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Cl^- Channels in Intact Human T Lymphocytes

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Summary. We recently described a large, multiple-conductance Cl^- channel in excised patches from normal T lymphocytes. The properties of this channel in excised patches are similar to maxi-Cl^- channels found in a number of cell types. The voltage dependence in excised patches permitted opening only at nonphysiological voltages, and channel activity was rarely seen in cell-attached patches. In the present study, we show that Cl^- channels can be activated in intact cells at physiological temperatures and voltages and that channel properties change after patch excision.

Maxi-Cl^- channels were reversibly activated in 69% of cell-attached patches when the temperature was above 32°C, whereas fewer than 2% of patches showed activity at room temperature. Upon excision, the same patches displayed large, multiple-conductance Cl^- channels with characteristics like those we previously reported for excised patches. After patch excision, warm temperatures were not essential to allow channel activity; 37% (114/308) of inside-out patches had active channels at room temperature. The voltage dependence of the channels was markedly different in cell-attached recordings compared with excised patches. In cell-attached patches, Cl^- channels could be open at cell resting potentials in the normal range. Channel activation was not related to changes in intracellular Ca^{2+} since neither ionomycin nor mitogens activated the channels in cell-attached patches, Ca^{2+} did not rise in response to warming and the Cl^- channel was independent of Ca^{2+} in inside-out patches. Single-channel currents were blocked by internal or external Zn^{2+} (100–200 μM), 4-acetamido-4′ isothiocyanostilbene-2,2′-disulfonate (SITS, 100–500 μM) and 4,4′-diisothiocyanostilbene 2,2′-disulfonate (DIDS, 100 μM). NPPB (5-nitro-2-(3-phenylpropylamino)-benzoate) reversibly blocked the channels in inside-out patches.

Key Words chloride channel · cell-attached patches · lymphocyte · T cell · temperature · voltage dependence

Introduction

There is indirect evidence that conductive Cl^- channels play roles in lymphocyte functions. The regulatory volume decrease (RVD) that follows lymphocyte swelling in hypotonic media depends on Cl^- and K^+ efflux through separate conductive pathways (Cahalan & Lewis, 1988; Deutsch & Lee, 1988; Grinstein & Dixon, 1989). Cl^- efflux and RVD are blocked by some drugs known to block Cl^- channels in other tissues, including 4-acetamido-4′isothiocyano-2,2′disulfonic acid stilbene (SITS) and 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene (DIDS), and by dipyridamole. A role for Cl^- channels in T lymphocyte proliferation has been proposed (Rosoff et al., 1988) since either DIDS or the removal of external Cl^- inhibits the antibody-stimulated influx of Ca^{2+} that is necessary for lymphocyte activation. Killing by cytotoxic T lymphocytes (Gray & Russell, 1986) and by natural killer cells (L.C. Schlichter, *unpublished results*) is also inhibited by SITS, DIDS or removal of extracellular Cl^-. The contribution of Cl^- permeability to the membrane potential at rest, during lymphocyte activation or during killing is not well understood; however, there is evidence that Cl^- permeability is significant and can contribute to the resting potential of B and T cell lines (Wilson & Chused, 1985; Bosma, 1989) and normal T cells (Deutsch et al., 1979; Felber & Brand, 1982).

A large multiconductance Cl^- channel has been characterized in excised, inside-out patches from normal human T lymphocytes (Schlichter et al., 1990). Similar, but not identical, maxi-Cl^- channels have been seen in mouse splenic B lymphocytes (McCann et al., 1989) and in a mouse B-cell hybridoma (Bosma, 1989). In the human T lymphocyte we observed Cl^- channel activity in 40–50% of inside-out patches at room temperature (Schlichter et al., 1990), and the basic properties are as follows. The channel has multiple-conductance levels producing the most common and largest conductance of 365 pS in symmetrical 150 mM Cl^- solutions. Less frequently observed subconductance states may be multiples of a 45-pS state. The voltage dependence in excised patches displayed a maximum probability of opening around 0 mV, and all channels closed when the voltage was outside the range of about ±30 mV. This voltage dependence was modeled as two

gates in series and described by two Boltzmann equations. The channel was highly selective for anions over Na^+ or K^+ and moderately selective among anions. From reversal potential measurements the anion permeability sequence was $I^- > NO_3^- > Br^-, Cl^- > F^-$, isethionate, $HCO_3^- > SO_4^{2-}$ > gluconate, propionate > aspartate, and from single-channel conductance the selectivity was $I^- > NO_3^- > Br^- > Cl^- > F^-$ > isethionate, HCO_3^-. In excised patches channel activity and conductance were independent of intracellular Ca^{2+} over the range 25 nM to 2.5 mM and pH over the range 6.5 to 8.5.

Two common observations regarding maxi-Cl^- channels in most tissues call into question the likelihood that they play a role in cell function. First, in almost all cases channel activity is not seen under normal conditions in cell-attached recordings (Nelson, Tang & Palmer, 1984; Kolb, Brown & Murer, 1985; Woll et al., 1987; Bosma, 1989; McCann et al., 1989; Schlichter et al., 1990, and additional references cited therein); however, most previous recordings were made at room temperature. Second, in excised patches Cl^- channels are usually active over a narrow voltage range that does not extend to the cells' normal resting potential (Schlichter et al., 1990; *see also* literature cited in McCann et al., 1989). During our earlier studies of the maxi-Cl^- channel (Schlichter et al., 1990) we had only observed Cl^- channel activity in excised, inside-out membrane patches. In cell-attached patches we observed activity in fewer than 2% of patches recorded at room temperature, regardless of the voltage tested. In the present study we have re-examined Cl^- channels in normal human T lymphocytes under more physiological conditions (i.e., intact cells at physiological temperature) and have compared voltage dependence and effect of channel blockers in different recording configurations.

Materials and Methods

Methods

Cells

Normal human blood was enriched for T cells either by separation on Ficoll Hypaque (Pharmacia) or on a discontinuous Percoll (Pharmacia) gradient, followed by depletion of B lymphocytes by adhering them to nylon wool. No differences in electrophysiological properties were detected in T cells separated by these two methods. Cell suspensions obtained with this procedure were ≥98% T cells as judged by fluorescence-activated cell sorter (FACS) analysis using OKT3 antibody (Ortho Pharmaceuticals). Cells were ≥95% viable in all batches, as judged by Trypan blue exclusion or by a commercial LIVE/DEAD assay (Molecular Probes, OR). Moreover, there was very little leakage of the fluorescent Ca^{2+} indicator, Fluo-3 (*see below*).

Electrophysiology

Single-channel currents were recorded in cell-attached or excised inside-out patch configurations using an Axopatch 1B (Axon Instruments) patch-clamp amplifier. Currents were low-pass filtered at 10 kHz, played through a pulse-code modulator (PCM-2, Medical Systems) and stored on a video cassette recorder (Sony). During playback for inspection or analysis, currents were filtered with a 4-pole or 8-pole Bessel filter, and all records shown are at 500–1000 Hz unless otherwise indicated. In addition, single-channel currents were recorded in the whole-cell configuration as described previously (Pahapill & Schlichter, 1990), stored on floppy disks using a Nicolet storage oscilloscope and subsequently transferred to an IBM PC-AT computer for analysis. Current records were displayed on a Gould chart recorder or on a Hewlett-Packard plotter. Ionic-current polarities are reported according to physiological convention; that is, positive charges leaving the cell (cation efflux, anion influx) correspond to upward deflections in the illustrated single-channel and whole-cell recordings. Membrane potentials are reported as intracellular with respect to ground on the extracellular side. For cell-attached recordings the bath and pipette usually contained an NaCl saline solution consisting of (in mM): 150 NaCl (occasionally 140), 5 KCl, 2.5 $CaCl_2$ and 10 HEPES adjusted to pH 7.4 with NaOH. Patches were usually excised into this solution, and anion selectivity was later confirmed by varying the NaCl and KCl concentrations reciprocally or by replacing some of the NaCl with sucrose (*see* Results). In some cases inside-out patches were exposed to a solution more similar to cytoplasm containing (in mM): 65 KH_2PO_4, 5 $KHCO_3$, 20 KCl, 55 KOH, 10 NaCl, 5 Na_2HPO_4, 35 NaOH, 0.5 $CaCl_2$, 10 EGTA, 10 aspartic acid, 10 glucose, pH = 7.2. For whole-cell recordings, the bath contained normal NaCl saline solution and the pipette usually contained 140 KF, 10 EGTA, 1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES adjusted to pH 7.4 with KOH. The reference agar bridge contained normal NaCl saline; hence, liquid junction potentials changed with changes in anions in the bath. These junction potentials were measured directly by a 3 M KCl electrode that had been zeroed with normal saline in the bath. Where appropriate, current-*versus*-voltage relations and reversal potentials were corrected for these offsets (*see* Schlichter et al., 1990). The temperature of the bathing solution was controlled by a home-made Peltier device as described previously (Pahapill & Schlichter, 1990).

Materials

The Cl^- channel blockers, 4-acetamido-4'isothiocyano-2,2'-disulfonic acid stilbene (SITS), 4,4'-diisothiocyano-2,2'-disulfonic acid stilbene (DIDS) and $ZnCl_2$ were purchased from Sigma Chemical. The Cl^- channel blocker, 5-nitro-2-(3-phenylpropylamino)-benzoate) (NPPB), is made by Hoechst (Frankfurt, FRG) and was a gift from Merck-Frosst, Canada. Stock solutions of DIDS and SITS (10 mM) were prepared in 0.1 M $KHCO_3$ (pH = 8); $ZnCl_2$ (10 mM) was dissolved in the 150 NaCl saline; NPPB (100 mM) was dissolved in DMSO. The mitogenic lectins, concanavalin A and phytahemagglutinin (PHA), were purchased from Sigma and dissolved in NaCl saline.

Calcium Measurements

To test the effect of temperature on intracellular Ca^{2+} levels, the Ca^{2+}-sensitive fluorescent dye Fluo-3 acetoxymethyl ester (Fluo-3 AM, Molecular Probes, OR) was used. Its advantages include a near zero fluorescence in the absence of binding by divalent cations, excitation by visible light (thereby reducing photobleaching) and enhanced quantum yield and absorbance, thus greatly lowering buffering effects encountered with dyes such as quin-2 (Kao, Harootunian & Tsien, 1989). Fresh T cells (prepared as above) were loaded in RPMI culture medium in 1 μM fluo-3 AM at 37°C for 20 min. Cells were then washed twice and resuspended in RPMI (without fluo-3) and incubated at 37°C for at least 30 min to ensure complete hydrolysis of the dye. Fluorescence measurements were made with cells in suspension (1×10^5 cells/ml) using an SLM Fluorometer (SLM Instruments, Urbana, IL). Calcium levels were calculated using the formula $[Ca^{2+}]_i = 400$ nM $(F - F_{min})/(F_{max} - F)$ where F_{max} is the maximal fluorescence obtained after addition of digitonin. To determine the background fluorescence (F_{min}) calcium was first displaced from the fluo-3 with 500 μM $MnCl_2$ which gives a fluorescence level (F_{Mn}) about fivefold lower than that of Ca^{2+}. F_{min} was calculated as $1.25 F_{Mn} - 0.25 F_{max}$. Due to variations in dye loading and loss, calibration was done at the end of each experiment.

Results

Chloride Channel Activity in Excised Patches

Figure 1*A* shows typical activity of the maxi-Cl^- channel in an excised inside-out patch bathed in symmetric 150 mM NaCl saline. At a transmembrane potential of +20 mV outward-going currents corresponding with channel openings were observed. This current record shows one predominant current amplitude and two sublevels which were about one-third and two-thirds of the main level. On an expanded time scale (traces *1* and *2*) these sublevels are more easily seen. A high occurrence of direct transitions between the large amplitude level and the baseline was seen (four such transitions in trace *1*) as well as long openings to the smallest current level (top trace and trace *2*). Figure 1*B* shows typical current fluctuations in these solutions at the membrane potentials indicated between the two traces. The currents were inward at negative potentials and outward at positive potentials. This is reflected in the unitary current-*versus*-voltage (*I-V*) relationship (Fig. 1*C*) which was linear in these symmetrical solutions with a slope of 380 pS. The mean value of the channel conductance for the large predominant level was 365 ± 13 pS (n = 37). The conductances of the two main sublevels were 265 and 140 pS. In this patch the channel was open most of the time between ±15 mV and closed soon after the voltage was stepped to ±20 mV. Because of this voltage-depen-

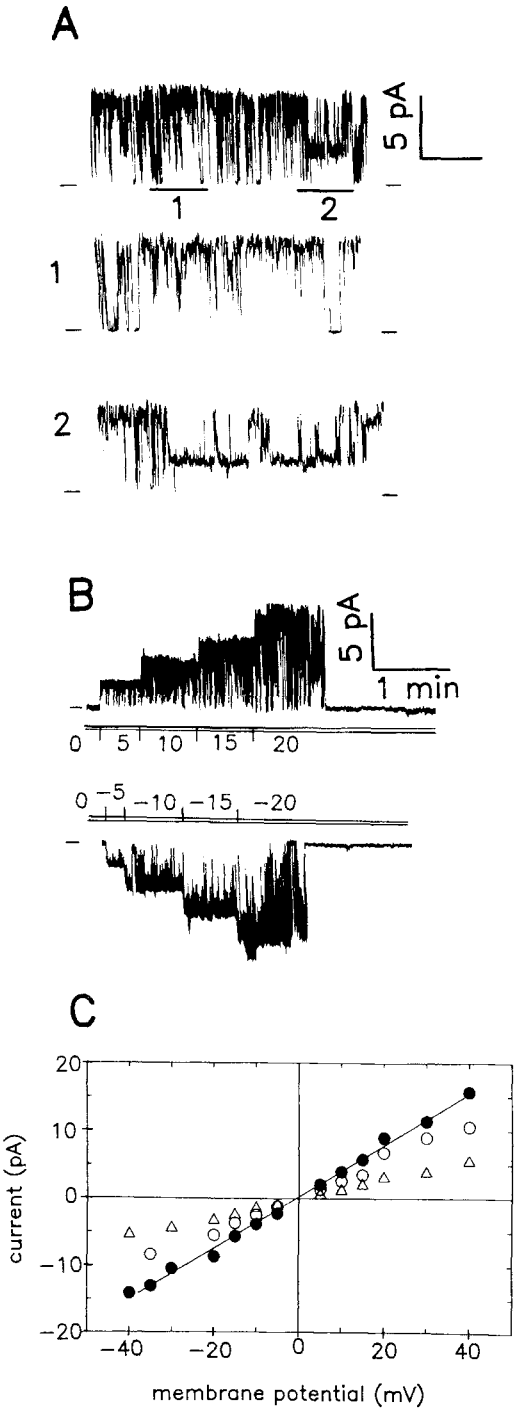

Fig. 1. Multiple conductance states of the maxi-Cl^- channel. (*A*) Current fluctuations in an excised, inside-out patch at +20-mV membrane potential. Time scale bar: upper trace, 10 sec; lower traces, 1 sec. Lower two traces are expansions of regions marked *1* and *2*. Dashes adjacent to the current traces represent the zero-current level. (*B*) Voltage-dependent opening and closing in an inside-out patch during voltage steps to the membrane potentials indicated between the traces. (*C*) Current-*versus*-voltage (*I-V*) relations for the patch in *B*. The straight line is a least-squares fit to the data for the most prevalent open state (●, 380 pS). Bath and pipette contained 150 mM NaCl saline (*see* Materials and Methods).

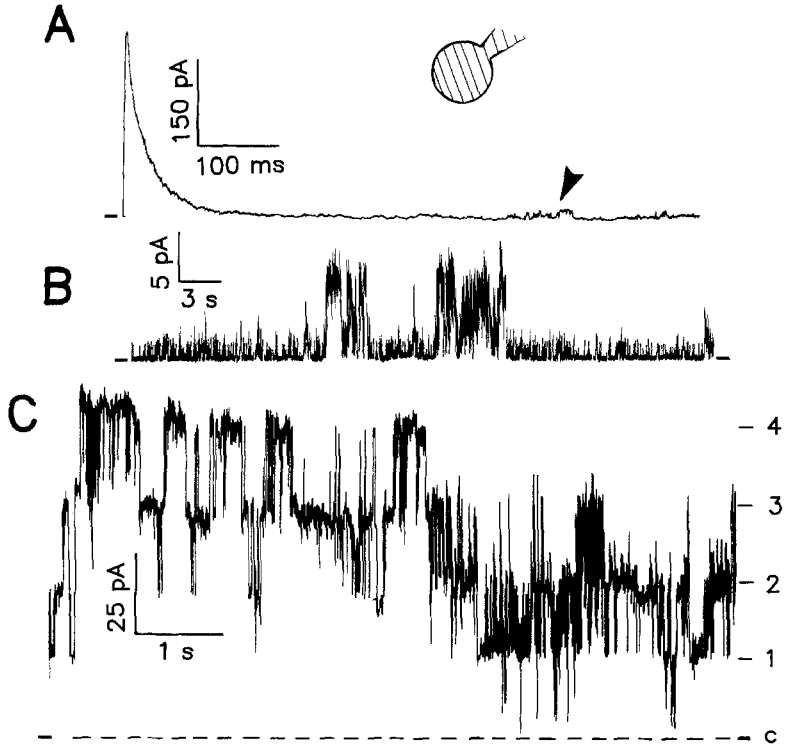

Fig. 2. Maxi-Cl^- channel activity in whole-cell recordings at 37°C. (*A*) The membrane potential was held for several minutes at −90 mV then stepped to 0 mV at the beginning of the trace. After the K^+ current inactivated, single Cl^- channel currents were discernible in the whole-cell configuration (arrow). (*B*) Steady-state current after holding the membrane potential for several minutes at 0 mV. Note the change in amplitude and time scales. Same cell as *A*. (*C*) Steady-state currents after holding for 60 sec at +50 mV in a different cell from *A* and *B*. Numbers at right of trace indicate 1 to 4 channels simultaneously open. The dashes adjacent to each trace mark the zero-current level. Pipette, 140 KF saline; bath, 140 NaCl saline.

dent closing, *I-V* relationships that extended beyond ±30 mV were constructed by holding the potential at voltages that permitted openings, then stepping to potentials where channels closed and measuring amplitudes of single-channel events during channel closing.

Similar currents were seen in 37% (114/308) of inside-out patches from normal human T lymphocytes. Following patch excision Cl^- channels either activated spontaneously within a couple of minutes or the patch remained quiescent for the remainder of a recording under control conditions (30–90 min). These basic characteristics of the maxi-Cl^- channel in excised patches have been reported (Schlichter et al., 1990). In our previous work, the selectivity of this channel among 11 anions and Na^+ and K^+ was determined. Among the halides the relative permeabilities were $I^- : Br^- : Cl^- : F^- = 1.38 : 1.04 : 1.00 : 0.57$. Moreover, the channel was about 30 times more selective for Cl^- than for Na^+ or K^+.

An important observation is that the Cl^- channel can conduct physiologically relevant anions such as phosphate, bicarbonate and aspartate. Current could also be carried by anions that are sometimes used as nonpermeant Cl^- substitutes such as isethionate, propionate and gluconate. For example, excised inside-out patches were exposed to 150 mM of any of these anions on the cytoplasmic side. If the membrane potential was held at 0 mV to activate Cl^- channels (*see* Fig. 6) then stepped to a potential more negative than −20 mV, distinctly inward currents were seen. Hence, in the membrane potential range of −50 to −70 mV organic anion efflux from the cytoplasmic side to the outside was significant.

Activity in Whole-Cell Recordings

Activity of Cl^- channels could be resolved in most whole-cell recordings. Single or multiple Cl^- channels were seen in about 39% (22/56) of whole-cell recordings at room temperature and 84% (16/19) of recordings made soon after establishing a whole-cell recording at temperatures above 32°C. For example, Fig. 2*A* shows a whole-cell current recorded at 37°C during a step to 0 mV. The large transient outward (upward) current that peaked in 10–15 msec and decayed to the zero-current level during the first 200 msec is the voltage-dependent K^+ current that is the most prevalent seen in whole-cell recordings from T lymphocytes. The kinetics of this K^+ current have been extensively described by several groups, including ours (Cahalan et al., 1985; Pahapill & Schlichter, 1990, 1991). After the K^+ current inactivated there was little remaining steady-state current and the single-channel events underlying the K^+ current were too small to be seen on this scale.

However, single Cl^- channels were seen near the end of the trace (Fig. 2*A*, arrow). These were subsequently identified as Cl^- channels with about 500-pS conductance at 37°C and a reversal potential of about −14 mV with Cl^- in the bath and F^- in the pipette. The calculated $P_F : P_{Cl}$ ratio would be about 0.57 as we have previously shown for the maxi-Cl^- channel (Schlichter et al., 1990). There was no change in reversal potential when 140 mM KCl saline was perfused into the bath. However, the reversal potential shifted to +14 mV when the bath Cl^- was reduced to 50 mM (sucrose substituted), as predicted for a Cl^- channel with the observed F^- permeability. In Fig. 2*B* the membrane potential was held at 0 mV for several minutes to inactivate most of the K^+ current and to observe channel activity in the steady state. The large events are Cl^- channel openings, and the small (~20-pS) events are the K^+ channels which underly the macroscopic K^+ current in Fig. 2A. In most whole-cell recordings more than one Cl^- channel was active at 37°C, and four overlapping openings, each with about 500-pS conductance, can be seen in Fig. 2*C*. The existence of multiple channels and subconductance states in whole-cell recordings made measurements of single-channel properties difficult. One whole-cell recording was obtained in which channel activity was low enough so that the amplitude of the largest single-channel events at each voltage was measurable. Figure 3*A* shows *I-V* relations for this Cl^- channel at 5, 20 and 37°C. The single-channel conductance was temperature sensitive, increasing from about 125 pS at 5°C to 250 pS at 20°C and 350 pS at 37°C. From these values, the Q_{10} for conductance was about 1.6 between 5 and 20°C and 1.2 between 20 and 37°C. However, the shapes and zero crossings of these *I-V* relations are not precise and these records were not corrected for junction potentials; hence, details of channel selectivity cannot be extracted from these records. Nevertheless, the predicted reversal potentials of −80 to −87 mV for K^+, −10 to −15 mV for Cl^- and > +80 mV for Na^+ or Ca^{2+} are consistent with Cl^- selectivity. Figure 3*B* shows that channel activity increased with temperature. The open-state probability for this channel at −60 mV, calculated from 60-sec long records at each temperature, increased from about 0.0005 at 5°C to 0.072 at 20°C and to 0.091 at 37°C.

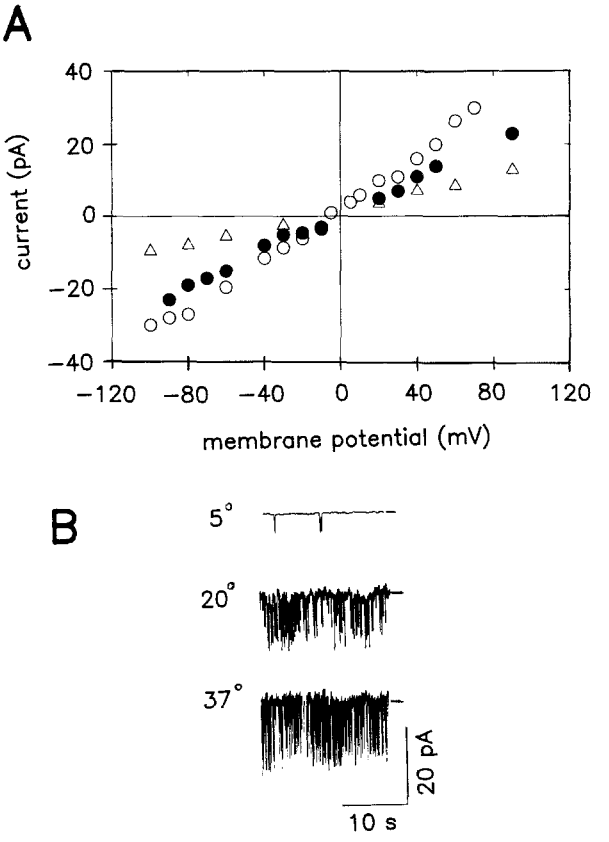

Fig. 3. Maxi-Cl^- channel activity at different temperatures in a whole-cell recording. (*A*) Single-channel current-*versus*-voltage (*I-V*) curves at 5°C (△), 20°C (●) and 37°C (○). (*B*) Cl^- current at −60 mV at 5, 20 and 37°C. Each 20-sec segment is representative of steady-state activity after 60 sec at −60 mV. Pipette, 140 KF saline; bath, 140 NaCl saline. The dashes adjacent to each current trace mark the zero-current level.

Activity in Cell-Attached Recordings

Figure 4*A* shows a cell-attached recording at the cell's normal resting potential. Unlike excised or whole-cell recordings Cl^- channels were rarely active in cell-attached patches at room temperature (<2% or 6/>300 patches). However, in 20/29 (69%) of previously quiescent patches, Cl^- channels were activated by increasing the temperature to 32°C or higher. The patch in Fig. 4*A* was quiescent for 20 min at 22°C and for several minutes at temperatures below 32°C. However, after 2 min at 32°C a maxi-Cl^- channel spontaneously activated (end of trace) and remained active until the temperature was lowered. Figure 4*B* shows steady-state activity at 37°C (left) at the resting potential followed by a decrease in activity 5 min after returning to 22°C (right). Note that the current amplitude decreased at 22°C. This was reflected by a decrease in the slope of the *I-V* relation, indicating a lower conductance (*not shown*). After 15 min at 22°C this patch became quiescent and remained so until the temperature was again raised (*not shown*). Figure 4*C* shows a different patch with low activity at 22°C (left) and very vigorous activity of several channels 15 min after the

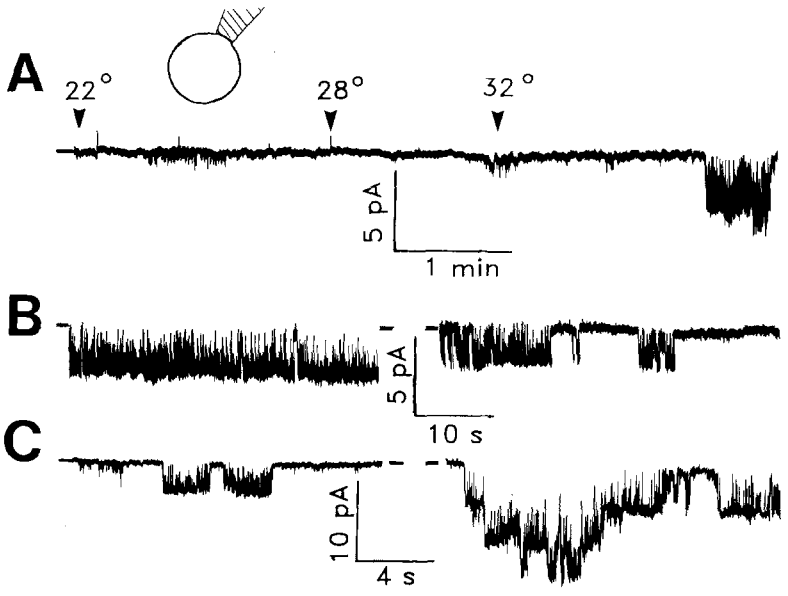

Fig. 4. Temperature-induced activation of maxi-Cl^- channels in a cell-attached recording. (*A*) The patch was held at the cell's resting potential (pipette potential 0 mV), and the temperature was raised from 22 to 28 to 32°C. Channel openings result in Cl^- efflux from the cell, shown as downward deflections. (*B*) Steady-state channel activity at the resting potential at 37°C (left) and 5 min after cooling to 22°C (right). (*C*) Cl^- currents in a different patch at 10 mV hyperpolarized from the resting potential, 15 min after cooling to 22°C (left) and 15 min after warming to 37°C (right). Bath and pipette contained 150 NaCl saline solution. The dash adjacent to each trace marks the zero-current level.

temperature was raised to 37°C (right). Again, the current amplitude was smaller at 22 than at 37°C.

I-V relations were obtained from 19 cell-attached patches. Figure 5*A* shows an example of the procedure used to measure *I-V* relations at potentials where the Cl^- channel open probability was reduced. At the beginning of the record shown, the patch was hyperpolarized by 60 mV and a single Cl^- channel activated within 30 sec. Current amplitudes could then be measured at 60, 50 and 40 mV hyperpolarized before the channel spontaneously closed. The channel was then rapidly reopened by a hyperpolarizing prepulse (−60 mV) before each step to less negative potentials; i.e., −30, −20, −10 (and +20 mV, inset in Fig. 5*B*). This procedure could then be repeated several times and the average current measured at each potential. Figure 5*B* shows single-channel *I-V* relations from three cell-attached patches at 37°C, including the cell from Fig. 5*A* (▲). The *I-V* relations were linear within the voltage range tested, with reversal potentials 8 to 12 mV depolarized from the resting potential. (The channel in Fig. 5*A* was apparently a smaller subconductance state—*see* Fig. 1 and Schlichter et al., 1990). Average reversal potentials with respect to the resting potential were 10.5 ± 1.0 mV (n = 9) with 150 NaCl saline in the pipette and 11.9 ± 4.0 mV (n = 4) with 150 KCl saline in the pipette. Moreover, in recordings with 140 KF saline in the pipette, the reversal potential was 29.0 ± 0.6 mV (n = 6) depolarized from the resting potential. Since the equilibrium potential for Cl^- in these cells is estimated to be between −30 and −35 mV (Grinstein & Dixon, 1989) and we have previously shown a permeability ratio for F : Cl of 0.57 (Schlichter et al., 1990), these measurements would imply a resting potential of −40 to −50 mV. This is in excellent agreement with the resting potential calculated from the reversal potential of K^+ channels in cell-attached patches from these cells (Pahapill & Schlichter, 1991) where we showed that with normal NaCl saline in the bath, the K^+ current reversed at a pipette potential (V_p) of −51 mV under symmetrical K^+ concentrations and 0 mV when the cell was depolarized by 150 mM KCl saline in the bath. With normal NaCl saline in the pipette and bath the K^+ current reversed at V_p +34 mV. Moreover, the voltage dependence of the K^+ channel in cell-attached patches was as predicted for a membrane potential of about −50 mV. The agreement between the K^+ and Cl^- current reversal potentials gives us confidence that the membrane potential is about −50 mV in these cells with the normal Na^+, K^+, and Cl^- gradients across the membrane.

In order to compare conductances from cell-attached recordings at 37°C with our previous inside-out patches at 22°C, we first determined the Q_{10} for single-channel conductance of this channel. The value calculated from one whole-cell recording (Fig. 3), three excised patches and two cell-attached patches was 1.21 ± 0.02 (n = 6) for temperatures between 22 and 37°C. Using this Q_{10} value, the calculated conductances for cell-attached patches at 22°C would be significantly less than the 350 to 450 pS seen routinely in excised patches at room temperature. Moreover, our earlier study (Schlichter et al., 1990) showed that Cl^- channel conductance increased with Cl^- concentration according to a Michaelis-Menten relation with a K_m of 120 mM. Intracellular Cl^- activity is lower than the 150-mM Cl^- used in our excised patches; hence, a further reduction in conductance is predicted due to Goldman

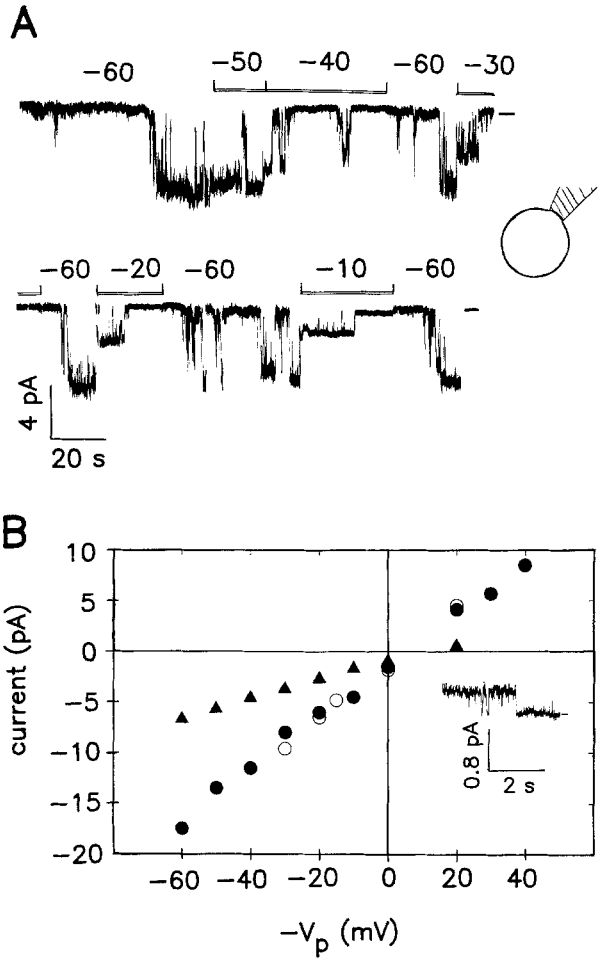

Fig. 5. Single-channel conductance in cell-attached patches at 37°C. (*A*) A continuous 6-min recording of maxi-Cl^- channel activity at different potentials. The numbers above the traces indicate the voltage (in mV) with respect to the cell's membrane potential. (*B*) *I-V* relations for single-channel currents from *A* (▲) and two other cells. The bath contained 150 NaCl saline for all three cells, and the pipette contained 150 NaCl (▲, ●) or 150 KCl saline (○). *Inset:* Channel closing after stepping the pipette potential from +60 to −20 mV (same cell as in *A*).

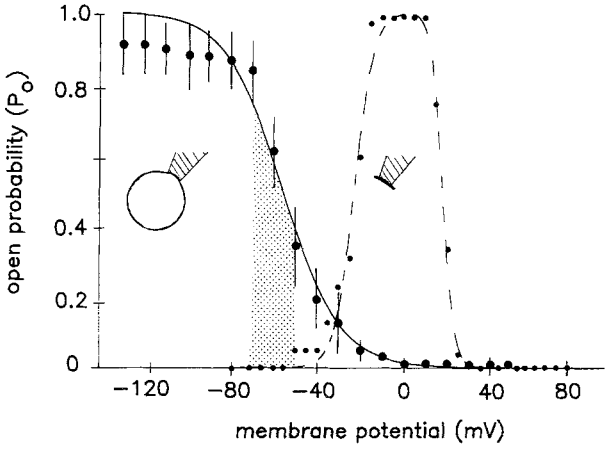

Fig. 6. Voltage dependence of the steady-state probability (P_o) of the maxi-Cl^- channel being in the open state for both cell-attached (solid curve) and inside-out (broken curve) patches. P_o was calculated from the proportion of time the channel was conducting compared to the total time of recording at a given voltage. For cell-attached patches (solid curve) data points represent the mean ± SEM, $n = 16$ (error bars at voltages above −20 mV are smaller than the symbols). A membrane potential of −50 mV was assumed, based on reversal potentials for Cl^- and K^+ currents. For excised, inside-out patches (broken curve) data are from Schlichter et al. (1990) (error bars are omitted for clarity). The solid curve was fitted to a single Boltzmann distribution, and the broken curve was fitted to the sum of two Boltzmann distributions of the form:

$$P(V) = P_{max}/[1 + e^{(V - V1/2)/k_n}]$$

where $V_{1/2}$ is the voltage producing half-maximal probability and k_n is the slope factor corresponding to the voltage sensitivity of activation. For excised patches (broken curve) the fitted values of $V_{1/2}$ and k_n were −22.8 and −4.4 mV for the rising curve and 18.0 and 2.6 mV for the falling curve. The bath and pipette contained 150 NaCl saline. For cell-attached patches, the fitted values were −55.8 mV for $V_{1/2}$ and +13.1 mV for k_n; the bath contained 150 NaCl saline and the pipette contained 150 NaCl, 150 KCl or 150 KF saline. No difference in voltage dependence was seen with these different pipette solutions. The shaded area represents the membrane potential range of intact cells.

rectification in the region of the *I-V* relation where net Cl^- efflux dominates. The most common conductance in cell-attached patches (248 pS at 37°C or 186 pS at 22°C) would be predicted from an intracellular Cl^- activity of about 60 mM. This value is well within the range thought to be typical of T lymphocytes (Grinstein & Dixon, 1989) and predicts a Nernst potential of about −24 mV at 37°C.

Voltage Dependence in Intact Cells

We have previously described the voltage dependence of the Cl^- channel in excised patches with symmetrical 150 mM NaCl saline solutions (Schlichter et al., 1990). Under these conditions, the steady-state open probability (P_o) *versus* holding potential produced a steep bell-shaped curve with P_o values reaching a maximum of 1.0 at 0 mV and falling off to 0 at about ±30 mV (Fig. 6, broken curve). This was modeled as two gates in series with 5.7 and 9.6 equivalent charges and half-maximal activation at −22.8 and +18.0 mV. Because the channels were open most of the time around 0 mV multiple channels would be seen as overlapping events, and we could be reasonably sure of the number of Cl^- channels in each patch. Hence, for the Cl^- channel the activity (sum of the open times/total time) was a good estimate of open probability. An important result of the present study is that following patch excision the voltage dependence is dramati-

cally altered (Fig. 6). Open probability was measured in 16 cell-attached patches. Channels activated with hyperpolarization and closed with depolarization. The voltage dependence in cell-attached recordings could be described by a single Boltzmann relation with a half-maximal probability at −55.8 mV and with a slope of voltage dependence, suggesting a gate with 3.2 equivalent charges.

To confirm that the Cl^- channels observed in cell-attached patches were the same as those we have observed in inside-out patches, comparisons were made before and after excision of the same patches. Three cell-attached patches were chosen that showed channel activity typical of cell-attached recordings at 37°C, i.e., typical reversal potentials, conductance and voltage dependence. After each patch was excised (inside-out) it then showed characteristics typical of the maxi-Cl^- channel we have previously described (*see* Fig. 1 and Schlichter et al., 1990); that is, conductance (377 ± 15 pS, 22°C), voltage dependence (full activation within ±20 mV, *see* Fig. 6), anion selectivity (expected shift in reversal potential after Cl^- was substituted by less permeant anions) and block by Zn^{2+}. Our criteria for estimating the membrane potential in cell-attached patches using reversal potentials of Cl^- and K^+ channels in normal solutions or with the cell fully depolarized by KCl saline (*see also* Pahapill & Schlichter, 1991) are in good agreement with the literature (Deutsch et al., 1979; Felber & Brand, 1982; Wilson & Chused, 1985; *see also* Grinstein & Dixon, 1989). The main point is that, unlike excised patches (Fig. 6, broken curve), significant Cl^- channel activity can occur in intact cells at 37°C in the normal resting potential range for lymphocytes (−40 to −70 mV; Fig. 6, shaded area).

To investigate this dramatic shift in voltage dependence following excision we first examined simple physical and ionic factors that were different from our previous excised-patch recordings. Temperature differences did not produce a change in the P_o-V relation either in cell-attached or inside-out patches (*data not shown*). To test the effects of anion differences on the cytoplasmic face, inside-out patches (n = 4) were exposed to a solution resembling cytoplasm (*see* Materials and Methods). This solution contained phosphate and bicarbonate instead of HEPES buffer and only 32 mM Cl^-, yet no difference in the voltage dependence was observed. The P_o-V curve was centered around 0 mV, and channels closed with voltage excursions beyond ±20 mV. Neither could the shift in voltage dependence be explained by intracellular calcium or pH differences since the channel in excised patches was unaffected by Ca^{2+} between 25 nM and 2.5 mM and pH 6.5 to 8.5 (Schlichter et al., 1990). To test Ca^{2+} sensitivity in intact cells we added 10–500 nM (n = 14) Ca^{2+} ionophore, ionomycin, or 10–20 μg/ml of the mitogenic lectins, Con A or PHA (n = 4) during cell-attached recordings at room temperature. No Cl^- channels were activated (0/18 patches) although excising 9 of these patches confirmed Cl^- channels were present in 4 of them. Evidence that Ca^{2+} rose in the cells following ionomycin or mitogens is that voltage-dependent K^+ channels (when present) were inhibited by these treatments and Ca^{2+}-dependent K^+ channels were often activated. Fluo-3 measurements in cell batches confirmed Ca^{2+} rises following mitogen treatment at room temperature. With 10–20 μg/ml Con A or PHA added Ca_i rose from 109 ± 6 nM (n = 12) to a peak of 192 ± 34 nM (n = 12). To further test whether activation by warming during cell-attached recordings was due to changes in intracellular Ca^{2+}, we measured free Ca^{2+} levels at different temperatures in four batches of cells. The resting Ca^{2+} level at 22°C was 103 ± 28 nM (n = 4), in excellent agreement with a recent publication using fluo-3 on human peripheral T cells (107 ± 18 nM; Vandenberghe & Ceuppens, 1990). There was virtually no change in fluorescence when the temperature was raised from 22–38°C or lowered back to 22°C. The largest change observed was from 123 nM at 22°C to 137 nM at 37°C in one cell batch. It was important to ensure complete hydrolysis of the acetoxymethyl ester (AM) form of the dye as any residual reaction will lead to an irreversible rise in fluorescence. For this reason all cells were incubated for 30 min at 37°C (5% CO_2) after dye loading. Once hydrolyzed, fluo-3 itself has very little temperature dependence, with fluorescence decreasing ≈1% between 22 and 37°C. Time-dependent loss of the dye during perfusion (10–15%/hr) was not a significant factor during the 15–20 min recording period. The <20 nM increase in Ca_i^{2+} we observed in the most extreme case is minuscule compared with the two- to threefold increase that is measured in cell batches during T cell activation and is required for activation of the Ca^{2+}-dependent K^+ channels (Mahaut-Smith & Schlichter, 1989).

Therefore, the altered voltage dependence upon patch excision is not due to the ionic content of our saline solutions. Clearly the Cl^- channel can be active in the resting, intact cell at physiological temperatures. This is not predicted from recordings on isolated patches and suggests that the ability to activate the channel, in addition to its conductance and voltage dependence, is modulated by intracellular constituents.

Cl^- Channel Blockers

In functional assays ion-channel blockers are added to the bath, whereas in most previous patch-clamp studies blockers have been added to the cytoplasmic

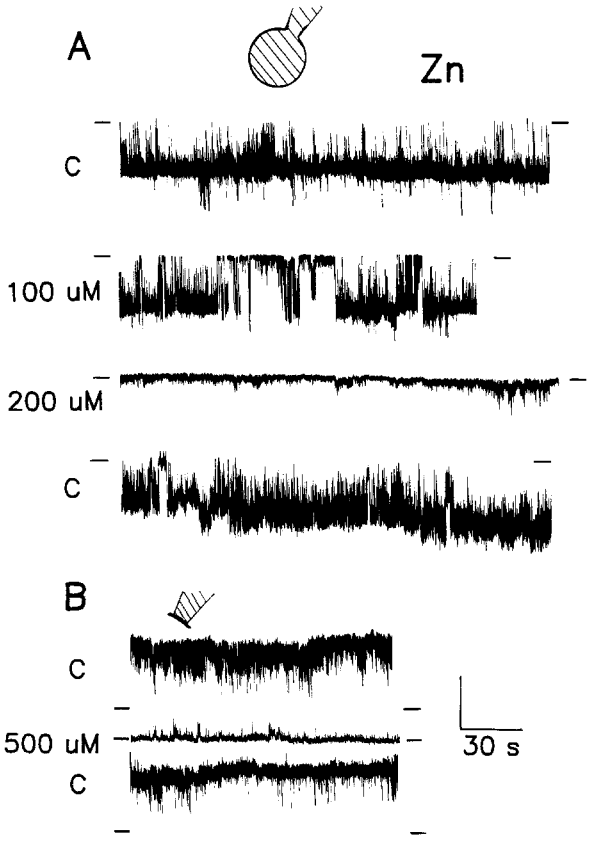

Fig. 7. Reversible block of single Cl^- channels by Zn^{2+} from the inside or outside. (*A*) Whole-cell configuration; V_m −10 mV. Pipette, 150 KCl saline; bath, 150 NaCl saline. First trace, control; second trace, 100 μM Zn^{2+}; third trace, 200 μM Zn^{2+}; fourth trace, 3 min after washout of Zn^{2+}. (*B*) Inside-out patch; V_m +10 mV. Pipette and bath, 150 NaCl saline. First trace, control; second trace, 500 μM Zn^{2+}, third trace 5 min after washout of Zn^{2+}. Room temperature. Scale bars are 30 sec and 4 pA (*A*), 5 pA (*B*). Dashes at the beginning of each trace mark the zero-current level.

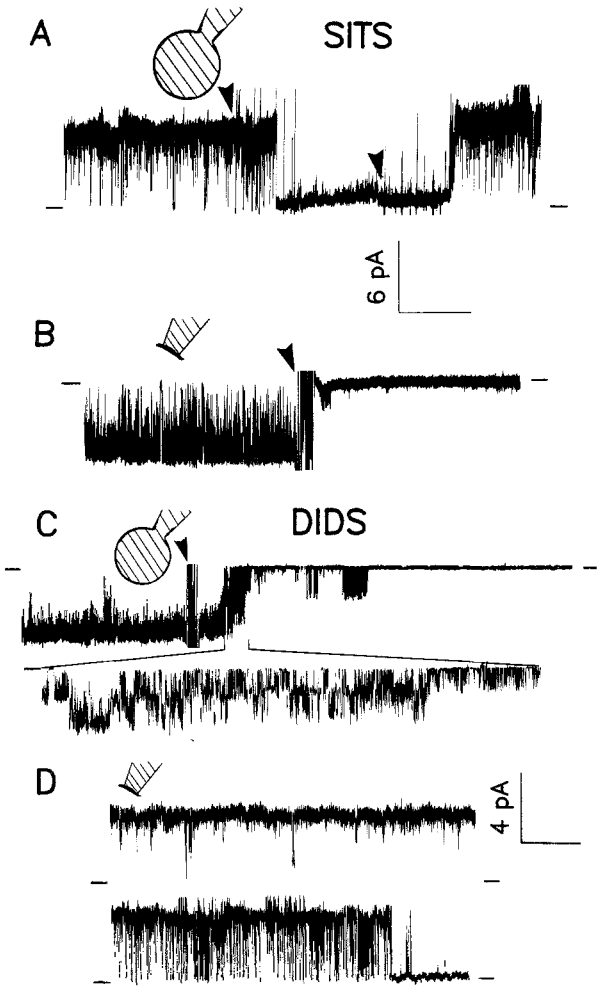

Fig. 8. Reversible block by internal or external SITS or DIDS. (*A*) Whole-cell recording; V_m +5 mV. Pipette, 150 KF saline; bath, 150 KCl saline. SITS (500 μM) was added (first arrow), then washed out (second arrow). (*B*) Inside-out patch; V_m 0 mV. Pipette, 80 mM F^- and 40 mM Cl^-; bath, 150 KCl saline. SITS (100 μM) added at arrow. (*C*) Whole-cell recording; V_m −5 mV. Pipette, 150 KCl saline; bath, 150 NaCl saline. DIDS (100 μM) was added in NaCl saline at the arrow. Below is a segment of the record on an expanded time scale. (*D*) Inside-out patch; V_m +10 mV. Pipette, 150 KCl saline; bath, 150 NaCl saline. Top trace, control; lower trace, 1 min after 100 μM DIDS was added. All recordings at room temperature. Dash adjacent to each trace marks the zero-current level. Time scale bars are 1 min in *A–C* (upper), 3 sec in *C* (lower), and 30 sec in *D*.

face. We took advantage of the ability to record single Cl^- channels in the whole-cell configuration to test the effects of external Zn^{2+} (Fig. 7), SITS (Fig. 8*A* and *B*) and DIDS (Fig. 8*C* and *D*). We also added these blockers (Figs. 7 and 8) or NPPB (*not shown*) to the cytoplasmic face of inside-out patches. Figure 7*A* shows the effects of Zn^{2+} added to the outside of the cell. In whole-cell recordings complete block was achieved within 60 sec at 200 μM Zn^{2+} but was reversed within 5 min after washing out the Zn^{2+}. For inside-out patches (Fig. 8*B*) we previously reported that 1 mM Zn^{2+} completely blocks maxi-Cl^- channels (Schlichter et al., 1990), and we now find almost complete, reversible block at 500 μM.

We tested effects of two Cl^- transport inhibitors that are known to inhibit volume regulation, activation, and killing by lymphocytes: SITS and DIDS (Gray & Russell, 1986; Rosoff et al., 1988; Grinstein & Dixon, 1989; L.C. Schlichter, *unpublished results*). These stilbene disulfonates were originally classified as inhibitors of the Band 3 anion exchanger in red blood cells, but have been found to block a wide variety of Cl^- channels, including maxi-Cl^- channels (Nelson et al., 1984; Bosma, 1989; *see also* literature cited in Gogelein, 1988) and medium-conductance Cl^- channels in epithelia (Gogelein, 1988). Figure 8 shows that SITS also blocked the channels in both whole-cell and excised-patch recordings. When added from the outside (Fig. 8*A*)

500 μM SITS rapidly (<1 min) blocked the Cl^- channel and this was quickly reversed upon washing the SITS from the bath (second arrow). In excised patches complete and rapid block (<30 sec) was obtained with only 100 μM SITS (Fig. 8*B*). Inhibition was reduced to a partial, flickery block at 10 μM SITS, and there was essentially no block at 5 μM SITS (*data not shown*). In other patches washing out the 100 μM SITS completely restored channel activity. In contrast to Zn^{2+} and SITS, block by DIDS was irreversible from both the inside and outside face. Addition of 100 μM DIDS (Fig. 8*C*, arrow) to the outside completely blocked active channels within about 1 min. On an expanded time base (lower trace) two channels that were previously open most of the time fluctuated between open and closed before closing completely. This block was not reversible even after 30 min washing (*not shown*). Figure 8*D* shows a similar block from the inside. In the upper trace a Cl^- channel is open most of the time. The lower trace begins 1 min after 100 μM DIDS was added, and the current was completely blocked within 2.5 min. Channel activity did not recover even after 10 min washing in normal solution.

NPPB has been found to block some Cl^- channels, especially in epithelia (Gogelein, 1988). NPPB added to inside-out patches produced a flickery block at 100–200 μM which was rapidly reversed upon washing. At 300–500 μM complete channel block occurred and there was no apparent voltage dependence. The solvent, DMSO, (0.5%) neither enhanced nor inhibited Cl^- channel activity. NPPB (3/3), Zn^{2+} (8/8), SITS (2/2), and DIDS (3/3) all caused increased flickering from conducting to nonconducting without a decrease in amplitude. Thus they can be classified as "intermediate" blockers according to their binding kinetics, suggesting that they have faster association and dissociation rates than the normal closing rate of the channel. It has been reported that the anionic pH buffer, HEPES, can block Cl^- channels and produce flickery events or subconductance states (Yamamoto & Suzuki, 1987) such as those described for the T lymphocyte channel. This cannot account for the subconductance states we observed since replacement of the HEPES buffer with a phosphate-bicarbonate buffer (*see* Materials and Methods) did not increase the prevalence of subconductance states.

Discussion

Channel Activation

Large-conductance Cl^- channels have been found in a number of cell types, including lymphocytes and lymphocytic cell lines (Cahalan & Lewis, 1988; Bosma, 1989; McCann et al., 1989; Schlichter et al., 1990) and several nonimmune cells (Nelson et al., 1984; Geletyuk & Kazachenko, 1985; Kolb et al., 1985; Falke & Misler, 1989). In almost all cases, channel activity was extremely rare in cell-attached recordings but appeared after patch excision, usually after several minutes. This behavior calls into question a physiological role for maxi-Cl^- channels; however, our present results show that failure to observe these channels in cell-attached patches may be a result of working at room temperature rather than physiological temperature. We have recently shown that K^+-channel behavior in T lymphocytes is temperature dependent and that nonactive channels can be recruited at warmer temperatures (Pahapill & Schlichter, 1990). This observation stimulated us to test the effect of temperature on Cl^- channels in intact cells. Cl^- channel activity was observed in <2% of cell-attached patches at room temperature but in about 70% of patches at temperatures $\geq$32°C. Raising the temperature dramatically increased the incidence of observing channel activity and also increased the number of channels active in some cell-attached patches. An important observation is that this activation by warming was reversible upon cooling the cells. Hence it does not represent an irreversible artefact of membrane instability or vesicle fusion to the membrane in the patch. Moreover, we have found that recordings of K^+ channels in cell-attached patches are quite stable at 37°C (Pahapill & Schlichter, 1991). We have found no previous reports of all-or-none recruitment of channel activity by raising temperature in intact cells. A recent paper (Welsh, Li & McCann, 1989) reported an increase in activity of a 50-pS Cl^- channel in inside-out patches from airway epithelial cells when the temperature was raised from room temperature to 37°C; however, there was no activation in cell-attached recordings. Although warming the cells activated Cl^- channels in intact T lymphocytes we do not think it is a direct effect of temperature since channels were often active at room temperature in whole-cell or excised-patch recordings. However, under these conditions raising the temperature further increased the probability of opening and increased the single-channel conductance. In general, single-channel conductances increase with temperature and display Q_{10} values $\leq$1.5. The increase we observed in the present study corresponds with a Q_{10} of about 1.2 between 22 and 37°C. We previously reported a Q_{10} of 1.18 in this temperature range for the single-channel conductance of the voltage-gated K^+ channel in whole-cell recordings from human T lymphocytes (Pahapill & Schlichter, 1990).

An important observation from the present study of T lymphocytes is that in cell-attached recordings at temperatures that permit channel activ-

ity (≥30°C), Cl^- channels can be active at the resting potential (e.g., Fig. 4). Activity increased at hyperpolarized potentials and decreased with depolarization. Our results show a dramatic change in voltage dependence after excision when channels become active only within a narrow voltage range, centered around 0 mV (e.g., Fig. 6). A similar narrow range of permissive voltages has been seen for maxi-Cl^- channels in excised patches from a number of cell types (*see* literature cited in McCann et al., 1989). The change in voltage dependence we observed after patch excision was not due to simple physical or ionic factors since changing temperature (20–37°C), cytoplasmic Ca^{2+} (25 nM–2.5 mM), pH (6.5–8.5) or Cl^- concentration (30–500 mM) did not affect the voltage dependence in inside-out patches. Changes in voltage dependence of channels do generally occur during whole-cell recordings (Fernandez, Fox & Krasne, 1984; Cahalan et al., 1985) usually appearing as a 10- to 20-mV shift of the conductance-*versus*-voltage (or P_o-V) curve toward more negative potentials. Such a shift is opposite to the more positive potentials for activation we observed after excision; moreover, it could not account for the apparent loss of voltage-dependent closing at hyperpolarized potentials in intact cells. We do not yet have an explanation for the dramatic shift in voltage dependence after excision. Our results on intact cells at 37°C show that maxi-Cl^- channels *can* be active under normal conditions at physiologically relevant voltages and temperatures. The question is, under what circumstances are these channels normally activated?

Possible Roles for the Cl^- Channel

The multiple-conductance Cl^- channel could contribute to, as well as respond to, the membrane potential. The resting potential of T lymphocytes (−40 to −70 mV) lies between the equilibrium potentials for K^+ and Cl^-. Reversal potential measurements of K^+ and Cl^- channels in the intact cell with cells bathed in normal NaCl saline or depolarized with KCl saline are consistent with intracellular K^+ and Cl^- concentrations of 140 to 150 mM and 35 to 40 mM, respectively. In the normal cell E_K would be about −85 mV and E_{Cl} −30 to −35 mV; hence, Cl^- channel activity should depolarize the resting cell. During our study of K^+ channels in cell-attached patches we observed an increase in single-channel K^+ conductance at 37°C but no significant change in the reversal potential. This suggests that the membrane potential changed by less than 5 or 10 mV (limit of resolution of E_{rev}). If Cl^- channels are activated at 37°C then why does the membrane potential not significantly depolarize? From results in this paper and Pahapill and Schlichter (1991), the voltage and temperature dependence of the K^+ and Cl^- currents makes it likely that the membrane potential will remain ~−50 mV. This can be illustrated with a sample calculation. The total K^+ or Cl^- current at any potential is given by

$$I = \gamma \cdot N \cdot P_o \cdot (E - E_j)$$

where γ is the single-channel conductance, N is the total number of channels in the cell, P_o is the open probability at a particular potential and $E - E_j$ is the driving force, i.e., the deviation of the membrane potential from the Nernst potential. The approximate number of Cl^- channels in each cell can be calculated based on the prevalence of seeing channels in cell-attached patches (69% at warm temperatures). In all but one cell-attached patch (Fig. 4*C*) only one channel was seen at 37°C. If 1–5% of the cell surface was sampled during each cell-attached recording, then each cell should have 15–70 active Cl^- channels. (Excised, inside-out patches often had more than one channel in each patch, suggesting that patch excision removes some endogenous deactivation. To test this we are now examining the response of these Cl^- channels to kinases and phosphatases. For the calculation of relative current the activity in the intact cell is the most relevant.) Considering the voltage dependence in intact cells (Fig. 6, P_o ~0.3 at −50 mV), an average conductance of 250 pS and a driving force of 10–15 mV (E_{Cl} −35 to −40 mV), the total Cl^- current would be 10 to 80 pA at 37°C. For the voltage-dependent K^+ channel, the conductance is about 10 pS at −50 mV, there are about 400 channels in the cell, the driving force is about 40 mV (E_K −89 mV at 37°C), and P_o is about 0.3 at 37°C. The K^+ current would be about 50 pA at −50 mV. In addition, we have found small conductance Ca^{2+}-dependent K^+ channels that can contribute to the membrane potential (P.A. Pahapill & L.C. Schlichter, *unpublished results*). Given the similarity in total K^+ and Cl^- current amplitudes, it is not surprising the membrane potential changes little with temperature. Moreover, any forces that tend to change the membrane potential will be opposed by the dual tendency for K^+ channels to close and Cl^- channels to open with hyperpolarization and for K^+ channels to open and Cl^- channels to close with depolarization. The membrane potential will tend to be "clamped" in a narrow range. This may be advantageous since imposed changes in membrane potential tend to inhibit lymphocyte functions (Schlichter & MacCoubrey, 1989; *see also* Grinstein & Dixon, 1989). This interpretation is further supported by a failure to observe Cl^- current "drooping" during cell-attached recordings at 37°C. When a Cl^- channel opens the cell will tend to depolarize,

but the current through one such channel (~1.1 pA) will be much smaller than the K^+ current through the rest of the cell (i.e., 250 pS · 15 mV · 0.3 P_o · 1 channel = 1.1 pA, compared with I_K ~50 pA).

A conductive Cl^- pathway is involved in the regulation of cell volume in lymphocytes and other cells (*see* Cahalan & Lewis, 1988; Deutsch & Lee, 1988; Falke & Misler, 1989; Grinstein & Dixon, 1989). In response to cell swelling in hypotonic media, conductance pathways for K^+ and Cl^- efflux are activated, water follows and normal cell volume is restored (RVD). Deutsch and Lee (1988) proposed that RVD involves an activation of Cl^- conductance which depolarizes the cell toward E_{Cl} and in turn induces gradual activation of $I_{K(V)}$ conductance. The voltage-dependent properties of the K^+ channels (Pahapill & Schlichter, 1991) and maxi-Cl^- channels in intact cells are ideally poised to prevent large excursions of membrane potential and to maintain the outward driving forces necessary for RVD. However, we have no direct evidence for a role for the maxi-Cl^- channel in RVD.

Interestingly, the maxi-Cl^- channel is permeable to some organic anions, including HCO_3^- and phosphates. If these channels open then internal organic anionic species may partly substitute for Cl^-, particularly if intracellular Cl^- becomes significantly depleted. If HCO_3^- is lost through Cl^- channels then pH_i should fall. Low pH_i might serve as a negative feedback signal since low pH inhibits $I_{K(V)}$ channels in normal human T lymphocytes (Deutsch & Lee, 1989). The anion channel blockers, SITS and DIDS, inhibit T lymphocyte activation and the cell-mediated cytotoxicity produced by cytotoxic T lymphocytes (CTL) and natural killer (NK) cells (Gray & Russell, 1986; Rosoff et al., 1988; L.C. Schlichter, *unpublished results*). Because the concentrations of SITS and DIDS required were ~100 times higher than needed to block the Cl^-/HCO_3^- exchanger, it was proposed that Cl^- channels are involved in each of these processes. Until we find highly specific inhibitors of the different types/conductances of Cl^- channels it will be difficult to prove which underlie a particular Cl^--dependent event such as activation, volume regulation or cellular cytotoxicity.

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