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Voltage-activated proton currents in human lymphocytes

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Voltage-activated proton currents are reported for the first time in human peripheral blood T and B lymphocytes and in the human leukaemic T cell line Jurkat E6-1. The properties of H^+ currents studied using tight-seal voltage-clamp recording techniques were similar in all cells. Changing the pH gradient by one unit caused a 47 mV shift in the reversal potential, demonstrating high selectivity of the channels for protons. H^+ current activation upon membrane depolarisation had a sigmoidal time course that could be fitted by a single exponential function after a brief delay. Increasing pH_o shifted the activation threshold to more negative potentials, and increased both the H^+ current amplitude and the rate of activation. In lymphocytes studied at pH_i 6.0, the activation threshold was more negative and the H^+ current density was three times larger than at pH_i 7.0. Increasing the intracellular Ca^{2+} concentration to 1 μM did not change H^+ current amplitude or kinetics detectably. Extracellularly applied Zn^{2+} and Cd^{2+} inhibited proton currents, slowing activation and shifting the voltage–activation curve to more positive potentials. The H^+ current amplitude was 100 times larger in CD19+ B lymphocytes and in Jurkat E6-1 cells than in CD3+ T lymphocytes. Following stimulation with the phorbol ester PMA, the H^+ current density in peripheral blood T lymphocytes and Jurkat T cells increased. In contrast, the H^+ current density of phorbol ester (PMA)-stimulated B lymphocytes was reduced and activation became slower. The pattern of expression of H^+ channels in lymphocytes appears well suited to their proposed role of charge compensation during the respiratory burst.

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Lymphocytes express a variety of ion channels, including K^+, Ca^{2+} and Cl^- channels, that play important roles in cellular functions, such as proliferation, cytokine release, volume regulation and migration of lymphocytes (Lewis & Cahalan, 1995; Garber & Cahalan, 1997; DeCoursey & Grinstein, 1999; Cahalan *et al.* 2001; Lewis, 2001). Other leukocytes, if anything, express even more types of ion channel than lymphocytes (DeCoursey & Grinstein, 1999). The expression of ion channels is often up- or downregulated in various functional states of the cells.

In this study we demonstrate for the first time the presence of voltage-activated proton currents in human leukaemic Jurkat T cells and in human peripheral blood B and T lymphocytes. The existence of H^+ channels in pig and human lymphocytes was proposed previously on the basis of arachidonic acid-stimulated, Zn^{2+}-sensitive pH changes (Káldi *et al.* 1994, 1996). In fact, if one accepts the proposal that the $gp91^{phox}$ component of NADPH oxidase is itself a voltage-gated proton channel (Henderson *et al.* 1995), then B lymphocytes must express these channels, because B lymphocytes and B lymphocyte-derived cell lines can generate the superoxide anion, O_2^-, evidently by an nicotinamide adenine dinucleotide phosphate (NADPH) oxidase similar to that in phagocytes (Volkman *et al.* 1984; Maly *et al.* 1988; Hancock *et al.* 1990; Leca *et al.* 1990; Leca *et al.* 1991). We characterise the voltage-gated proton currents in Jurkat cells. We also describe alterations in channel expression and properties after stimulation with PMA (phorbol 12-myristate 13-acetate), which stimulates lymphocytes to express cytokine receptors and to proliferate (Mastro, 1983; Aman *et al.* 1984; Suzuki & Cooper, 1985).

In other leukocytes, the function of proton channels is well established. The NADPH oxidase enzyme complex that produces microbicidal reactive oxygen species is electrogenic, and H^+ efflux through voltage-gated proton channels compensates for this charge translocation (Henderson *et al.* 1987, 1988). H^+ channel inhibition by polyvalent cations blunts the respiratory burst and the generation of reactive oxygen species (Henderson *et al.* 1988; Lowenthal & Levy, 1999). The proton currents in B lymphocytes and Jurkat cells are large, whereas those in T lymphocytes are quite small. Thus, voltage-gated proton channels are highly expressed in the cells that are capable of superoxide anion production, B lymphocytes (Volkman *et al.* 1984; Maly *et al.* 1988; Leca *et al.* 1990, 1991) and

Jurkat cells (Benichou *et al.* 1989; Gulbins *et al.* 1996), but sparingly expressed in T lymphocytes which lack this capability (van Reyk *et al.* 2001). We show here that the levels of H^+ channel expression in human B lymphocytes and in Jurkat cells are quantitatively more than adequate to sustain NADPH oxidase activity in these cells.

METHODS

Cells

Jurkat T cells. The human T leukaemia cell line Jurkat E6-1 was obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). Jurkat T cells were grown in RPMI 1640 medium (GibcoBRL, Eggenstein, Germany) supplemented with 10 % fetal bovine serum and 2 mM glutamine at densities of $1–9 \times 10^5$ in a 37 °C humidified incubator with 5 % CO_2, and split three times weekly.

Human lymphocytes. Venous blood was drawn from healthy adult volunteers after informed written consent was obtained according to procedures approved by the local Institutional Review Board and in accordance with Federal regulations. Lymphocytes were obtained from the whole blood of the donors. The suspension was layered on top of ficoll-hypaque (Amersham-Pharmarcia, Freiburg, Germany) after dilution with an equal volume of RPMI 1640 (GibcoBRL, Eggenstein, Germany). After centrifugation (30 min, 500 *g*, room temperature), mononuclear cells were harvested from the interphase, washed twice with low-speed centrifugation (200 *g*, 10 min, 20 °C) to remove platelets, and resuspended at 1.5×10^8 ml^{-1} in degassed RPMI1640 containing 0.5 % bovine serum albumin and 2 mM EDTA. B cells were separated by negative depletion using the MACS B cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturer's instructions. Briefly, after blocking Fc receptors with aggregated human immunoglobulin, cells were incubated with murine hapten-conjugated monoclonal antibodies directed against human CD2, CD4, CD11b, CD16, CD36 and anti-IgE at 10 °C for 10 min, washed twice, and then incubated with anti-hapten microbeads at 10 °C for 15 min. The washed cell suspension was then placed on a LS+/VS+ column (Miltenyi), and the unlabelled cells passing through were collected from the effluent. This fraction consisted of 97 % CD19+ cells, as judged by flow cytometry after staining with fluoresceinisothiocyanate-conjugated Leu-12 monoclonal antibody (Becton Dickinson, Heidelberg, Germany). Subsequently, the LS+/VS+ column was placed outside the magnetic field, and the retained cells were eluted. This B cell-depleted fraction contained 80–90 % of CD3+ cells as judged by flow cytometry after staining with fluoresceinisothiocyanate-conjugated anti-CD3 monoclonal antibody (Dako, Hamburg, Germany).

In some experiments, Jurkat T cells, CD3+ T lymphocytes and CD19+ B lymphocytes were stimulated with 50 ng ml^{-1} PMA, and H^+ currents were measured 24 h after PMA treatment.

Electrophysiological recordings

Whole-cell recordings. Whole-cell membrane currents were measured using an EPC-9 patch-clamp amplifier (HEKA, Lambrecht/Pfalz, Germany). The amplifier was interfaced to an IBM computer for pulse application and data recording. Series resistance compensation was routinely used to reduce the effective series resistance by about 70 %. Patch electrodes of 5–8 MΩ were fabricated on a two-stage puller (Narishige PP-83, Tokyo, Japan) from borosilicate glass (o.d. 1.5 mm and i.d. 1 mm; Hilgenberg, Malsfeld, Germany). For K^+ current recordings, electrodes were filled with solution I_1 which contained (mM): 120 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 Hepes, 11 EGTA. This solution was adjusted to pH 7.3 with KOH. The extracellular solution E_1 contained (mM): 130 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 Hepes, 8 D-glucose. The pH of the extracellular solutions was adjusted to 7.4 with NaOH. For measurements of isolated H^+ currents, the following solutions were used: I_2 (mM): 80 $TMAMeSO_3$, 100 Mes, 2 $MgCl_2$, 1 BAPTA. This solution was adjusted to pH 6.0 with TMAOH. When the intracellular solution I_2 was adjusted to pH 7.0, the buffer Hepes (100 mM) was used instead of Mes. The extracellular solution E_2 contained 80 mM $TMAMeSO_3$, 2 mM $CaCl_2$, and 100 mM Mes (at pH_o 5.5, 6.0 and 6.5) or 100 mM Hepes (at pH_o 7.0 and 7.5) or 100 mM Tricine (at pH_o 8.0). Except for H^+ current measurements of the influence of Zn^{2+} or Cd^{2+}, 1 mM EGTA was added to the extracellular solution. (In the metal studies, the control solution also had no EGTA.) In order to study the effects of Zn^{2+} or Cd^{2+} on H^+ currents, $ZnCl_2$ (10, 50 μM) or $CdCl_2$ (10, 50 μM) was added to the extracellular solution E_2. The pH of the extracellular solutions was adjusted with TMAOH. Measurements of solutions similar to those used here revealed that there was very little liquid junction potential between them (Cherny *et al.* 1995). This is understandable as the constituents of the solutions were identical except for the buffer used, and the buffers used probably have similar diffusion coefficients. Therefore no liquid junction potential correction has been applied. During all recordings a four-barrel microperfusion pipette was used, which was positioned at a distance of about 30–50 μm from the recorded cell to permit a rapid and permanent exchange of solutions outside the cell. The flow rate was adjusted by hydrostatic pressure. All recordings were made at room temperature (20–23 °C). Whole-cell H^+ currents were filtered at 3 kHz and stored on computer disk for subsequent analyses. Analyses were performed on IBM computers with the Pulse/PulseFit program (HEKA, Lambrecht/Pfalz, Germany). Data are presented as mean values ± standard error of the mean (S.E.M.). The numbers of experiments are indicated, *n*. If not stated otherwise, all chemicals were obtained from Sigma, Germany. The statistical significance of differences between experimental groups was evaluated by Student's *t* test using the SPSS program. Differences were considered to be statistically significant at $P < 0.05$.

Recordings from inside-out patches. Inside-out patches of Jurkat T cells were formed by lifting the pipette briefly into the air. In these measurements, pipettes were filled with the extracellular solution (E_2) at pH_o 7.5. The bath contained intracellular solution (I_2) at pH_i 6.0. In some experiments, the intracellular solution I_2 additionally contained 0.647 mM $CaCl_2$ in order to obtain a free Ca^{2+} concentration of 1 μM. The total Ca^{2+} concentration was calculated using the program WINMAXC (Chris Patton, Stanford University, CA, USA).

Determination of H^+ current density. Cells were depolarised for 4 s to various test potentials, and amplitudes of the corresponding H^+ currents were measured at the end of the pulse. H^+ current amplitude was determined following leak current subtraction. The leak conductance was estimated by measuring the current evoked by a −20 mV pulse from the holding potential, where no time-dependent current was present. Leak current was subtracted from each H^+ current recording assuming an ohmic leak conductance. In order to estimate H^+ current density, cell surface area was evaluated as cell capacitance, which was read from the C_{slow} dial on the EPC-9 amplifier. We determined a mean value for C_{slow} of 1.68 ± 0.07 pF ($n = 79$) for peripheral blood B lymphocytes, 1.56 ± 0.07 pF ($n = 77$) for peripheral blood T

lymphocytes and 5.79 ± 0.26 pF (n = 52) for Jurkat T cells.

Control of pH_i by pipette buffer. It is clearly important to control pH when studying proton currents. Three types of evidence suggest that pH_i was well controlled in this study. Firstly, the time constant of diffusional equilibration of substances in the patch pipette into a cell studied in whole-cell configuration has been determined empirically (Pusch & Neher, 1988). Based on the least favourable assumptions, i.e. with an 8 MΩ pipette resistance, the time constant of equilibration of buffer (Hepes or Mes) into the largest cells studied, Jurkat, is < 30 s. For human B and T lymphocytes, which are substantially smaller, the calculated equilibration time constant is < 5 s. Secondly, direct measurement of the time course of pH_i changes in the much larger (~15 pF) macrophages studied in whole-cell configuration with 100 mM buffer indicated a time constant of ~1 min (Kapus *et al.* 1993). Finally, direct measurement of V_{rev} (Fig. 2) shows that pH_i deviates at most by ~0.4 unit with a 2.0 unit applied pH gradient, ΔpH (= pH_o − pH_i), and the deviation is less at smaller ΔpH.

RESULTS

Depolarisation-induced outward currents in Jurkat T cells

When leukaemic Jurkat T cells were studied in solutions containing physiological ions they exhibited voltage-gated outward currents during depolarising pulses (Fig. 1*A*). Transient outward K^+ currents were activated at potentials of −20 mV or more positive. The current reached its maximum amplitude within tens of milliseconds and then inactivated with a time constant of about 600 ms at potentials positive to 0 mV. The underlying voltage-gated K^+ channels in Jurkat T cells have been studied extensively, and have been described elsewhere (for review, see Lewis & Cahalan, 1995).

In addition to the transient K^+ current, a slowly activating outward current component was detected in Jurkat T cells at depolarising potentials more positive than +20 mV at 'physiological' pH, pH_o 7.4 and pH_i 7.3 (Fig. 1*A*). Slowly activating outward currents were observed more clearly in Jurkat T cells when K^+ was omitted from the intracellular solution by equimolar substitution with TMA^+ (Fig. 1*B*). These currents were unaffected by substitution of extracellular Cl^- with $MeSO_3^-$, suggesting that the voltage-gated slowly activating outward current in Jurkat T cells was carried neither by K^+ nor by Cl^-.

Permeability of slowly activating outward currents in Jurkat T cells

We suspected that the slowly activating outward current in Jurkat T cells was carried by protons. In order to minimise changes in pH_i and pH_o during the course of the measurements, intracellular and extracellular solutions contained 100 mM buffer in all of the following measurements (solutions I_2 and E_2). In order to examine H^+ selectivity of the slowly activating outward currents, the reversal potential, V_{rev}, was estimated by measuring tail currents. A 2 s depolarising prepulse was applied to activate the H^+ conductance, and tail currents were observed upon repolarisation to a range of test potentials. Determination of V_{rev} at pH_o 6.0, 7.0, 7.5 and 8.0 is illustrated in Fig. 2 *A*–*D*, all in the same cell at pH_i 6.0. The average V_{rev} from similar experiments is plotted against the pH gradient (ΔpH = pH_o − pH_i) in Fig. 2*E*. The continuous line calculated by linear regression indicates a 47 mV shift in the reversal potential for a ten-fold change in H^+ concentration. This slope is close to the Nernst value of 58 mV (dashed line). Since V_{rev} was near the Nernst potential and varied over a 100 mV range when pH was changed and other ions were kept constant, these channels are highly selective for H^+. The deviation of V_{rev} from the Nernst potential for H^+ at ΔpH = 2.0 reflects a permeability ratio, $P_H/P_{TMA} > 5 \times 10^6$, calculated with the Goldman–Hodgkin–Katz voltage equation (Goldman, 1943; Hodgkin & Katz, 1949). Every V_{rev} value in Fig. 2*E* indicates that the permeability of H^+ relative to TMA^+, $P_H/P_{TMA} > 10^6$.

pH dependence of H^+ current activation in Jurkat T cells

Figure 3 illustrates H^+ currents in the same Jurkat cell studied at pH_i 6.0 and pH_o 6.0, 7.0, 7.5 and 8.0 (*A*, *B*, *C* and *D*, respectively). The threshold for H^+ current activation shifted to more negative potentials and the H^+ current amplitude increased upon alkalinisation of the extracellular solution. H^+ current was activated only at potentials positive to +20 mV at pH_o 6.0, whereas activation occurred at −60 mV at pH_o 8.0.

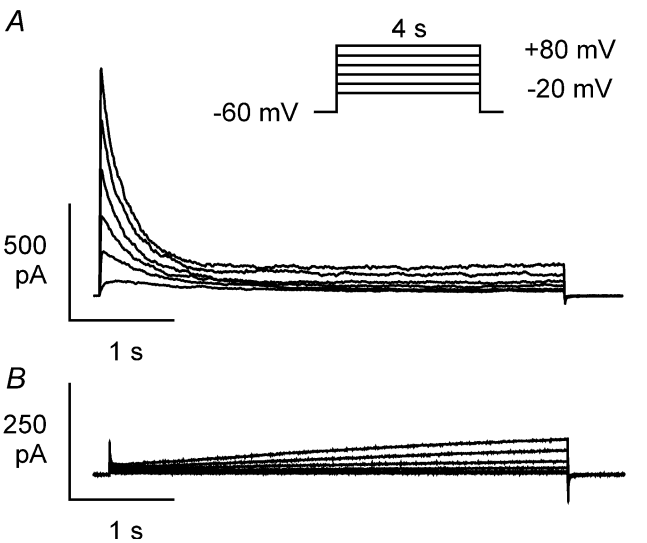

Figure 1. Voltage-gated outward K^+ and H^+ currents in Jurkat T cells at 'physiological' pH

Depolarising pulses lasting 4 s were applied from the holding potential (−60 mV) to potentials between −20 and +80 mV in 20 mV increments at 40 s intervals. *A*, outward currents in a Jurkat T cell measured with K^+-containing pipette solution I_1 and extracellular solution E_1. *B*, slowly activating outward currents measured at pH_o 7.5 and pH_i 7.0 with K^+-free intracellular solution I_2, while the cell was bathed in the extracellular solution E_2. The same family of pulses was applied.

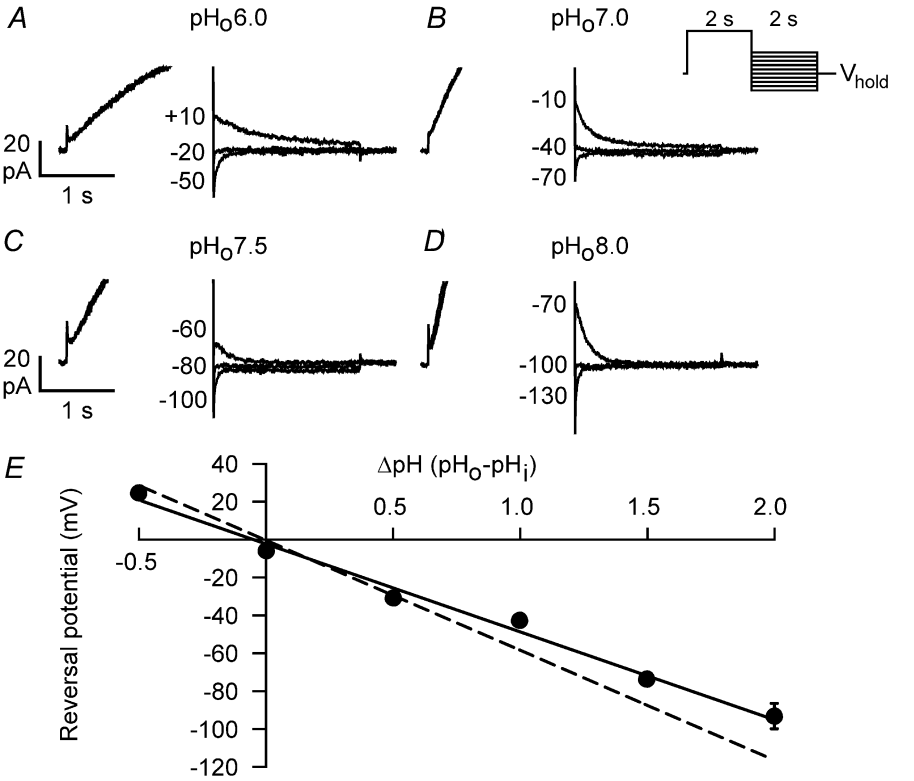

Figure 2. Determination of reversal potentials by tail currents in Jurkat T cells

After depolarising prepulses to +80 mV (*A*) or +60 mV (*B–D*) that activated H^+ current (peak currents are off-scale), the membrane was clamped to various potentials between +10 and −130 mV. Holding potentials (V_{hold}) were −60 mV (*A–C*) or −80 mV (*D*). Selected tail currents are illustrated in *A–D*, as indicated. pH_i was 6.0, while pH_o was changed from 6.0 to 8.0 as indicated. *E*, mean values of V_{rev} (± S.E.M.) at several ΔpH values ($pH_o - pH_i$; n = 5, 6, 10, 4, 10 and 3 for ΔpH of −0.5, 0, 0.5, 1, 1.5 and 2, respectively). Data were fitted by a continuous line with a slope of −47 mV (pH unit)$^{-1}$. The Nernst potential for H^+ has a slope of −58 mV (pH unit)$^{-1}$ (dashed line).

Average current–voltage relationships for H^+ currents measured at the end of the 4 s test pulses like those in Fig. 3 are shown in Fig. 4. Measurements were performed at pH_o 6.0, 7.0, 7.5 or 8.0, both at pH_i 6.0 (Fig. 4*A*) and at pH_i 7.0 (Fig. 4*B*). The corresponding conductance–voltage curves are shown in Fig. 4*C* and *D*. As demonstrated in Fig. 4, proton currents in Jurkat T cells were profoundly sensitive to both extracellular and intracellular pH. H^+ currents studied at pH_i 6.0 were larger and the activation threshold was roughly 40 mV more negative than at pH_i 7.0. However, the activation threshold of H^+ currents studied at the same ΔpH was similar. When ΔpH was 0 (◆), H^+

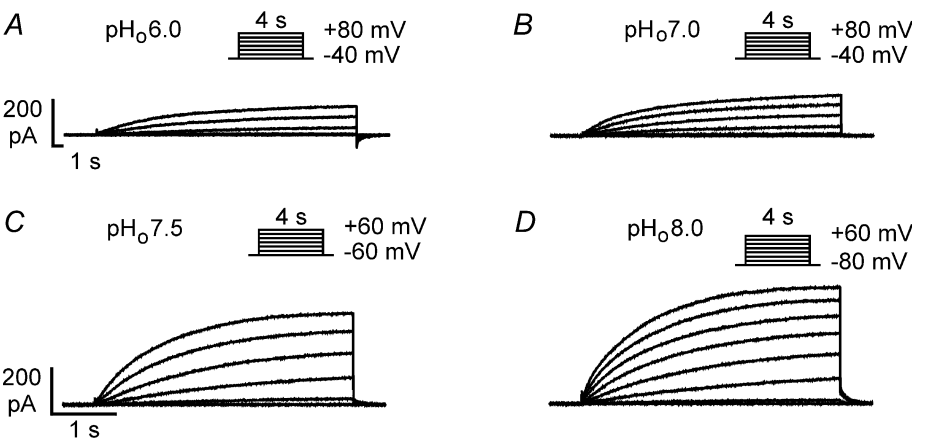

Figure 3. Families of outward H^+ currents in Jurkat T cells at several pH_o values

H^+ currents were measured at pH_i 6.0 and at pH_o as indicated. Currents were elicited by 4 s voltage pulses in 20 mV increments with a 40 s interval between pulses.

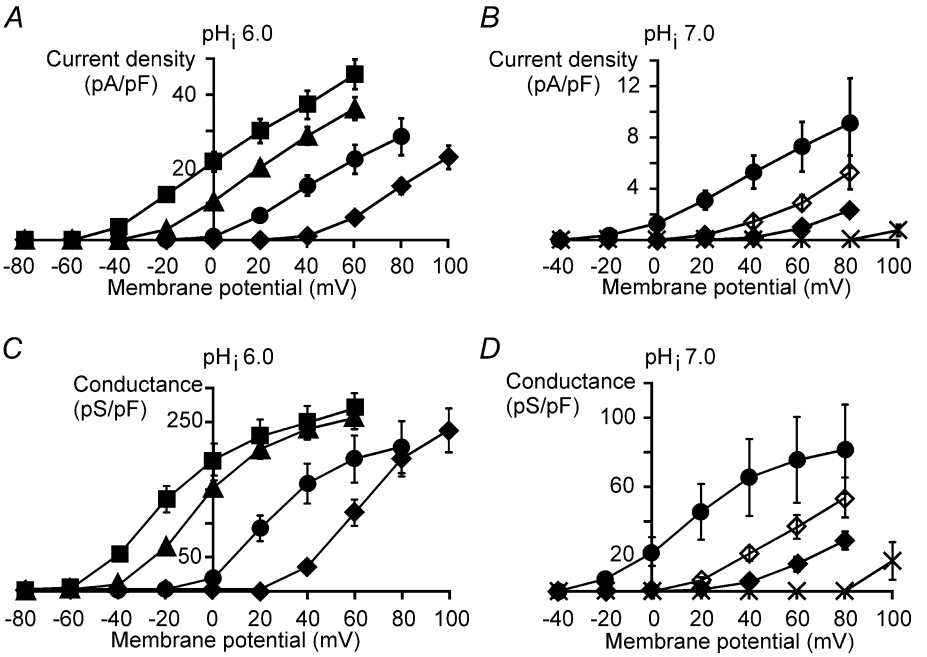

Figure 4. Effects of pH$_i$ and pH$_o$ on the H$^+$ current–voltage and H$^+$ conductance–voltage relationships in Jurkat T cells

A, H$^+$ current densities (± S.E.M.) determined at pH$_i$ 6.0 and pH$_o$ 6.0 (◆, $n = 15$), 7.0 (●, $n = 6$), 7.5 (▲, $n = 52$) or 8.0 (■, $n = 14$). *B*, H$^+$ current densities (± S.E.M.) at pH$_i$ 7.0 and pH$_o$ 6.0 (×, $n = 4$), 7.0 (◆, $n = 7$), 7.5 (◇, $n = 16$) or 8.0 (●, $n = 4$). *C* and *D*, H$^+$ chord conductance–voltage relationships calculated from the data in *A* and *B*, respectively, assuming reversal at E_H. Measurements at identical ΔpH are illustrated using identical symbols (◆: ΔpH = 0; ●: ΔpH = 1).

currents activated positive to +20 mV, while the activation threshold was about −20 mV for H$^+$ currents at ΔpH of 1 (●). In general, the activation threshold was close to the empirical formula:

$$V_{\text{threshold}} = 20 - 40\,(\text{pH}_o - \text{pH}_i), \qquad (1)$$

where $V_{\text{threshold}}$ is the activation threshold of the proton current in mV (Cherny *et al.* 1995).

The H$^+$ chord conductance g_H appeared to approach a similar limiting maximal value at all values of pH$_o$ studied at pH$_i$ 6.0 (Fig. 4*C*). The voltage range of the measurements did not permit a similar comparison at pH$_i$ 7.0 (Fig. 4*D*). The H$^+$ current density at pH$_i$ 6.0 was significantly larger than at pH$_i$ 7.0 even when compared at identical ΔpH values (Fig. 4*C vs.* 4*D*). At pH$_i$ 6.0, the mean H$^+$ current density at +80 mV was three times larger than at pH$_i$ 7.0 ($P < 0.05$) when ΔpH was 1 unit (●).

Time-dependent activation of H$^+$ currents in Jurkat T cells

Figure 5 illustrates time-dependent activation of proton currents in Jurkat T cells studied at different values of pH$_o$, all at pH$_i$ 6.0. H$^+$ currents were activated with a sigmoid time course that was slow compared with most other ion channels. Activation became faster with increasing

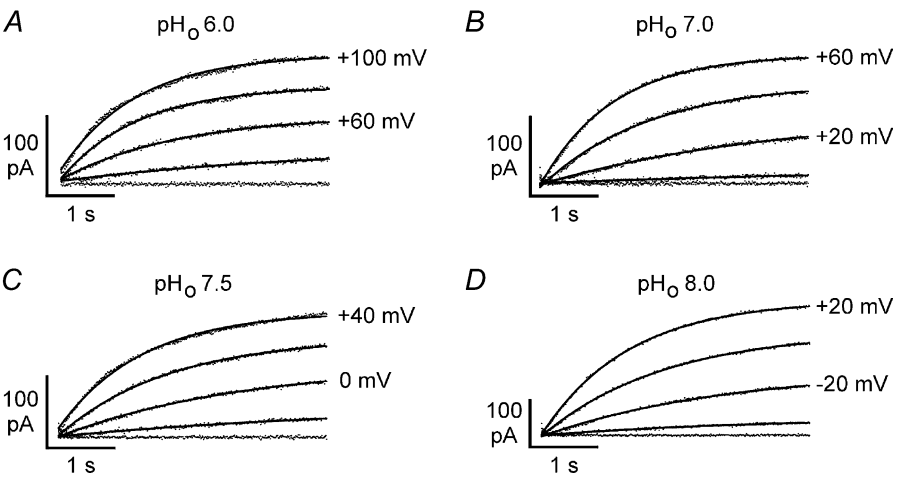

Figure 5. Time dependence of H$^+$ current activation in Jurkat T cells

The time course of activation of H$^+$ currents was fitted mono-exponentially after a brief delay. *A–D*, H$^+$ currents were evoked by 4 s pulses in 20 mV increments to the potentials indicated. The extracellular pH was 6.0 (*A*), 7.0 (*B*), 7.5 (*C*) or 8.0 (*D*) and pH$_i$ was 6.0. The continuous curves show the best fitting single exponential to the activation time course, at potentials which evoked H$^+$ current.

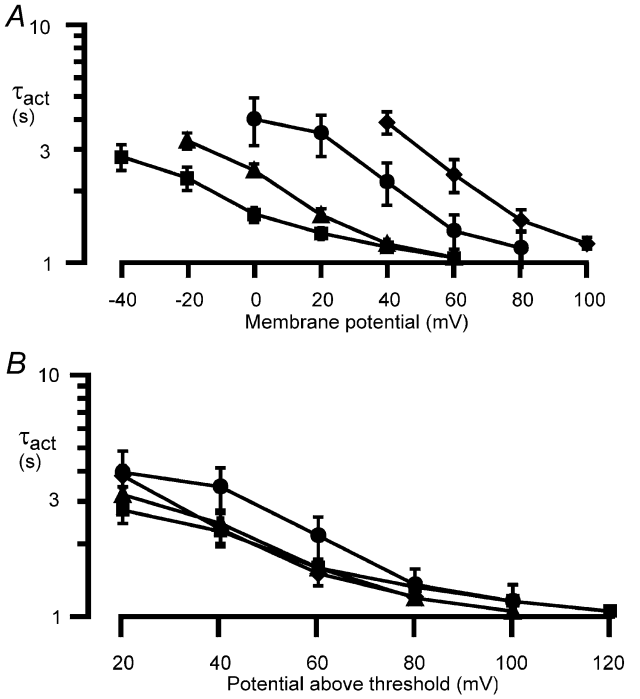

Figure 6. Mean activation time constant, τ_{act}, of H^+ currents in Jurkat T cells

H^+ current activation time constants (± S.E.M.) were determined at pH_i 6.0 and pH_o 6.0 (◆; $n = 6$), 7.0 (●; $n = 6$), 7.5 (▲; $n = 44$) or 8.0 (■; $n = 9$). Cells were depolarised for 4 s to potentials between −40 and +100 mV. *A*, τ_{act} plotted as a function of membrane potential. *B*, τ_{act} re-plotted as a function of the potential above the theoretically determined activation threshold (see text for details).

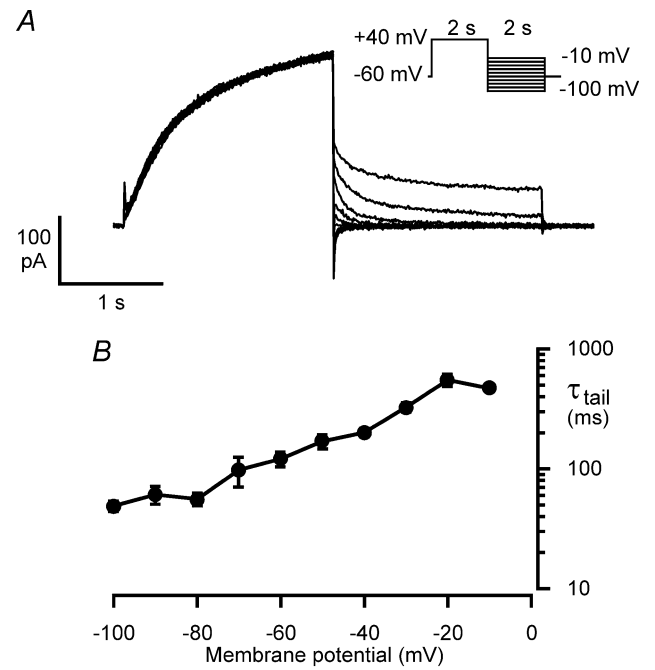

Figure 7. Deactivation of H^+ currents in Jurkat T cells

A, tail currents at pH_i 6.0 and pH_o 7.5 elicited by 2 s voltage pulses to potentials between −10 and −100 mV following a depolarising prepulse to +40 mV. *B*, voltage dependence of tail current time constants, τ_{tail}, at pH_i 6.0 and pH_o 7.5, obtained by fitting the tail current by a single exponential.

membrane depolarisation, and could be fitted fairly well by a single exponential function (continuous lines in Fig. 5). Average activation time constants of H^+ currents, τ_{act}, are shown in Fig. 6. The τ_{act} of H^+ currents depended on voltage and pH_o, and was faster at higher pH_o and at more positive voltages. The mean τ_{act} at +60 mV was 2.28 ± 0.36 s ($n = 6$), 1.34 ± 0.21 s ($n = 6$), 1.04 ± 0.03 s ($n = 44$) and 1.03 ± 0.05 s ($n = 9$) for proton currents at pH_o values of 6.0, 7.0, 7.5 and 8.0, respectively.

As alkalinisation of the external solution shifts $V_{threshold}$ in a negative direction (Fig. 4), we replotted τ_{act} relative to $V_{threshold}$, calculated according to eqn (1). This procedure effectively 'corrects' for the shift in the g_H–V relationship. As demonstrated in Fig. 6*B*, to a first approximation, τ_{act} and $V_{threshold}$ were shifted equally by changes in pH_o.

Deactivation of H^+ currents in Jurkat T cells

To characterise the time dependence of H^+ current deactivation, Jurkat T cells at pH_o 7.5 and pH_i 6.0 were depolarised to +40 mV, and subsequently stepped to potentials between −100 mV and −10 mV for 2 s, as illustrated in Fig. 7*A*. The time constant of deactivation, τ_{tail}, was obtained by fitting the tail current with a single exponential. The fit was excellent at all subthreshold voltages, but a slower component became evident at more

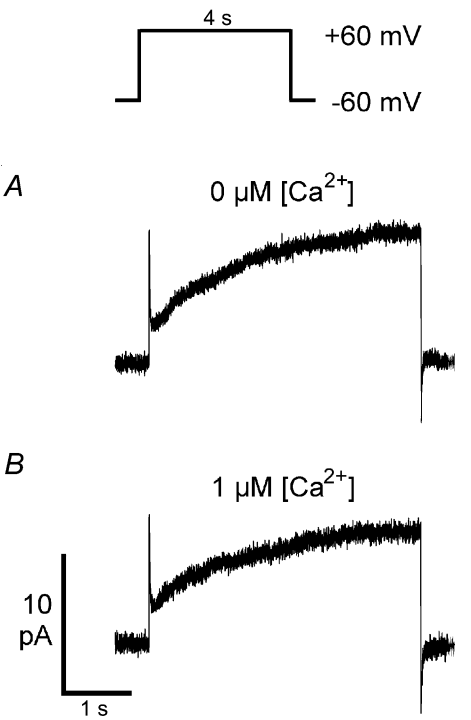

Figure 8. Insensitivity of H^+ currents in Jurkat T cells to $[Ca^{2+}]_i$

H^+ currents in an inside-out patch from a Jurkat T cell were evoked by 4 s pulses from −60 to +60 mV. The pipette contained solution E_2 at pH 7.5. The bath contained solution I_2 at pH 6.0, which was either nominally Ca^{2+} free (*A*, 0 μM $[Ca^{2+}]$) or contained 1 μM free Ca^{2+} (*B*).

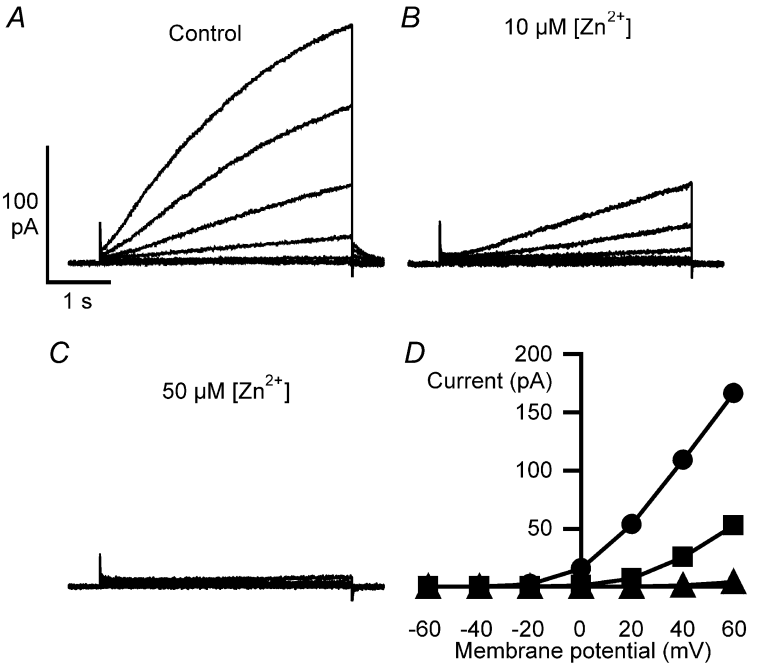

Figure 9. Effects of Zn^{2+} on H^+ currents in a Jurkat T cell

Families of H^+ currents from the same cell evoked by 4 s voltage steps from the holding potential (−60 mV) up to +60 mV in 20 mV increments in the same cell are shown before (*A*) and during superfusion with an extracellular solution containing 10 μM Zn^{2+} (*B*), or 50 μM Zn^{2+} (*C*). *D*, amplitude of H^+ currents (shown in *A*–*C*) at the end of the 4 s pulse in the absence (●) or presence of 10 μM (■) or 50 μM (▲) Zn^{2+}.

positive voltages where the tail current did not decay fully (e.g. at −10 mV in Fig. 7*A*). As shown in Fig. 7*B*, τ_{tail} varied exponentially with voltage, and was slower at more positive voltages. On average τ_{tail} changed e-fold in 26 ± 4 mV (n = 13).

Ca^{2+} dependence of H^+ currents in Jurkat T cells

The intracellular calcium concentration, $[Ca^{2+}]_i$ is an important signalling mechanism in many cells, including lymphocytes. We explored the sensitivity of H^+ currents in Jurkat T cells to $[Ca^{2+}]_i$ in inside-out patches. Figure 8 illustrates currents elicited by 4 s voltage steps from −60 to +60 mV, in a membrane patch bathed in Ca^{2+}-free solution (Fig. 8*A*) or in solution containing 1 μM Ca^{2+} (Fig. 8*B*). The intracellular pH was 6.0, while the extracellular pH was 7.5 in these experiments. H^+ currents (I_H) did not increase in amplitude when $[Ca^{2+}]_i$ was increased

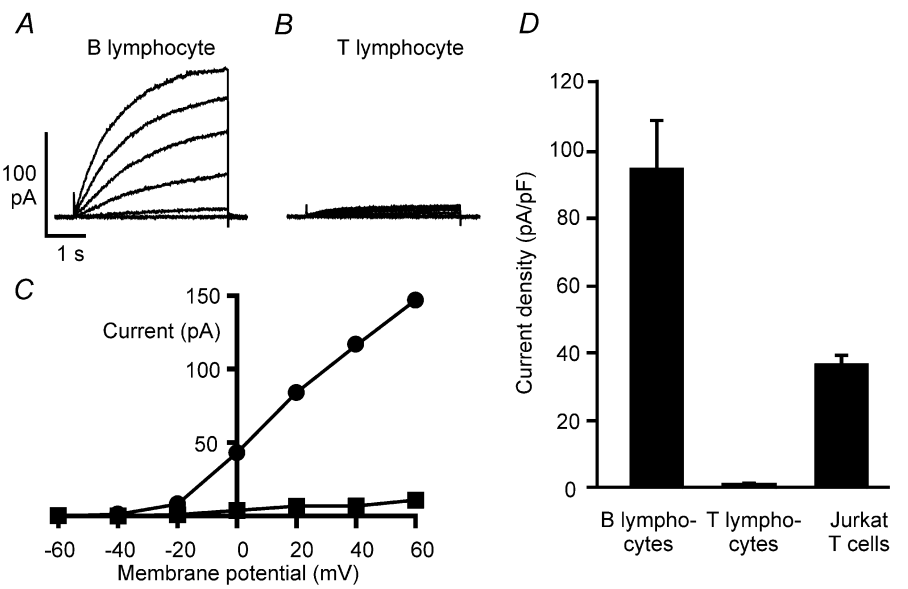

Figure 10. H^+ currents in human peripheral blood lymphocytes

Superimposed H^+ currents in a CD19+ B lymphocyte (*A*) and a CD3+ T lymphocyte (*B*) elicited by identical families of 4 s voltage pulses from the holding potential of −60 mV to potentials between −40 and +60 mV, both at pH_i 6.0 and pH_o 7.5. *C*, current–voltage relationships for H^+ currents at the end of the pulses shown in *A* and *B* (●, B lymphocyte; ■, T lymphocyte). *D*, average (± S.E.M.) H^+ current densities in lymphocyte subpopulations. H^+ current densities were calculated from the H^+ current measured at the end of a 4 s pulse to +60 mV, and normalised to the capacity in each cell. Data are from 63 human B lymphocytes, 46 human T lymphocytes, and 52 Jurkat T cells at pH_i 6.0 and pH_o 7.5.

from nominally Ca^{2+}-free to 1 μM (Fig. 8). The average ratio I_H(1 μM Ca^{2+})/I_H(Ca^{2+}-free) was 0.90 ± 0.04 (n = 10), when the H^+ current was measured at the end of pulses to +60 mV before and after the solution change. Intriguingly, during measurements in inside-out patches from Jurkat T cells, H^+ currents exhibited rundown behaviour, which was not observed in whole-cell recordings. The H^+ current amplitude decreased progressively in either high or low [Ca^{2+}] solutions. The average decrease was 37 ± 6 % (n = 12) during recordings in inside-out patches that lasted on average 12 ± 1 min.

Pharmacological properties of H^+ currents in Jurkat T cells

As in other cells, H^+ currents in Jurkat T cells were potently inhibited by extracellular divalent cations. The effects of 10 μM Zn^{2+} applied to a Jurkat cell at pH_i 6.0 and pH_o 7.5 are shown in Fig. 9. Zn^{2+} reduced the current amplitude, shifted $V_{threshold}$ to more positive voltages on average by 44 ± 6 mV (n = 10), and slowed τ_{act}. In the presence of 50 μM Zn^{2+}, proton currents were not seen during 4 s pulses to potentials less than +60 mV. The H^+ current at the end of a 4 s pulse to +60 mV was reduced by 93 ± 2 % (n = 9) in the presence of 50 μM Zn^{2+}. Qualitatively similar effects on proton currents were observed following extracellular application of Cd^{2+}, although Cd^{2+} was less potent than Zn^{2+}. At 50 μM Cd^{2+}, $V_{threshold}$ was shifted by 26 ± 4 mV (n = 13) in the depolarising direction, leading to a decrease in H^+ current amplitude by 37 ± 6 % (n = 8) when measured at the end of a 4 s test pulse to +60 mV.

H^+ currents in unstimulated human peripheral blood lymphocytes

Voltage-gated proton currents were also studied in freshly isolated human CD3+ T lymphocytes and CD19+ B lymphocytes. Both of these lymphocyte populations express H^+ channels. Figure 10 shows typical H^+ current families in a B lymphocyte (Fig. 10*A*) and in a T lymphocyte (Fig. 10*B*), and the corresponding current–voltage curves (Fig. 10*C*). Intriguingly, the H^+ current amplitudes differed dramatically. Measured at +60 mV with pH_i 6.0 and pH_o 7.5, the average H^+ current amplitude was 170 ± 24 pA (mean ± S.E.M., n = 63) in CD19+ B lymphocytes; 1.46 ± 0.37 pA (n = 46) in CD3+ T lymphocytes and 236 ± 21 pA (n = 52) in Jurkat T cells. Normalised for capacity, the mean H^+ current density was 94.7 ± 14.5 pA pF^{-1} (n = 63) in human B lymphocytes; 0.9 ± 0.2 pA pF^{-1} (n = 46) in human T lymphocytes, and 36.3 ± 2.4 pA pF^{-1} (n = 52) in Jurkat T cells (Fig. 10*D*).

H^+ currents of freshly isolated human B lymphocytes studied at pH_i 6.0 and pH_o 7.5 did not differ in their voltage dependence, gating kinetics, or sensitivity to Zn^{2+} from those of Jurkat T cells. On average, in B lymphocytes $V_{threshold}$ was −20 ± 2.5 mV (n = 47), and shifted by 60 ± 6 mV (n = 5) following application of 50 μM Zn^{2+}. The mean V_{rev} was −7 ± 2 mV (n = 5) at ΔpH 0 and

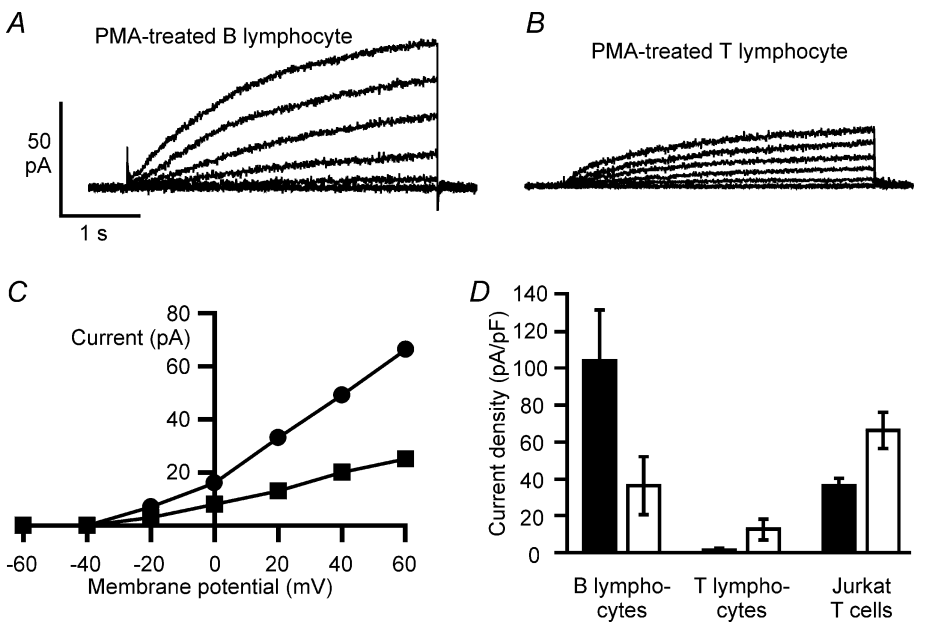

Figure 11. H^+ currents in PMA-stimulated human peripheral blood lymphocytes

Superimposed H^+ current families in a PMA-stimulated CD19+ B lymphocyte (*A*) and a PMA-stimulated CD3+ T lymphocyte (*B*), both at pH_i 6.0 and pH_o 7.5. Currents were elicited by identical 4 s voltage pulses from the holding potential of −60 mV to potentials between −40 and +60 mV. *C*, current–voltage curves from the cells in *A* (●) and B (■). *D*, mean (± S.E.M.) H^+ current densities in lymphocyte subpopulations before and after PMA stimulation. H^+ current density was measured at the end of 4 s pulses to +60 mV at pH_i 6.0 and pH_o 7.5. Unstimulated cells are shown as filled bars, PMA-treated cells as open bars. Numbers of cells are given in the text.

−69 ± 2 mV (n = 9) at ΔpH 1.5, respectively. Due to the extremely small amplitude of H^+ currents in human peripheral blood T lymphocytes, it was difficult to compare their properties with those in Jurkat and other cells.

H^+ currents in PMA-stimulated human peripheral blood lymphocytes

H^+ channel expression in lymphocytes changed markedly 24 h after stimulation with 50 ng ml^{-1} PMA. In Fig. 11, H^+ current records in PMA-stimulated B and T lymphocytes are illustrated. We compared the amplitude of H^+ currents in PMA-stimulated and unstimulated lymphocytes from the same donors (thus the numbers of cells were smaller than above). In PMA-stimulated peripheral blood T lymphocytes, the H^+ current amplitude was 13 times larger than in untreated cells, reaching a mean value of 32 ± 11 pA (n = 16) when measured at +60 mV. In contrast, the mean amplitude in PMA-stimulated B lymphocytes was decreased by 54 % (90 ± 33 pA, n = 19) in comparison with untreated cells (193 ± 49 pA, n = 24). As T and B lymphocytes increased in size by about 30 % following stimulation with PMA, H^+ currents were normalised to cell capacity. The H^+ current density at +60 mV increased 8.4-fold, from 1.5 ± 1.0 pA pF^{-1} (n = 19) to 12.6 ± 5.4 pA pF^{-1} (n = 16) in CD3+ T lymphocytes following stimulation with PMA. The mean H^+ current density in Jurkat T cells also increased after treatment with PMA, from 36.3 ± 3.9 pA pF^{-1} (n = 24) to 66.4 ± 9.9 pA pF^{-1} (n = 23). In contrast, the mean H^+ current density of CD19+ B lymphocytes decreased from 104.1 ± 27.6 pA pF^{-1} (n = 24) to 36.3 ± 15.9 pA pF^{-1} (n = 19) after PMA stimulation.

The gating kinetics of H^+ currents in B lymphocytes changed subtly after PMA treatment. Measured at +60 mV, the mean τ_{act} increased significantly ($P < 0.05$) from 0.81 ± 0.14 s (n = 14) in unstimulated human B lymphocytes, to 1.73 ± 0.26 s (n = 14) in PMA-stimulated B lymphocytes. In contrast, the mean τ_{act} of H^+ currents in untreated Jurkat T cells, 1.0 ± 0.06 s (n = 24) was not significantly different after treatment with PMA, 0.87 ± 0.08 s (n = 23).

DISCUSSION

Comparison of H^+ currents in lymphocytes with those of other cell preparations

In the present paper we describe for the first time voltage-gated proton currents in the human leukaemic T cell line Jurkat E6 and in human peripheral blood lymphocytes, including both CD3+ T lymphocytes and CD19+ B lymphocytes. Proton currents in lymphocytes exhibit the main features of proton currents in other mammalian cells (Eder & DeCoursey, 2001), including high selectivity for protons, slow time-dependent activation of outward currents in response to depolarising voltage commands, dependence of gating on voltage, pH_i and pH_o, and sensitivity to divalent cations such as Zn^{2+} and Cd^{2+}. The selectivity of H^+ channels is astounding. Calculated with the Goldman–Hodgkin–Katz voltage equation (Goldman, 1943; Hodgkin & Katz, 1949), the relative permeability of H^+ to TMA+ is > 10^6, based on V_{rev} measurements. Although we show here that voltage-gated proton currents can be observed in lymphocytes in solutions with approximately physiological ionic composition and pH, H^+ currents have not been reported previously in lymphocytes, despite hundreds of patch-clamp studies on these cells. It is most likely that this failure is attributable to the small amplitude of H^+ currents in T lymphocytes, the most frequently studied variety. In B lymphocytes and Jurkat cells, H^+ currents are much larger and can be seen during prolonged depolarising pulses after the omnipresent delayed rectifier K^+ current inactivates. Under normal ionic conditions, voltage-gated proton currents might be mistaken for a 'non-inactivating' component of K^+ current.

H^+ channels can be divided into at least four distinct types, based mainly on differences in activation and deactivation kinetics (DeCoursey, 1998). Within this nomenclature, H^+ currents of lymphocytes resemble the type p variety found in phagocytic leukocytes (DeCoursey & Cherny, 1993; Kapus *et al.* 1993; Gordienko *et al.* 1996; Schrenzel *et al.* 1996) and microglia (Eder *et al.* 1995). Like type p currents, lymphocyte H^+ currents exhibit extremely slow time-dependent activation and deactivation, both of which can be fitted reasonably well by one exponential. In contrast, neuronal (type n) H^+ channels activate three orders of magnitude faster (Byerly *et al.* 1984). In our studies of inside-out patches, increasing $[Ca^{2+}]_i$ had no effect on the amplitude or activation kinetics of H^+ currents in lymphocytes. Neuronal (type n) H^+ channels are similarly insensitive to $[Ca^{2+}]_i$ (Byerly *et al.* 1984). However, the Ca^{2+} sensitivity of type p H^+ channels is controversial. Our observations are in agreement with the lack of Ca^{2+} sensitivity of the H^+ conductance in neutrophils (Nanda & Grinstein, 1995), but in contrast to observations in eosinophils where high $[Ca^{2+}]_i$ modestly enhanced H^+ currents (Gordienko *et al.* 1996; Schrenzel *et al.* 1996). As in alveolar epithelial cells (Cherny *et al.* 1995), the voltage dependence of H^+ current activation in lymphocytes can be predicted from the gradient between pH_o and pH_i, according to eqn (1). Zn^{2+} and Cd^{2+} are effective inhibitors of proton currents in lymphocytes, as in other cells (Thomas & Meech, 1982; Barish & Baud, 1984; Meech & Thomas, 1987; Mahaut-Smith, 1989; DeCoursey & Cherny, 1993; Kapus *et al.* 1993; Eder *et al.* 1995; Gordienko *et al.* 1996; Schrenzel *et al.* 1996; Cherny & DeCoursey, 1999). In one respect, the properties of H^+ currents in Jurkat T cells differed from those reported previously. Lowering pH_i from 7 to 6 tripled the g_H in Jurkat T cells, whereas previous studies have been consistent with a two-fold increase per unit decrease in pH_i

in many types of cells (DeCoursey, 1998). Because, based on V_{rev} measurements (Fig. 2*E*), when pH_i is nominally 6.0 it evidently is closer to 6.2–6.3, the difference in g_H might have been even greater had perfect pH_i control been achieved. Although g_H is not proportional to H^+ concentration, pH_i distinctly affects the H^+ current amplitude.

H^+ channel expression in Jurkat T cells and in peripheral blood lymphocytes

H^+ currents were much larger in B than in T lymphocytes. The average whole-cell H^+ current was 70 times larger in B cells than in T cells, and the H^+ current density was 105 times larger. H^+ currents of human Jurkat T cells were much larger than in human peripheral blood T lymphocytes. The H^+ current when measured at +60 mV, pH_o 7.5 and pH_i 6.0, was 97 times larger in Jurkat T cells than in T lymphocytes, and the H^+ current density was 40 times larger. The higher expression of H^+ channels in Jurkat T cells may reflect a more activated state of these cells in comparison with unstimulated T lymphocytes.

Effects of PMA stimulation on H^+ currents in lymphocytes

Lymphocyte activation is accompanied by an increase in the expression of several ion channels, including voltage-gated K^+ and Na^+ channels (DeCoursey *et al.* 1987; Sutro *et al.* 1989; Lewis & Cahalan, 1995), Ca^{2+}-activated K^+ channels (Grissmer *et al.* 1993; Ghanshani *et al.* 2000), and calcium release-activated calcium (CRAC) channels (Fomina *et al.* 2000). Our experiments in T lymphocytes are consistent with this pattern, because the H^+ current density increased 8-fold following stimulation with PMA. H^+ currents were upregulated 6-fold in HL-60 cells during differentiation into granulocytes (Qu *et al.* 1994). The implication is that T lymphocytes have a much greater need for H^+ channels after activation.

The phorbol ester PMA had opposite effects on human B and T lymphocytes. H^+ current amplitude and current density decreased after PMA stimulation of B lymphocytes. A decrease in H^+ current density was observed previously during macrophage differentiation induced by PMA (DeCoursey & Cherny, 1996) and in microglial cells following activation with lipopolysaccharide or treatment with astrocyte-conditioned medium (Klee *et al.* 1999). A possible explanation for the different effects of PMA on H^+ currents in T and B lymphocytes is that PMA differentially modulates the expression and activation of protein kinase C (PKC) isoforms in both cell populations. For example, high levels of PKC-θ mRNA were found in human T lymphomas and normal T lymphocytes, but lower PKC-θ mRNA levels were detected in B lymphocytes (Mischak *et al.* 1993). PKC-θ mRNA is the major PKC isoform expressed in T lymphocytes, whereas the B cell phenotype is associated with high expression of PKC-θ (Mischak *et al.* 1991). Whatever the signalling pathway, PMA reduced the differences in H^+ channel expression among lymphocyte populations.

Possible functions of H^+ currents in lymphocytes

The main role of voltage-gated proton channels in phagocytes is to mediate H^+ efflux during the respiratory burst (DeCoursey & Grinstein, 1999, Eder & DeCoursey, 2001). The NADPH oxidase produces superoxide anions extracellularly by transporting electrons across the plasma (or phagosome) membrane. The continued function of this electrogenic enzyme requires counterion flux, and most or all of this flux is mediated by H^+ (Henderson *et al.* 1987; Henderson *et al.* 1988; Nanda & Grinstein, 1991; DeCoursey & Cherny, 1993; Reeves *et al.* 2002). The patterns of expression of voltage-gated proton channels in lymphocytes are compatible with this function. T lymphocytes apparently do not generate significant amounts of superoxide anion (van Reyk *et al.* 2001), and in fact may not even express the required components of NADPH oxidase (Káldi *et al.* 1996). Apparent increases in intracellular reactive oxygen species (ROS) levels in activated T lymphocytes were attributed to ROS generation by cells other than T lymphocytes in heterogeneous cell preparations (van Reyk *et al.* 2001). The low level of H^+ channel expression in T lymphocytes is compatible with the absence of metabolic stress in the resting state. However, the 8-fold upregulation in PMA-stimulated T lymphocytes suggests that H^+ channels may become functionally more important in processes of lymphocyte activation. H^+ channels may contribute to the stabilisation of an alkaline pH_i during antigen presentation, proliferation and cytokine release of T lymphocytes. Intracellular acidification has been shown to inhibit interleukin-2 (IL-2) release in T lymphocytes (Bental & Deutsch, 1994), which may subsequently lead to inhibition of IL-2-dependent proliferation. Following activation with mitogens, significant increases in pH_i have been reported in a variety of T lymphocyte preparations and T cell lines (Gerson *et al.* 1982; Bental & Deutsch, 1994). Functional H^+ channels thus may contribute to the observed intracellular alkalinisation in activated T lymphocytes.

B lymphocytes and Jurkat cells expressed much larger H^+ currents than T lymphocytes. Human peripheral blood B lymphocytes are capable of generating the superoxide anion upon cross-linking of surface antigens such as IgM, IgD, IgG, HLA-DR and CD19, or following treatment with PMA or pansorbin (Volkman *et al.* 1984; Maly *et al.* 1988; Leca *et al.* 1990, 1991). Generation of ROS by Jurkat T cells was observed following treatment with the Fas antigen (Gulbins *et al.* 1996) or with several mitogens (Benichou *et al.* 1989). Voltage-gated H^+ channels may be better suited than voltage-gated K^+ channels to repolarise the membrane during superoxide anion generation in B lymphocytes and Jurkat T cells, because the Kv1.3 channels expressed in both Jurkat T cells and B lymphocytes are inhibited by

reactive oxygen species (Duprat *et al.* 1995; Szabo *et al.* 1997) and during intracellular acidification (Deutsch & Lee, 1989). Furthermore, K^+ efflux would have severe osmotic consequences (Reeves *et al.* 2002) producing cell shrinkage, whereas H^+ efflux that balances NADPH oxidase-mediated electron efflux would not.

Are the levels of expression of voltage-gated proton channels adequate to compensate for NADPH-mediated electron flux?

The rate of superoxide anion production by human B lymphocytes stimulated with PMA is 12 times smaller than that in adherent monocytes (Leca *et al.* 1991). Human monocytes stimulated with PMA produce O_2^- at a rate equivalent to −1.7 pA of electron current (Tarsi-Tsuk & Levy, 1990), thus the NADPH oxidase-generated electron current would be −0.14 pA in human B lymphocytes. Activation of even a small fraction of the H^+ conductance in B cells would be enough to compensate this small electron current. The next question is whether the g_H would be activated in an intact cell. Depolarisation of human neutrophils reaches +58 mV during the respiratory burst (Jankowski & Grinstein, 1999), which is certainly sufficient to activate the g_H. Although analogous measurements have not been made in B lymphocytes, the fact that the NADPH oxidase is electrogenic (Henderson *et al.* 1987) means that it will depolarise the membrane unless the charge is compensated. Calculated from the rate of superoxide anion production just discussed, and the average capacity of B lymphocytes, 1.68 pF, the activity of NADPH oxidase would depolarise the membrane at a rate of 83 mV s^{-1}, or 5 V in one minute. Obviously this charge separation must be compensated or the oxidase would shut down within a few seconds. Equally obviously, voltage-gated proton channels would be activated massively within a few seconds if there were no other mechanism available to compensate charge. Protons mediate the charge compensation during the respiratory burst in all other leukocytes that have been studied (Henderson *et al.* 1987; 1988; Nanda & Grinstein, 1991; DeCoursey & Cherny, 1993; Reeves *et al.* 2002). This can be understood teleologically because a sustained respiratory burst compensated by any other ion would result in severe osmotic consequences. The ubiquitous Na^+–H^+-antiporter is electroneutral and cannot compensate charge. We are unaware of any evidence that H^+-ATPase exists in the plasma membrane of human lymphocytes. The hypothesis that voltage-gated proton channels serve this function in lymphocytes therefore seems quite reasonable. In summary, the levels of expression of voltage-gated proton channels in lymphocytes are entirely consistent with their function in sustaining the respiratory burst.

The superoxide-generating system of B lymphocytes, NADPH oxidase, appears to be identical to that in phagocytes (Maly *et al.* 1988; Hancock *et al.* 1989; Cohen-Tanugi *et al.* 1991). Intracellular ROS are able to modulate activation and differentiation processes of lymphocytes. Thus, ROS might serve as intracellular messengers mediating the activation of the transcription factor nuclear factor kappa B (NFκB) and thereby the expression of genes involved in inflammatory and immune responses (Schreck *et al.* 1991). Since H^+ channel inhibition blunts ROS generation, the use of drugs specifically acting on H^+ channels may serve as a good tool to modulate ROS generation and subsequent activation processes of lymphocytes.

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