contain recirculating peripheral T cells, rather than cells solely in the intrathymic cell lineage. Consistent with this suggestion is the observation that B2A2⁻ thymocytes are enriched for antigen-specific responsive cells after priming with Moloney murine sarcoma virus (32).

CRT are a distinct subpopulation of mature phenotype thymocytes (33). Phenotypically, CRT appear to be enriched for the J11d⁻ subpopulations of Lyt-2⁺/L3T4⁻ and Lyt-2⁻/L3T4⁺ thymocytes (Fig. 4). The reduced expression of J11d among CRT appears to be in conflict with the data of Scollay et al. (34) using B2A2, but is in agreement with Bruce et al. (11), where CRT were found to be expressing low levels of J11d, as determined by mAb and C cytotoxicity. Electrophysiologically, CRT appear to be very similar to the J11d⁻ subpopulations.

For both the J11d⁻ and CRT subpopulations, the Lyt-2⁺/L3T4⁻ cells expressed, on average, more K⁺ channels than did the Lyt-2⁻/L3T4⁺ cells (Table III). This result is consistent with the reports of size differences between these cells (20, 21), in which Lyt-2⁺ cells were found to be, on average, larger than the L3T4⁺ cells. The physiological significance of this difference between the two cell populations is uncertain.

In conclusion, it is clear that K⁺ channel expression in T cells is developmentally regulated. Increased expression of the channel is apparently induced in response to mitogenic signals throughout the T cell lineage, and therefore, serves as a useful marker in defining the steps of T cell development. T cell differentiation provides an ideal model system for studying the genetic mechanisms controlling ion channel expression, and further studies should provide additional information about ion channel expression; information that has, so far, proven difficult to obtain from other mammalian systems.

Summary

In this report we have combined the whole-cell electrophysiological recording technique with flow microfluorometry to isolate phenotypically defined thymocytes and T lymphocytes. Results obtained showed that J11d⁻/Lyt-2⁻/L3T4⁻ cells express none or very few delayed rectifier K⁺ channels, whereas most other Lyt-2⁻/L3T4⁻ cells, as well as typical cortical thymocytes (Lyt-2⁺/L3T4⁺), do express K⁺ channels. Mature (Lyt-2⁺/L3T4⁻ or Lyt-2⁻/L3T4⁺) thymocytes, which are heterogeneous for J11d expression, were also found to be heterogeneous for K⁺ channel expression. Consistent with this finding was the observation that the cortisone-resistant subpopulation of thymocytes, which express low levels of J11d, were enriched for cells expressing low levels of K⁺ channels. Mature phenotype peripheral T lymphocytes expressed very low levels of K⁺ channels, but upon activation with Con A were found to express high levels of K⁺ channels.

The results suggest that K⁺ channel expression in T cells is developmentally regulated. Increased expression of the channel is induced in response to mitogenic signals throughout the T cell lineage. Expression of the channel, therefore, serves as a useful marker in defining steps in the T cell differentiation pathway.

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