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Outwardly Rectifying Chloride Channels in Lymphocytes

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Summary. Outwardly rectifying Cl^- channels in cultured human Jurkat T-lymphocytes were activated by excising a patch of membrane using the inside-out (*i/o*) patch-clamp configuration and holding at depolarized voltages for prolonged periods of time (1–6 min at +80 mV, 20°C). The single-channel current at +80 mV was 4.5 ± 0.3 pA and at −80 mV, it was 1.0 ± 0.4 pA. After activation, the probability of being open (P_o) for the lymphocyte channel was voltage independent. Activation of the Cl^- channel in lymphocytes was temperature dependent. Nineteen percent of *i/o* recordings from lymphocytes made at 20°C exhibited Cl^- channel activity. In contrast, 49% of recordings made at 30°C showed channel activity. The number of channels in an active patch was not significantly different at the two temperatures. Channel activation in excised, depolarized patches also occurred 20-fold faster at 30°C than at 20°C. There was no marked change in the single-channel conductance at 30°C. Open-channel conductance was blocked by 200 μM indanyloxyacetic acid (IAA) or 1 mM SITS when applied to the intracellular side of the patch. The characteristics of this channel are similar to epithelial outwardly rectifying Cl^- channels thought to be involved in fluid secretion.

Key Words chloride channel · lymphocyte · outward rectification · temperature · regulation

Introduction

Apical Cl^- channels are vital to basic physiological processes involved in salt and water secretion by the intestine, trachea and exocrine glands (for review, *see* Gögelein, 1988; Frizzell & Halm, 1990). In exocrine glands, fluid secretion provides a vehicle for macromolecular secretions. In airways, fluid secretion provides a liquid layer necessary for mucociliary clearance. In the intestine, Cl^- permeability regulates the fluid content of the lumen. In Cl^- secreting epithelia, second-messenger regulated Cl^- and K^+ conductances control the rate of secretion. Several types of Cl^- channels have been described at the single-channel level in epithelial and other cell types (for review *see* Frizzell & Halm, 1990). One or more of these channel types is likely to be involved in the regulation of epithelial fluid secretion and absorption.

Outwardly rectifying Cl^- channels have received much attention. These channels have been described in epithelial cells, fibroblasts, lymphocytes, and other cell types (Schoumacher et al., 1987; *see also* Frizzell & Halm, 1990; Bear, 1988; Li et al., 1988; Chen, Schulman & Gardner, 1989). When recorded using the inside/out patch-clamp configuration, this channel can be activated in the presence of exogenously applied catalytic subunit of protein kinase A (PKA) or protein kinase C (Schoumacher et al., 1987; Bear, 1988; Li et al., 1988, 1989; Chen et al., 1989; Hwang et al., 1989). Activation of the outwardly rectifying Cl^- channel may drive fluid secretion directly, or it may do so indirectly, by stimulating bicarbonate secretion via the Cl/HCO_3 anion exchanger that is present in the apical membranes of secretory epithelia (Hanrahan & Tabcharani, 1989). The abnormal regulation of this channel in tissues of cystic fibrosis (CF) origin suggests that it may contribute to the abnormal regulation of fluid secretion that is characteristic of this disease.

The physiological function of this outwardly rectifying Cl^- channel in cell types other than secretory epithelia, such as lymphocytes, remains unclear. As an initial step towards understanding the physiological role of this channel in lymphocytes, I have investigated the effects of two pharmacological agents, IAA and SITS, and of temperature on channel activity. I have found that the characteristics of this channel are generally similar to that of the outwardly rectifying, epithelial Cl^- channel. The two channel types are, however, distinct at the biophysical level. These differences

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suggest that there may be a family of outwardly rectifying Cl^- channels which serve different cellular functions.

A preliminary form of this work has been presented in abstract form (Garber, 1990).

Materials and Methods

Recordings were made from Jurkat cells, a human leukemic T-lymphocyte line, using standard patch-clamp techniques (Hamill et al., 1981). Cells were maintained in suspension with RPMI 1640 medium supplemented with 10% fetal calf serum at 5% CO_2, and they were split and fed every other day. Small aliquots of cells were washed 2–3 times in recording bath solution and allowed to adhere to a glass coverslip 5–10 min before recording.

Cl^- channels were activated and recorded using the inside/out recording conformation. Cl^- channel activity was elicited after excising a patch of membrane and holding at a depolarized voltage for a prolonged period of time (e.g., +80 mV for 1–6 min). If no channel activation occurred, the voltage was increased in 20 mV increments to a maximum of +120 mV and held for a similar amount of time. Time required to activate channels was taken from the time of patch excision (t = 0 sec), at a holding voltage of +80 mV, to activation of stable unitary channel amplitude.

Experiments were performed with a LSI 11/73 (Digital Equipment, Maynard, MA) based minicomputer system (Indec Systems, Sunnyvale, CA). The output of an EPC-7 patch-clamp amplifier (List-Medical Systems, Greenvale, NY) was low-pass filtered through an 8-pole Bessel filter (Frequency Devices, Haverhill, MA) and digitized as indicated. Junctional potential was nulled immediately before obtaining a seal. Records were not corrected for junctional potential errors (≤5 mV).

Electrophysiological data were obtained and analyzed as described by Hoshi and Aldrich (1988*a*,*b*). Briefly, amplitude histograms were constructed from nonleak subtracted, non-idealized traces. Peak P_o was determined from ensemble averages of single-channel 'idealized' traces. (Traces were 'idealized' after leak subtraction.) Opening and closing transitions were detected using as criterion a level of 50% of the unitary current amplitude at a particular voltage. Single-channel recordings were made from >50 cells.

Pipette contained (in mM): 154 NaCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES-NaOH, pH 7.3. Bath solution was either the same as in the pipette or (in mM) 140 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 11 EGTA, 10 HEPES-NaOH, pH 7.3. Thus, recordings of channel activity in *i/o* patches were made in symmetrical [Cl^-] solutions, unless otherwise indicated. Channel activity was not markedly affected by the composition of the bath solution. I will use normal cellular conventions when referring to the direction of current. In other words, *outward* current refers to *inward* Cl^- ion flow. The distinctive, rectifying *I/V* relationship was used as a marker for Cl^- channel activity.

SITS was obtained from Sigma. Indanyloxyacetic acid (IAA-94,95) was the generous gift of Dr. M. Akabas (Columbia University).

Results

The outwardly rectifying Cl^- channel from epithelial cells can be activated, in the absence of kinase, by depolarizing an inside/out patch of membrane (e.g., +80 mV) for several minutes (Frizzell & Halm, 1990). Excised, depolarization-activated channels remain active for the duration of experimental procedures (~30 min). This procedure, while nonphysiological, provides a convenient assay to test for channel activity in the patch. Outwardly rectifying Cl^- channels in lymphocytes can also be activated using this paradigm (Chen et al., 1989). Channel activity elicited using excision and depolarization appears similar to that elicited in the presence of the catalytic subunit of cAMP-dependent protein kinase (Chen et al., 1989). Channel activation may occur abruptly, with an initial channel opening to the full unitary conductance in a single step. Alternatively, opening of the channel to the full unitary conductance level may be preceded by poorly resolved current fluctuations lasting for 1–90 sec. There was no marked correlation between the manner of activation and any notable characteristic of the activated channel. Similar activity before 'full' activation of an ion channel to the unitary conductance level has been observed for the outwardly rectifying Cl^- channel in excised/depolarization-activated channels from epithelial cells (Halm et al., 1988*b*).

Single-Channel Conductance

Single-channel activity of excised and voltage-activated outwardly rectifying Cl^- channels from a Jurkat T-lymphocyte, in response to a steady-state command voltage, is shown in Fig. 1*A*. The anion selectivity of this channel in lymphocytes has been previously demonstrated (Chen et al., 1989). Altering the cation composition of either the bath or pipette solutions did not result in a shift of the reversal potential in either case (*not shown*). The current flowing through this channel at +80 mV was greater than the current flow at −80 mV, resulting in an outward rectification of the current. This rectification can be seen clearly in Fig. 1*B*, in which open-channel current elicited in response to a voltage ramp from −100 to +100 mV is shown. The single-channel current of the Cl channel at +80 mV is 4.5 ± 0.3 pA (n > 5). Recordings were made in symmetrical Cl^- solutions, so that the rectification cannot simply be due to different concentrations of the permeant anion on either side of the channel. Thus, rectification is likely to be an inherent property of ion permeation in this channel.

The ratio of single-channel conductance (γ_s) at +80 to −80 mV is 4.3 ± 0.7. Such a ratio serves as a reliable indicator of the degree of current rectification flowing through this channel. The value of γ_s at 0 mV is dependent on the slope of the line drawn tangent to the current at 0 mV, and thus, the mea-

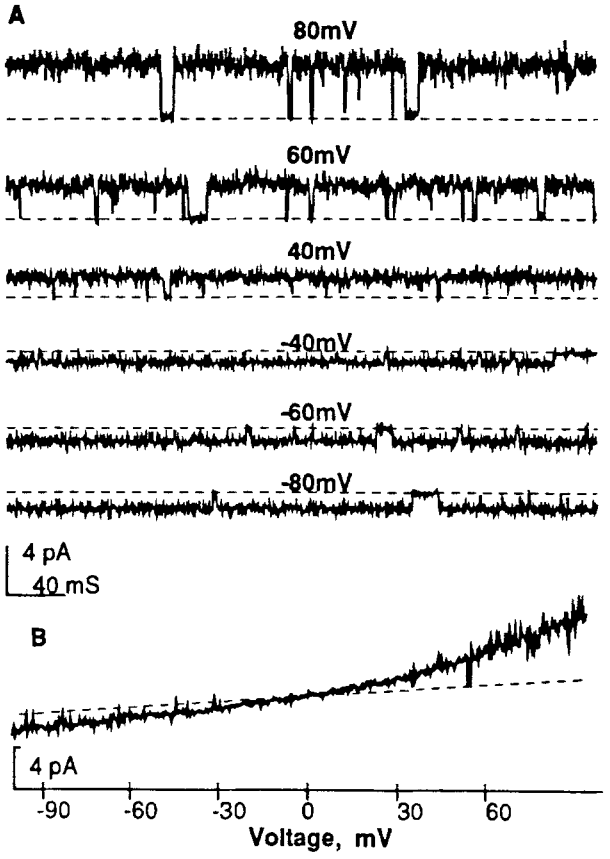

Fig. 1. Rectifying current/voltage relationship. (*A*) Representative traces of single Cl^- channel activity in response to indicated steady-state holding voltages. (*B*) Rectifying open channel current in response to a voltage ramp from −100 to +100 mV. Length of ramp was 250 mS. All traces were recorded from the same patch. Dashed lines indicate closed, nonconducting level. Experimental temperature was 20°C; filter 500 Hz.

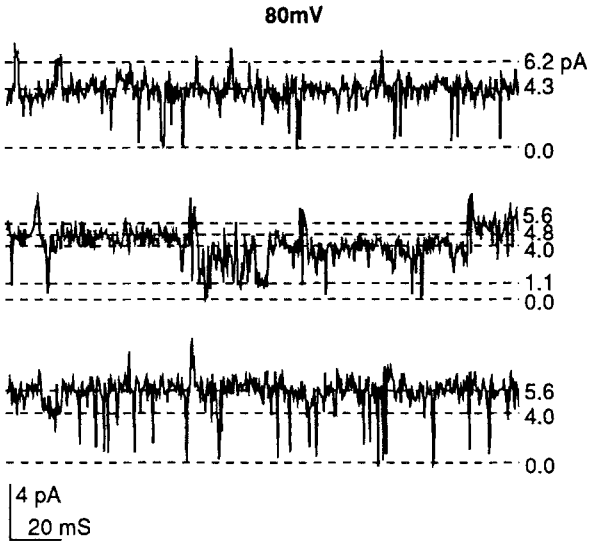

Fig. 2. Alternate conductance states. Sequential traces of the activity of a patch, containing at least one active Cl^- channel from lymphocyte cells, recorded at 20°C. Several conductance levels are apparent. Dashed lines indicate discernable levels of current amplitude as noted. (Filter, 2000 Hz.)

surement of γ_s in even a single experiment may vary. For example, measurement of the γ_s at 0 mV ranged from 40–50 pS over several experiments ($n > 5$). The γ_s reported by Chen and colleagues falls within this range. The *I/V* relationship reported by Chen et al. (1989), however, suggests that the unitary current of the channel is generally greater than that reported here. Recordings made of an outwardly rectifying Cl^- channel from airway epithelia made under similar conditions (in the same laboratory) revealed it to have a greater unitary current at +80 mV (6.0 ± 0.3 pA) but a lower degree of rectification ratio (2.4 ± 0.8) when comparing γ_s at +80 and −80 mV (Garber, 1990; Cozens et al., 1992).

Close inspection of single-channel records reveals the occasional presence of alternate conductance states. An example of this is shown in Fig. 2. Several different conductance levels can be discerned when the channel is open. When the predominant conductance level (i.e., 4.5 ± 0.3 pA at +80 mV) ceased, no other channel activity was observed. This suggests that the conductance levels are the result of ion permeation through a single ion channel protein. Alternatively, if a second smaller channel is present, its activity must somehow be coordinated with the large conductance channel.

Gating activity of the channel was unstable in that the channel sometimes remained mostly open for a period of time, then switched into a mode exhibiting fast opening and closing, and then returned to a mostly open state (*see* Fig. 2). Note that the trace in Fig. 2 was filtered at 2 kHz. Unstable gating activity of single-channel recordings was observed spontaneously for short periods of time (e.g., ~1 sec), independent of temperature or recording conditions. Amplitude histograms did not show extra peaks, however, indicating that such events contributed little to the average unitary conductance. Recordings made at slower speeds and with a lower cutoff frequency (e.g., Fig. 1) resulted in the average apparent unitary conductance that is reported above.

Voltage Dependence of Opening

Channel activity showed long-duration openings which were interrupted, at all voltages, by either brief or longer-lasting closing events (*see* Fig. 1).

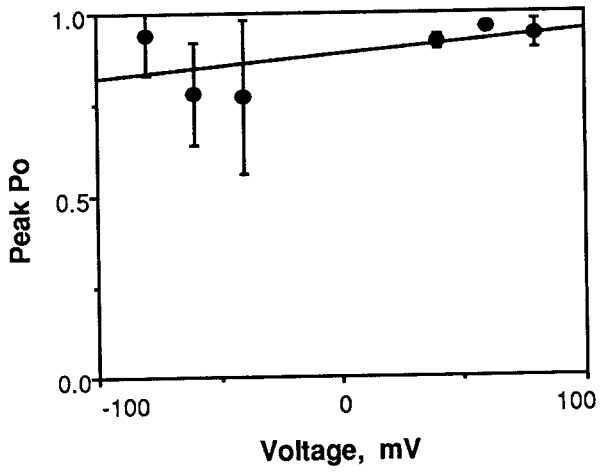

Fig. 3. P_o is independent of voltage. The open probability of outwardly rectifying Cl^- channels in lymphocytes is independent of voltage. The peak P_o from three experiments was averaged and plotted against voltage (SEM are shown). The solid line indicates a simple linear regression fit to the data with $y = 0.89 + 0.0007x$ ($r = 0.33$).

Figure 3 shows the average probability of the channel being open (P_o) in response to voltage (V) for three experiments, including the experiment shown in Fig. 1. The average P_o for these three experiments does not change appreciably over a voltage range from −80 to +80 mV for outwardly rectifying Cl^- channels from Jurkat lymphocytes. In contrast, the P_o of outwardly rectifying Cl^- channels from epithelia is voltage dependent (Hayslett et al., 1987; Halm et al., 1988*a*; Garber, 1990).

Temperature Dependence of Activity

Figure 4*A* shows the activity of a patch containing at least three active Cl^- channels elicited at 30°C. When the temperature of the patch was decreased to 20°C only two channels appeared active (Fig. 4*B*). The single-channel conductance was not significantly different over this temperature range (2.2 pA ± 0.1 at 20°C and 2.3 pA ± 0.1 at 30°C). A further decrease in temperature (15°C) resulted in further decrease in the number of active channels in the patch (*not shown*). These observations were notable in that, when maintaining a constant temperature, the number of active channels in a patch was not normally observed to decrease during the course of an experiment. If the number of active channels in a patch did change during an experiment at constant temperature, it was most commonly via an increase in the number of active channels. Recordings were made at 30°C rather than 35–37°C because these latter recordings were invariably unstable and short-lived, despite showing a large number of events from channels of many different single-channel amplitudes. It was not clear whether this increased activity resulted from distinct ion-channel proteins or was the result of a single ion channel becoming unstable. Patch recordings at 30°C showed both sufficient stability and clearly identifiable channel fluctuations throughout each experiment.

The most significant effect of temperature on outwardly rectifying Cl^- channel activity in lymphocytes was the time required to hold a patch at a depolarized voltage (≥ +80 mV) before activation. The time preceding activation decreased from >200 sec at 20°C to <20 sec at 30°C (Fig. 5*A*). The number of patches containing active channels also increased (from 19 to 49%) with higher experimental temperature (Fig. 5*B*). The average number of channels per active patch, however, did not increase significantly (Fig. 5*C*). No appreciable channel activity in cell-attached patches was observed at either 20 or 30°C.

Blocking Agents

Indanyloxyacetic acid (IAA) was used by Landry and colleagues to block Cl^- flux in vesicles made from bovine kidney cortex (Landry et al., 1987). Kunzelmann and colleagues have also shown that IAA is effective in blocking outwardly rectifying Cl^- channels in several types of epithelial cells (Kunzelmann, Tilmann & Greger, 1990). Application of 20 μM IAA to the intracellular face of single-channel currents from Jurkat lymphocytes resulted in appearance of fast-closing events interrupting normally long-duration open events (Fig. 6*A*; $n = 3$). The frequency of these events increased with application of 200 μM IAA (Fig. 6*B*; $n = 3$). The endogenous, complex gating activity of the channel in the absence of blocking agents prevented a straightforward quantitation and interpretation of the block. This is the subject of a future study.

Disulfonic stilbenes, such as SITS, are considered to be classic, albeit nonspecific, blockers of Cl^- transport proteins. Application of the stilbene disulfonate, SITS, to the intracellular face of an outwardly rectifying Cl^- channel from lymphocytes resulted in a fast 'flickery-type' block of the single-channel conductance similar to that observed with IAA but at a 10-fold higher concentration (1 mM; $n = 3$) (Fig. 7).

Open-channel activity of the outwardly rectifying Cl^- channel in inside-out patches was not affected by applications (to the intracellular face of the channel) of bath solutions over a pH range of 6.8 to 7.9 or bath solutions containing $[Ca^{2+}] = 1$ nM to 1 mM, 1 mM ATP (alone), 1 mM Mg^{2+} (alone, or with

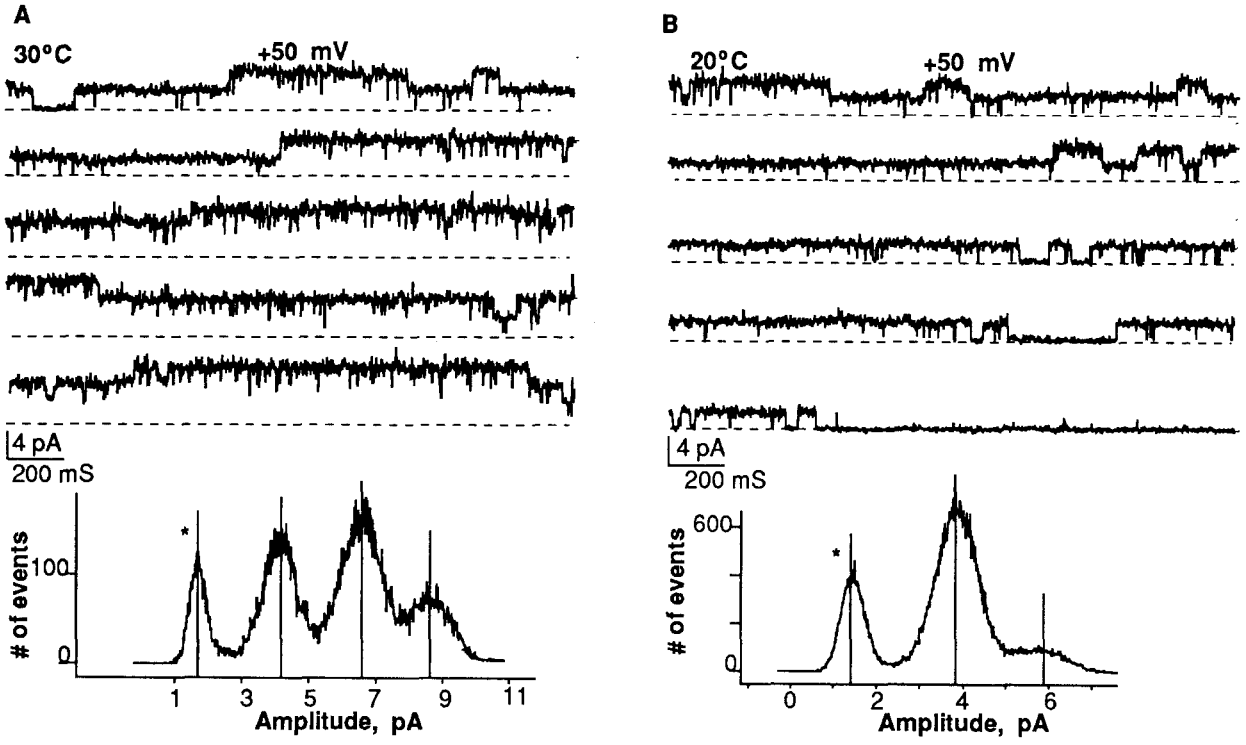

Fig. 4. Cl^- channel activity increases with temperature. Sequential, steady-state recordings (at +50 mV) made from an inside/out patch containing at least three Cl^- channels from a lymphocyte cell. (*A*) Patch was formed and channels activated at 30°C. (*B*) Channel activity in the same patch decreased as the temperature was lowered to 20°C. Amplitude histograms (*below*) indicate there was no change in the single-channel amplitude over this temperature range (2.2 pA ± 0.1 at 20°C and 2.3 pA ± 0.1 at 30°C). Asterisk indicates nonconductance level in histogram. Dashed lines indicate closed, nonconducting level. (Filter, 500 Hz)

ATP), 100 μM dibutryl- or 8-bromo-cAMP, NaCl, KCl, or N-methyl-D-glucosamine-Cl (*data not shown*).

Discussion

The characteristics of an outwardly rectifying Cl^- channel in Jurkat T-lymphocytes, presented here, were similar to outwardly rectifying Cl^- channels described in epithelial cells. Both channels, for example, are activated in inside-out patches with prolonged depolarization, exhibit an outwardly rectifying *I/V* relationship, and their open-channel conductance could be blocked by indanyloxyacetic acids and disulfonic stilbenes (Schoumacher et al., 1987; Li et al., 1988; Bridges et al., 1989; Greger, Kunzelmann & Gerlach, 1989; Frizzell & Halm, 1990; Garber, 1990; Kunzelmann et al., 1990; Cozens et al., 1992).

The activity of the lymphocyte channel is also dependent on experimental temperature, as has been reported for outwardly rectifying Cl^- channels recorded from epithelial cells (Greger et al., 1989; Welsh, Li & McCann, 1989). Temperature-dependent activity of a maxi-Cl^- channel in T-lymphocytes has also been reported by Pahapill and Schlichter (1990). There are several possible explanations to account for temperature dependence of channel activity. Regulation of ion channel activity by second messengers, for example, has been shown to be temperature dependent in some systems (Walsh, Begenisich & Kass, 1989). Kunzelmann and colleagues have suggested that the outwardly rectifying Cl^- channel in epithelia may be inhibited by a cytosolic factor (Kunzelmann et al., 1990). Such a factor may be separated from the channel on excision and would thus enhance channel activation. It is possible that this factor may also be membrane associated, and thus, able to regulate channel activity in a temperature-dependent manner. An alternative possibility is that the temperature dependence of activation may indicate the amount of free energy required to move the channel protein from an inactive protein conformation to one that is active and conducting.

Landry et al. (1987) have used IAA to inhibit chloride transport in membrane vesicles derived from bovine kidney cortex. Cl^- transport and binding inhibition assays both yielded an IC_{50} of <10 μM.

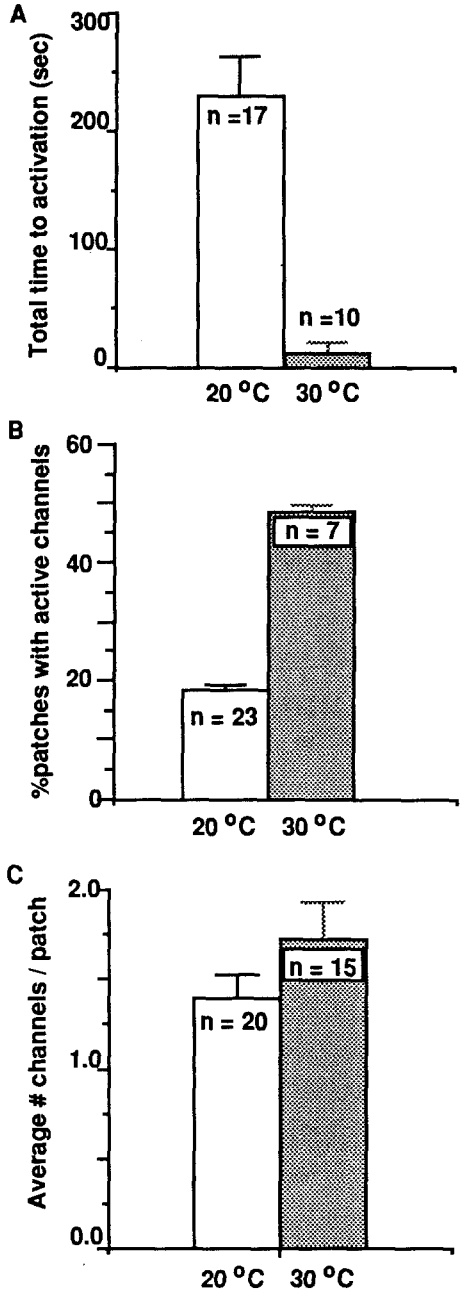

Fig. 5. Time to activation decreases with increasing temperature. (*A*) The time required to hold at a depolarized voltage in order to activate Cl^- channels at 20°C is 226 ± 33 sec. At 30°C, the time to activation is reduced to 11 ± 10 sec. (*B*) Nineteen percent (±2%) of inside/out, depolarized patches contained Cl^- channel activity when the temperature was 20°C. In contrast 49 ± 3% of the patches showed activity when the temperature was 30°C. (*C*) On average, excised patches contained at least one active channel (1.4 ± 0.1 channels at 20°C; 1.7 ± 0.2 channels at 30°C). The small increase in the average number of active channels/patch at 30°C is not significant. The number of individual patches from different lymphocyte cells used in averages is given as "*n*." Bars indicate standard errors.

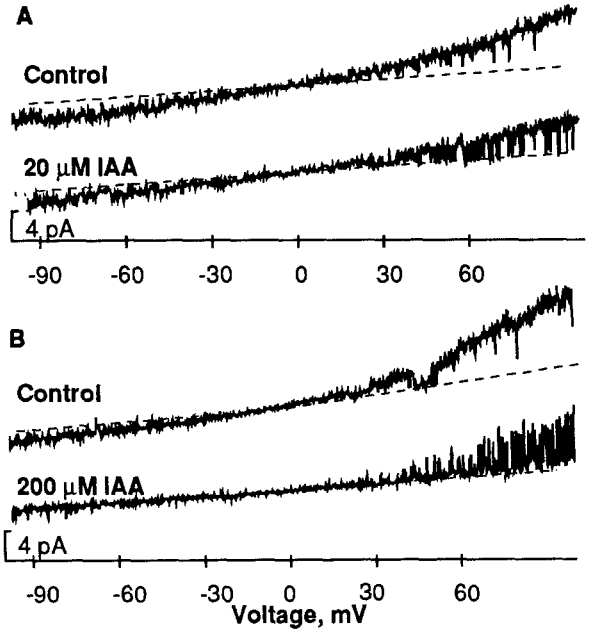

Fig. 6. IAA interrupts the open-channel conductance of the outwardly rectifying Cl^- channel with fast-closing events when bath applied to the intracellular face of the channel: (*A*) 20 μM IAA; (*B*) 200 μM IAA. Two different experiments are shown (control and + IAA from the same patch in each case). There is only a single channel in each patch. Voltage ramps, from −100 to +100 mV, were applied over 250 mS. Dashed lines indicate closed, nonconducting level. (Temperature, 20°C; filter, 500 Hz)

Reconstitution of these vesicles into planar lipid bilayers showed several different types of Cl^- channels but not one with an outwardly rectifying current-voltage relationship (Landry et al., 1989). The orientation of the channels in the vesicle population studied by Landry and colleagues was probably mixed, with the cytoplasmic face of some of the channel proteins facing the inside of the vesicle and some facing the outside of the vesicle. It is difficult to compare the affinity of IAA for Cl^- channels in the vesicle preparation to the block of the outwardly rectifying Cl^- channel described here. It is likely, however, that if the vesicle population contained cytoplasmically exposed outwardly rectifying Cl^- channels, that their contribution to the IAA inhibited Cl^- flux was minimal in part because of the high affinity of IAA in this preparation reported by Landry and colleagues (1989).

Differences between the lymphocyte and epithelial channel, however, do exist at the biophysical level. This includes differences in the unitary conductance and the dependence of the P_o on voltage. The unitary conductance of single epithelial channels, which I have recorded under similar conditions in the same laboratory, is higher than that of the

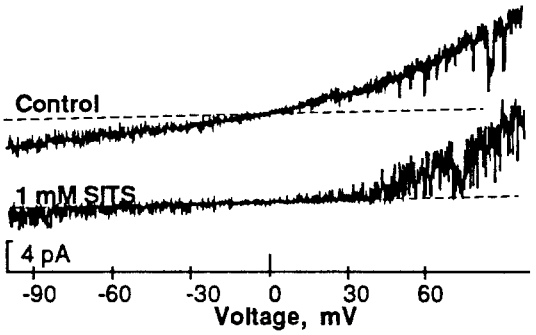

Fig. 7. SITS is effective at a 10- to 100-fold higher concentration than IAA in producing fast blocking events of the open-channel conductance. Control and treated traces are taken from a single experiment in which there are two channels in the patch. Dashed lines indicate closed, nonconducting level. (Temperature, 20°C; filter, 500 Hz)

lymphocyte channel (Garber, 1990; Cozens et al., 1992). Other reports also indicate that there is a greater unitary conductance in the epithelial channel (Hayslett et al., 1987; Lin & Gruenstein, 1987; Schoumacher et al., 1987; Halm et al., 1988*a*; Li et al., 1988). Although previous reports of the unitary current amplitude of the lymphocyte channel are greater than those reported here, such differences may be due to experimental technicalities such as leak subtraction techniques or shifts in junction potentials.

The difference in the unitary conductance of this channel in lymphocytes and epithelial cells, however, is relatively small and may not be of great physiological importance. A physiologically more relevant parameter is P_o, the probability of a channel being open in response to voltage. In the lymphocyte, this parameter is independent of voltage. The P_o of outwardly rectifying channels recorded from epithelia, however, is voltage dependent (Hayslett et al., 1987; Halm et al., 1988*a*; Garber, 1990; Cozens et al., 1992).

One likely physiological function of the outwardly rectifying Cl^- channel in epithelial cells is regulation of fluid secretion. Fluid secretion, however, appears less important to lymphocyte function except during volume regulation. Thus, the channel is likely to have a different physiological role in lymphocytes. Possible roles include housekeeping functions such as volume regulation, maintenance of the resting potential, and regulation of bicarbonate concentration, in addition to potential involvement in macromolecular secretion. Cahalan and Lewis (1988) have shown the activation of an osmotically induced outwardly rectifying Cl^- current in T-lymphocytes. Noise analysis of these currents, however, suggests that the unitary conductance of the channels underlying the macroscopic current is much smaller (<5 pS) than that of the channel studied here (Cahalan & Lewis, 1988). A pressure-sensitive, outwardly rectifying Cl^- channel from epithelia has a similar unitary conductance as the channel studied here but has different biophysical characteristics (Solc & Wine, 1991).

Predepolarization has been observed to facilitate the activation of a Ca^{2+} curent in chromaffin cells (Hoshi & Smith, 1987; Artalejo et al., 1991). This current is observed at both the macroscopic and single-channel levels and is mediated by a cAMP/PKA-dependent mechanism (Artalejo et al., 1990; 1991). The facilitation of this Ca^{2+} current may play a role in catecholamine secretion. The activation of this Ca^{2+} current by depolarization and its dependence on cAMP as a second messenger is reminiscent of the outwardly rectifying Cl^- channel described here. There are differences, however, in that the experimental activation of the Cl^- channel appears to require excision as well as greater and longer-lasting depolarization (+80 to +120 mV from rest for several minutes, rather then a fraction of a second). The normal physiological means of activating this Cl^- channel has not yet been determined. In fact, activation of an outwardly rectifying Cl^- current or channel in neither epithelial cells nor lymphocytes has been observed by depolarization using the whole-cell patch-clamp configuration and is rarely observed in cell-attached patch recording configurations (Schoumacher et al., 1987; Halm et al., 1988*b*; Cliff & Frizzell, 1990; S.S. Garber, *unpublished*). It is conceivable that under certain 'whole-cell' conditions, such as during a prolonged depolarization of the cell, the outwardly rectifying Cl^- channel would activate, thus allowing Cl^- ions to flow into the cell. This current flow would tend to repolarize the cell towards the resting potential and, in addition, could also regulate cellular volume or fluid secretion.

While the physiological role of this channel is likely to be specific to cell type, the gross similarity of the outwardly rectifying Cl^- channels in lymphocytes and epithelial cells suggests that these channels may belong to a structurally related family of ion channels, like that described for Shaker-type K^+ channels (Jan & Jan, 1990). Identification of the genes encoding these channel proteins, in the future, will provide an important basis for functional comparison at the molecular level.

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