The Tyrosine Kinase p56\textsuperscript{lek} Mediates Activation of Swelling-induced Chloride Channels in Lymphocytes

Albrecht Lepple-Wienhues, Ildikó Szabó, Tilmann Laun, Nubia Kristen Kaba, Erich Gulbins, and Florian Lang

Department of Physiology, University of Tübingen, D-72076 Tübingen, Germany

Abstract. Osmotic cell swelling activates Cl\textsuperscript{−} channels to achieve anion efflux. In this study, we find that both the tyrosine kinase inhibitor herbimycin A and genetic knockout of p56\textsuperscript{lek}, a src-like tyrosine kinase, block regulatory volume decrease (RVD) in a human T cell line. Activation of a swelling-activated chloride current (I\textsubscript{Cl\textsuperscript{−},swell}) by osmotic swelling in whole-cell patch-clamp experiments is blocked by herbimycin A and lavendustin. Osmotic activation of I\textsubscript{Cl\textsuperscript{−},swell} is defective in p56\textsuperscript{lek}-deficient cells. Retransfection of p56\textsuperscript{lek} restores osmotic current activation. Furthermore, tyrosine kinase activity is sufficient for activation of I\textsubscript{Cl\textsuperscript{−},swell}. Addition of purified p56\textsuperscript{lek} to excised patches activates an outwardly rectifying chloride channel with 31 pS unitary conductance. Purified p56\textsuperscript{lek} washed into the cytoplasm activates I\textsubscript{Cl\textsuperscript{−},swell} in native and p56\textsuperscript{lek}-deficient cells even when hypotonic intracellular solutions lead to cell shrinkage. When whole-cell currents are activated either by swelling or by p56\textsuperscript{lek}, slow single-channel gating events can be observed revealing a unitary conductance of 25–28 pS. In accordance with our patch-clamp data, osmotic swelling increases activity of immunoprecipitated p56\textsuperscript{lek}. We conclude that osmotic swelling activates I\textsubscript{Cl\textsuperscript{−},swell} in lymphocytes via the tyrosine kinase p56\textsuperscript{lek}.

When cells are exposed to hypotonic stress, initial swelling is followed by regulatory volume decrease (RVD).\textsuperscript{1} In lymphocytes, RVD involves activation of volume-sensitive anion channels, leading to membrane depolarization and thereby opening voltage-activated potassium channels (Deutsch and Lee, 1988). This produces a net loss of KCl, resulting in regulatory decrease of intracellular osmolarity and driving H\textsubscript{2}O out of the cell. Anion channels activated by osmotic stress are expressed in a wide variety of nonexcitable and excitable tissues (for review see Lang et al., 1997), and are thought to mediate an efflux of osmotically active anions in response to increased cellular volume (Cahalan and Lewis, 1988).

Biophysical and pharmacological differences have been observed between swelling-activated anion channels found in lymphocytes and other tissues (Kunzelmann et al., 1989; Solc and Wine, 1991; Sorota, 1992; Lewis et al., 1993). Most of these channels are outwardly rectifying chloride channels ranging from 20 to 90 pS. At least three different anion channels have been characterized at the single channel level in membrane patches from lymphocytes (Lewis and Cahalan, 1988; Nishimoto et al., 1991; Garber, 1992). However, a clear relationship between single-channel currents and whole-cell currents is lacking. Although the genes for a number of different Cl\textsuperscript{−} channel proteins have been cloned, the proteins forming swelling activated anion channels in lymphocytes have not yet been identified. Their activation mechanism is even less completely understood. In lymphocytes, neither an increase in cytosolic calcium nor in membrane area is necessary for activation of volume-sensitive anion channels (Ross et al., 1994; Ross and Cahalan, 1995). However, participation of cytoskeletal elements in channel activation has been demonstrated (Levitan et al., 1995). Most interestingly, the presence of intracellular ATP is necessary for maintenance or repeated activation of the swelling-activated anion current (Lewis et al., 1993; Ross et al., 1994). Recent work has shown inhibition of volume-sensitive chloride current in heart cells by tyrosine-kinase inhibitors (Sorota, 1995) and regulation of the cystic fibrosis transmembrane conductance regulator (CFTR) channel by tyrosine phosphorylation (Fischer and Machen, 1996). In this study we have examined the role of a src-like lymphocyte tyrosine kinase, p56\textsuperscript{lek}, in the activation of swelling-activated anion channels in lymphocytes.

\textsuperscript{1}Abbreviations used in this paper: RVD, regulatory volume decrease; I\textsubscript{Cl}, chloride current; I\textsubscript{Cl\textsuperscript{−},swell}, swelling-activated chloride current; CFTR, cystic fibrosis transmembrane conductance regulator; DIDS, diisothiocyanato-2-2-stilbene-sulfonic acid.

\textsuperscript{E. Gulbins and F. Lang contributed equally to this work.}

\textsuperscript{Address all correspondence to Albrecht Lepple-Wienhues, Physiology I, University of Tübingen, Gmelinstrasse 5, D-72076 Tübingen, Germany. Tel.: (49) 7071-2975285. Fax: (49) 7071-293073. E-mail: alepplew@uni-tuebingen.de}
Materials and Methods

Cell Culture and Stimulation

Jurkat and p56\(^{\text{ck}}\)-deficient JCaM1.6 cells were obtained from the American Type Culture Collection (Rockville, MD). p56\(^{\text{ck}}\) reconstituted JCaM1.6 (JCaM1.6Lck\(^{+}\)) were a gift from Dr. A. Weiss (University of California, San Francisco, CA). The cells were grown in RPMI-1640 medium supplemented with 10% FCS, 10 mM Hepes, pH 7.4, 2 mM \(\text{Ca}^{2+}\), 1 mM sodium pyruvate, 100 \(\mu\text{M}\) nonessential amino acids, 100 \(\mu\text{U}\) penicillin, 100 \(\mu\text{g}\) streptomycin (all purchased from Life Technologies, Eggenstein, Germany), and 50 \(\mu\text{M}\) \(\beta\)-mercaptoethanol. JCaM1.6Lck\(^{+}\) were cultured in 250 \(\mu\text{g}\)/ml hygromycin, which was removed 48–72 h before beginning of experiments. Stable expression of p56\(^{\text{ck}}\) was verified (data not shown). The specific src-like tyrosine kinase inhibitor herbimycin A (Calbiochem, Bad Soden, Germany) was added to the cells at a concentration of 10 \(\mu\text{M}\) 7–8 h before the experiments.

Solutions

Cells were superfused with modified Ringer’s containing 145 mM NaCl, 5 mM KCl, 2 mM CaCl\(_2\), 1 mM MgCl\(_2\), 10 mM glucose, and 10 mM Hepes, pH 7.4 (310 mOsm/kg). The internal pipette solution was used to separate Cl\(^-\) currents in lymphocytes (Ross et al., 1994) and contained 160 Cs\(^+\)–glutamate, 0.1 mM CaCl\(_2\), 2 mM MgCl\(_2\), 1.1 mM EGTA, 4 mM Na\(_2\)ATP, and 10 mM Hepes, pH 7.2 (330 mOsm/kg). For hypertonic intracellular conditions, this solution was diluted as indicated. For hyper- tonic conditions, sucrose was added to obtain the indicated osmolality. When varying ATP concentrations, 2 mM Na glutamate replaced 1 mM Na\(_2\)ATP, respectively. Purified p56\(^{\text{ck}}\) was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). All other chemicals were obtained from Sigma Chemical Co. (Deisenhofen, FRG). Osmolality was measured using a freezing point osmometer (Knauer, Berlin, FRG).

Volume Measurements

Volume changes were measured on the stage of an inverted microscope (Axiovert 135; Carl Zeiss, Oberkochen, Germany) by video imaging. Cells were loaded with 2 \(\mu\text{M}\) calcein-AM (Molecular Probes, Eugene, OR) for 15 min. Excitation was performed at 497 nm using a monochromator (Uhl, Munich, Germany). Video images were recorded at 521 nm and digitized (IMAGIT; Lindemann and Meiser, Homburg, Germany). The fluorescent cell area was analyzed using the PC version of the public domain NIH Image program (ImagePC; Scion Corp., Frederick, MD; available on the Internet via http://rsb.info.nih.gov/nih-image). Relative volume \((V/V_0)\) was calculated from the image area \((A)\) with the following relation:

\[
V/V_0 = A^{1.5}/A_0^{1.5}.
\]

Patch-Clamp Recordings

Whole-cell currents were recorded at 31°C using an EPC-9 patch-clamp amplifier and Pulse software (Heka, Lambrecht, Germany). Experiments were performed on an Axiovert 135 microscope and cells were observed using a video system to note volume changes. Pipettes were pulled to a resistance of 2–5 MΩ from borosilicate glass. For high resolution recordings, pipettes were coated with sylgard (Dow Corning, Midland, MI). Capacitive transients were cancelled using the C\(_{\text{ac}}\)–compensation of the amplifier. Series resistance was not compensated. Cells were held at 0 mV and voltage ramps or pulses of the indicated size were applied every 5 or 20 s. The current signal was filtered at 1 kHz and digitized at a 5-KHz sampling rate. By convention, anionic inward fluxes are shown as positive (outward) currents.

Immunoprecipitation and p56\(^{\text{ck}}\) Assay

Cell stimulation was terminated by lysis in 25 mM Hepes, pH 7.4, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 125 mM NaCl, 10 mM each of sodium fluoride, Na\(_2\)VO\(_4\), and sodium pyrophosphate and 10 \(\mu\text{g}\)/ml of each aprotinin and leupeptin (radioimmunoprecipitation assay buffer) for immunoprecipitation of p56\(^{\text{ck}}\). After lysis, DNA and cell debris were pelleted by centrifugation at 20,000 \(\times\) g for 15 min, and the supernatants were subjected to immunoprecipitation of p56\(^{\text{ck}}\) using an anti-p56\(^{\text{ck}}\) polyclonal antibody (Upstate Biotechnology Inc.). Control immunoprecipitates were performed with irrelevant affinity-purified polyclonal rabbit immunoglobulins. Immunoprecipitates were incubated overnight at 4°C. After addition of anti-rabbit, IgG-coupled agrose, incubation was continued for at least 60 min. Immunocomplexes were washed four times in lysis buffer, twice in kinase buffer (25 mM Hepes, pH 7.0, 150 mM NaCl, 10 mM MnCl\(_2\), 1 mM Na\(_2\)VO\(_4\), 5 mM DTT, and 0.5% NP-40), and then resuspended in the same buffer. The kinase reaction was initiated by addition of 10 \(\mu\text{Ci}\) \(^{32}\text{P}\)ATP (3,000 Ci/mmol; Du Pont-NEN, Boston, MA) and ATP (10 \(\mu\text{M}\)) in kinase buffer. The samples were incubated at 30°C for 20 min. The reaction was stopped with reducing 5× SDS sample buffer and 10% SDS-PAGE was performed, followed by autoradiography. An aliquot of the immunoprecipitates was analyzed by immunoblotting for detection of equal amounts of p56\(^{\text{ck}}\). Immunoblots were developed by incubation with HRP-conjugated protein G (BIO RAD Laboratories, Munich, Germany) and a chemiluminescence kit (Amersham, Braunschweig, Germany).

Results

RVD Depends on Tyrosine Kinase Activity

When Jurkat T lymphocytes were superfused with 80% Ringer’s (250 mOsm/kg), RVD restored the initial volume after 13 min in control cells (Fig. 1). RVD was completely blocked in cells preincubated with 10 \(\mu\text{M}\) herbimycin A and was defective in JCaM1.6 cells, a Jurkat subclone deficient for the src-like tyrosine kinase p56\(^{\text{ck}}\). Retransfection of p56\(^{\text{ck}}\) restored RVD in JCaM1.6 cells. To elucidate the mechanism of RVD block by inhibition of src-like tyrosine kinases, we studied the volume-sensitive anion current with the patch-clamp method.

Osmotic Activation of a Chloride Current (I\(_{\text{Cl}}\)) Requires a Tyrosine Kinase

Osmotic swelling of Jurkat T cells induced a Cl\(^-\) current 10–20 s after washing a hypertonic pipette solution into the cell. The current was characterized by strong outward rectification and a poor permeability to intracellular glutamate (Fig. 2A). The swelling-activated Cl\(^-\) current (I\(_{\text{Cl,swell}}\)) did not undergo visible inactivation using 200-ns pulses up to 80 mV, and was blocked by 500 \(\mu\text{M}\) disothiocyanato-2,2-stilbene sulfonic acid (DIDS), as previously described (Lewis et al., 1993).

![Figure 1. RVD is blocked by herbimycin A. Cells were superfused with hypertonic solution as indicated. After the initial swelling, control cells restored their volume within 13 min (\(\bullet\); \(n = 24\)). No RVD was seen in cells pretreated with herbimycin A (\(\square\); \(n = 13\)) or in p56\(^{\text{ck}}\)-deficient JCaM1.6 cells (\(\triangle\); \(n = 12\)). RVD was restored in JCaM1.6 cells retransfected with p56\(^{\text{ck}}\) (\(\Delta\); \(n = 30\)). Error bars show SEM.](image-url)
Osmotic activation of $I_{\text{Cl}}$-swell is defective in p56$^{lck}$-deficient JCaM1.6 cells. (A) Osmotic swelling activates an outwardly rectifying anion conductance. The reversal potential $<-40$ mV and the small inward current indicate poor permeability for intracellular glutamate. Traces are shown 1, 28, 42, 56, and 77 s after break-in. The lower trace (thin line) was recorded from a cell with symmetrical Cl$^-$/concentrations 35 s after break-in, demonstrating intrinsic outward rectification. No leak correction was performed. Instantaneous IV-plots were obtained using 200-ms voltage ramps. (B) Outward currents at 35 mV in Jurkat cells (■), p56$^{lck}$-deficient JCaM1.6 cells (▲), p56$^{lck}$-retransfected JCaM1.6/lck$^+$ cells (○), and Jurkat cells pretreated for 8 h with 10 μM herbimycin A (□; $n = 13, 10, 10,$ and 8, respectively). Error bars show SEM. Leak currents determined at $-55$ mV were subtracted.

In contrast, the development of the outwardly rectifying current was attenuated and delayed in JCaM1.6 cells. Osmotic activation of the current was rescued in JCaM1.6/lck$^+$ cells retransfected with p56$^{lck}$. Furthermore, $I_{\text{Cl}}$-swell was blocked in Jurkat cells by the inhibitors of src-like tyrosine kinases herbimycin A and lavendustin. The time course of outward currents is summarized in Fig. 2B. Table I shows dose-dependent block by lavendustin (IC$_{50} \sim 10$ nM) and compares outward currents using different genetic and pharmacological manipulations.

p56$^{lck}$ Activates Chloride Conductance without Swelling

If tyrosine phosphorylation is a crucial step in the activation of $I_{\text{Cl}}$-swell, addition of constitutively active p56$^{lck}$ to the cytosol should activate $I_{\text{Cl}}$-swell in the native as well as the lck$^-$ cell line without cell swelling. When using a hypotonic pipette solution (260 mOsmol/kg), Jurkat cells started to shrink shortly after break-in. However, a large $I_{\text{Cl}}$ could be observed when purified p56$^{lck}$ was added to the intracellular solution (Table I). This current shared with the swelling-activated conductance strong outward rectification, a poor permeability to glutamate and lack of inactivation (Fig. 3, A and B). Because Jurkat cells possess intrinsic p56$^{lck}$ activity, we attempted to activate $I_{\text{Cl}}$ in the p56$^{lck}$-deficient subclone JCaM1.6 using hypotonic pipette solutions. In five whole-cell recordings from JCaM1.6 cells patched with a slightly hypotonic pipette solution (300 mOsmol/kg), $I_{\text{Cl}}$ did not activate and cell volume decreased slowly during the experiment. However, when p56$^{lck}$ was added to the pipette solution, a small outwardly rectifying $I_{\text{Cl}}$ developed (Fig. 3C; Table I). The current was completely and reversibly blocked by 500 μM DIDS (Fig. 3C) and no fast inactivation could be observed (data not shown). Thus, purified lck activates $I_{\text{Cl}}$ with properties indistinguishable from $I_{\text{Cl}}$-swell.

### Table I. Tyrosine Kinase Inhibitors Block $I_{\text{Cl}}$-swell

| Osmolality | p56$^{lck}$ | Current | SEM | n   |
|------------|------------|---------|-----|-----|
| mOsmol/kg  | U/μM               | pA   | pA  |
| Jurkat     | + herbimycin A    | 370  | 0   | 45.7, 36.7 | 8 a |
| Jurkat     | + lavendustin 10 μM| 370  | 7.8 | 6.2, 4   | 4 b |
| Jurkat     | + lavendustin 100 nM| 370  | 20.3, 33.6 | 3 c |
| Jurkat     | + lavendustin 10 nM| 370  | 72.5, 11.1 | 5 d |
| Jurkat     | 370              | 210.5, 35.1 | 14 a–e |
| Jcam1.6    | 370              | 100, 47.9 | 12 e, f |
| Jcam1.6, p56$^{lck}$ retransfected| 370 | 247.3, 41 | 12 f |
| Jcam1.6    | 300              | 38.1, 8.3 | 9 g, h |
| Jcam1.6    | 300              | 8.9, 2 | 5 g |
| Jurkat     | 260              | 107.1, 44 | 5 i, h |
| Jurkat     | 260              | 6.9, 24 | 6 i |

Whole-cell $I_{\text{Cl}}$ currents 2 min after break-in. Osmolality of the pipette solution was varied to swell or shrink the respective cell types. Lavendustin added 5 min before recordings blocked the swelling-induced $I_{\text{Cl}}$ current in a dose-dependent manner: a–i, significantly different data pairs (t test, $P < 0.05$). Current activated by purified p56$^{lck}$ in the pipette was significantly larger in Jurkat cells when compared with Jcam1.6 cells. Extracellular osmolality was always 310 mOsmol/kg.

p56$^{lck}$ Activates Cl$^-$ Channels in Excised Patches

Addition of purified p56$^{lck}$ to the cytosolic surface of excised patches from Jurkat T cells activated an outwardly rectifying Cl$^-$ channel (Fig. 4, A and B). No spontaneous activation was observed in 55 excised patches held at 0 mV holding potential without enzyme added. The channel showed outward rectification, even with symmetrical Cl$^-$ concentrations, and was selective for Cl$^-$ over glutamate (Fig. 4D).

### Single-Channel Transitions in Whole-Cell Recordings

Single-channel opening and closing transitions observed in discrete whole-cell current levels were frequently seen, when $I_{\text{Cl}}$ was slowly activated by p56$^{lck}$ in JCaM1.6 cells using slightly hypotonic intracellular solution (300 mOsmol/kg; Fig. 3 C). Single-channel openings lasting for several seconds were observed in continuous recordings at constant voltage (Fig. 4 C). The transitions in current traces obtained with voltage ramps frequently spanned more than one channel level. A similar behavior has been described for swelling-activated chloride channels (Meyer and Korbmacher, 1996). With voltage ramps, the current often jumped several times between an open and closed state.

Single-channel transitions could also be observed when Jurkat cells were patched with slightly hypertonic intracellular solution (330 mOsmol/kg). Cell volume increased and $I_{\text{Cl}}$-swell activated slowly under these conditions. Tran-
and in vitro assays were performed on immunoprecipitated p56\textsuperscript{ck}. A transient increase in p56\textsuperscript{ck} activity was observed resembling the time course of RVD. p56\textsuperscript{ck} activation was detected 1 min after exposure to osmotic stress, peaked at 15 min, and then declined rapidly thereafter (Fig. 5). No activation of p56\textsuperscript{ck} was seen when cells were kept in isotonic solution (data not shown).

**Discussion**

In this study, we show for the first time that the tyrosine kinase, p56\textsuperscript{ck}, mediates RVD by activating I\textsubscript{Cl–swell}. T cells deficient for p56\textsuperscript{ck} are defective in RVD and activation of I\textsubscript{Cl–swell}, and retransfection of this kinase results in restoration of osmotic current activation.

Several observations suggesting involvement of a phosphorylation event in osmotic current activation have been reported. ATP in the pipette is required for maintenance and repeated activation of I\textsubscript{Cl–swell} in lymphocytes (Lewis et al., 1993; Ross and Cahalan, 1995). A similar ATP dependence has been demonstrated in many different endothelial and epithelial cell types (Gill et al., 1992; Jackson et al., 1994, 1996; Oiki et al., 1994; Meyer and Korbmacher, 1996), although some discrepancies exist between different cells, i.e., the ability of nonhydrolyzable ATP analogues to replace ATP. Furthermore, a delay between the volume change and current activation in the range of 1 min is typically observed, making a direct link between membrane stretch and channel gating unlikely (Lewis et al., 1993; Ross et al., 1994). Direct evidence for involvement of tyrosine phosphorylation in I\textsubscript{Cl–} activation came from pharmacological studies. Inhibitors of tyrosine phosphorylation have been recently reported to inhibit swelling-induced \textsuperscript{125}I efflux in intestinal epithelium (Tilly et al., 1993) and activation of I\textsubscript{Cl} in cardiac and endothelial cells (Sorota, 1995; Voets et al., 1998). To our knowledge, no specific tyrosine kinase has been linked to the activation of I\textsubscript{Cl–so far.

When compared with the native Jurkat cell line, activation of I\textsubscript{Cl–swell} in JCaM1.6 cells was not completely abolished, but reduced in size and delayed. This could conceivably indicate a permissive role for p56\textsuperscript{ck}-mediated tyrosine phosphorylation. However, the p56\textsuperscript{ck}-deficient cell line JCaM1.6 expresses other src-like kinases like fyn (August and Dupont, 1995). Herbimycin A and lavendustin, which inhibit all src-like kinases, blocked RVD and the osmotic current response. Therefore, we suppose that other src-like kinases can partially substitute for lck in lck-deficient cells. Strong evidence for a crucial role of p56\textsuperscript{ck} comes from the experiments using purified p56\textsuperscript{ck}. When added to the cytosol, p56\textsuperscript{ck} activates a whole-cell chloride current with properties indistinguishable from I\textsubscript{Cl–swell} without cell swelling and opens a Cl\textsuperscript{−} channel in excited patches. Both currents share lack of inactivation, block by 500 \textmu M DIDS, selectivity for Cl\textsuperscript{−}, and outward rectification. The smaller amplitude of the lck-activated current typically observed in JCaM1.6 cells could be attributed to amplification of kinase activity by phosphorylation of the native p56\textsuperscript{ck} present in Jurkat cells.

Several reports show that CFTR can be involved in the regulation of outwardly rectifying chloride channels (Gabriel et al., 1993; Schwiebert et al., 1995). Single-channel recordings from fibroblasts transfected with CFTR have re-

---

**Figure 3.** I\textsubscript{Cl} activated by p56\textsuperscript{ck}. (A) I\textsubscript{Cl} in a shrinking Jurkat cell with 10 U/ml purified p56\textsuperscript{ck} in the pipette. Representative traces are shown 1, 121, 218, 284, and 371 s after break-in (no leak subtraction). (B) Lack of inactivation of outward currents induced by purified p56\textsuperscript{ck}. 200-ms pulses beginning at -80 mV, with 20-mV increments, were applied every 5 s. Leak and capacitive currents were subtracted. (C) I\textsubscript{Cl} in a shrinking JCaM1.6 cell with 10 U/ml purified p56\textsuperscript{ck} in the pipette. Traces are shown 307, 373, and 482 s after break-in. The lowest trace shows block of the current with 500 \textmu M DIDS. Note the current steps indicating single-channel transitions. 250-ms voltage ramps, no leak subtraction.
revealed the activation of a fast gate by the tyrosine kinase p60<sup>tyk2</sup>, increasing this chloride channel's open probability (Fischer and Machen, 1996). Whereas I<sub>Cl-swell</sub> is easily distinguishable from CFTR channels by its outward rectification and sensitivity to DIDS, the CFTR protein could represent a target for tyrosine phosphorylation, regulating the I<sub>Cl-swell</sub> studied here. Identification, cloning, and purification of the channel protein underlying I<sub>Cl-swell</sub> in lymphocytes will be necessary to determine the phosphorylation target. Therefore, we attempted to characterize the single channel underlying I<sub>Cl-swell</sub> in whole-cell recordings.

The single channel responsible for I<sub>Cl-swell</sub> has not been identified so far. An outwardly rectifying, DIDS-sensitive 40–50 pS chloride channel can be activated by membrane excision from lymphocytes and prolonged depolarization (Garber, 1992). However, ~1 pS unitary conductance was estimated by stationary noise analysis of I<sub>Cl</sub> from an excised inside-out patch activated by purified p56<sup>lck</sup>-glutamate; ○, whole-cell current transitions activated by p56<sup>lck</sup> in a JCaM1.6 cell with slightly hypoosmotic intracellular solution (300 mOsmol/kg, Cs<sup>-</sup>-glutamate); ▲, excised inside-out patch activated by purified p56<sup>lck</sup> with Cs<sup>-</sup>-glutamate in the bath; △, excised patch activated by purified p56<sup>lck</sup> with NaCl in the bath. The extracellular solution always contained NaCl (310 mOsmol/kg).

Voltage-dependent inactivation hinders the use of nonstationary noise analysis. In the present study we observed single-channel events in high resolution whole-cell recordings. The channels show extremely slow gating even at 31°C and prolonged open states lasting for several seconds. Similar behavior of swelling-activated chloride channels has been recently described in glioma and kidney epithelial cells (Jackson and Strange, 1995; Meyer and Korbmacher, 1996). We obtain unitary conductances of 25 and 28 pS when constructing IV plots from single channel transitions in whole-cell recordings activated by swelling and by purified p56<sup>lck</sup>, respectively. Furthermore, we were able to activate a 31-pS, outwardly rectifying Cl<sup>-</sup> channel in excised membrane patches by adding purified p56<sup>lck</sup> to the cytosolic surface. Thus, cell swelling and p56<sup>lck</sup> seem to activate the same chloride channel. The 31-pS outwardly rectifying single channels observed in excised patches may well be responsible for I<sub>Cl-swell</sub> in lymphocytes.

Cell swelling has been previously described to induce tyrosine phosphorylation in an intestinal epithelial cell line (Tilly et al., 1993). Hypoosmotic stimulation of mitogen-activated protein kinase was blocked by tyrosine kinase inhibitors in astrocytes (Schliess et al., 1996). Tyrosine kinase activation is upstream of MAP kinase (Schliess et al., 1996). In Jurkat T lymphocytes we show for the first time activation by hypoosmotic cell swelling of a specific tyrosine kinase target. Therefore, we attempted to characterize the single channel responsible for I<sub>Cl</sub> in lymphocytes.

When current variance is analyzed to obtain information about unitary events, gating events could be missed because of their low frequency. Recording at room temperature further slows down gating kinetics and the lack of voltage-dependent inactivation hinders the use of nonstationary noise analysis. In the present study we observed single-channel events in high resolution whole-cell recordings. The channels show extremely slow gating even at 31°C and prolonged open states lasting for several seconds. Similar behavior of swelling-activated chloride channels has been recently described in glioma and kidney epithelial cells (Jackson and Strange, 1995; Meyer and Korbmacher, 1996). We obtain unitary conductances of 25 and 28 pS when constructing IV plots from single channel transitions in whole-cell recordings activated by swelling and by purified p56<sup>lck</sup>, respectively. Furthermore, we were able to activate a 31-pS, outwardly rectifying Cl<sup>-</sup> channel in excised membrane patches by adding purified p56<sup>lck</sup> to the cytosolic surface. Thus, cell swelling and p56<sup>lck</sup> seem to activate the same chloride channel. The 31-pS outwardly rectifying single channels observed in excised patches may well be responsible for I<sub>Cl-swell</sub> in lymphocytes.

Cell swelling has been previously described to induce tyrosine phosphorylation in an intestinal epithelial cell line (Tilly et al., 1993). Hypoosmotic stimulation of mitogen-activated protein kinase was blocked by tyrosine kinase inhibitors in astrocytes (Schliess et al., 1996). Tyrosine kinase activation is upstream of MAP kinase (Schliess et al., 1996). In Jurkat T lymphocytes we show for the first time activation by hypoosmotic cell swelling of a specific tyrosine kinase target. Therefore, we attempted to characterize the single channel responsible for I<sub>Cl</sub> in lymphocytes.
The activity of \( p56^{\text{ck}} \) is lacking kinase activity (munoprecipitate performed with irrelevant affinity-purified polyacrylamide gel electrophoresis and analyzed by autoradiography. Nonspecific control immunoprecipitation of immunoprecipitated \( p56^{\text{ck}} \) panel right lane, NS).

Figure 5. Cell swelling activates \( p56^{\text{ck}} \). The activity of \( p56^{\text{ck}} \) induced by hypoosmotic cell swelling is shown at two different time scales. Jurkat cells (4 \times 10^6 per sample) were incubated with 250 mM sodium chloride solution for the indicated time, lysed, and then \( p56^{\text{ck}} \) was immunoprecipitated from postcentrifugation supernatants. Activity of \( p56^{\text{ck}} \) was determined by autophosphorylation in the presence of \( [\text{32P}]\text{ATP} \). Samples were separated by 10% SDS-PAGE and analyzed by autoradiography. Nonspecific control immunoprecipitation performed with irrelevant affinity-purified polyacrylamide gel electrophoresis and analyzed by autoradiography. Nonspecific control immunoprecipitation is shown by the right lane, NS. The second blot in each panel shows an aliquot of immunoprecipitated \( p56^{\text{ck}} \) immunoblotted with anti-\( p56^{\text{ck}} \) antibody demonstrating comparable amounts of protein in each lane. The results are representative of four independent experiments.

This work was in part funded by Deutsche Forschungsgemeinschaft Le 792/3-1, La 315/4-3, and Bundesminister für Bildung, Wissenschaft, Forschung und Technologie/Interdisziplinäres Klinisches Forschungszentrum 01KS9665. L. Szabó is grateful for a European Molecular Biology Organization fellowship.

Received for publication 15 September 1997 and in revised form 2 January 1998.

References

August, A., and B. Dupont. 1995. Activation of extracellular signal-regulated protein kinase (ERK/MAP kinase) following CD28 cross-linking: activation in cells lacking \( p56^{\text{ck}} \). Tissue Antigens. 46:155–162.

Cahalan M.D., and R.S. Lewis. 1988. Role of potassium and chloride channels in volume regulation by T lymphocytes. In Cell Physiology of Blood. R.B. Gunn, and J.C. Parker, editors. Rockefeller University Press, New York.

281–301.

Deutsch, C., and S.C. Lee. 1988. Cell volume regulation in lymphocytes. Renal Physiol. Biochem. 3:260–276.

Doroshenko, P., and E. Neher. 1992. Volume-sensitive chloride conductance in bovine chromaffin cell membrane. J. Physiol. (Lond.). 449:197–218.

Fischer, H., and T.E. Machen. 1996. The tyrosine kinase \( p60^{\text{src}} \) regulates the fast gate of the cystic fibrosis transmembrane conductance regulator chloride channel. Biophys. J. 71:3073–3082.

Gabriel, S.E., L.L. Clarke, R.C. Boucher, and M.J. Stutts. 1993. CFT and outward rectifying chloride channels are distinct proteins with a regulatory relationship. Nature. 363:263–268.

Garber, S. 1992. Outwardly rectifying chloride channels in lymphocytes. J. Membr. Biol. 127:49–56.

Gill, D.R., S.C. Hyde, C.F. Higgins, M.A. Valverde, G.M. Mintenig, and F.V. Sepulveda. 1992. Separation of drug transport and chloride channel functions of the human multidrug resistance P-glycoprotein. Cell. 71:23–32.

Jackson, P.S., and K. Strange. 1995. Single-channel properties of a volume-sensitive anion conductance. Current activation occurs by abrupt switching of closed channels to open state. J. Gen. Physiol. 105:643–660.

Jackson, P.S., R. Morrison, and K. Strange. 1994. The volume-sensitive organic osmolyte-anion channel VSOAC is regulated by nonhydrolytic ATP binding. Am. J. Physiol. 267:C1203–C1209.

Jackson, P.S., K. Churchwell, N. Ballatori, J.L. Boyer, and K. Strange. 1996. Swelling-activated anion conductance in sea urchin spermatozoa: regulation by cell Ca2+ and ATP. Am. J. Physiol. 270:C57–C66.

Kunzelman, K., H. Pavelstädte, and R. Greger. 1989. Properties and regulation of chloride channels in cystic fibrosis and normal airway cells. Pflugers. Arch. 415:172–182.

Lang, F., G. Busch, M. Ritter, H. Voßk., S. Waldegger, E. Gulbins, and D. Häusinger. 1998. The functional significance of cell volume regulation mechanisms. Physiol. Rev. 78:247–306.

Levitan, I., C. Almonte, P. Mollard, and S.S. Garber. 1995. Modulation of a volume-regulated Cl− current by f-actin. J. Membr. Biol. 147:283–294.

Lewis, R.S., and M.D. Cahalan. 1988. Subset-specific expression of potassium channels in developing murine T lymphocytes. Science 239:771–775.

Lewis, R.S., P.E. Ross, and M.D. Cahalan. 1993. Chloride channels activated by osmotic stress in T lymphocytes. J. Gen. Physiol. 101:801–826.

Meyer, K., and C. Korbmacher. 1996. Cell swelling activates ATP-dependent voltage-gated chloride channels in M1 mouse cortical collecting duct cells. J. Gen. Physiol. 108:177–193.

Nishimoto, I., J.A. Wagner, H. Schulman, and P. Gardner. 1991. Regulation of Cl− channels by multifunctional CaM kinase. Neuron. 6:547–555.

Oiki, S., M. Kubo, and Y. Okada. 1994. Mg2+ and ATP-dependence of volume-sensitive Cl− channels in human epithelial cells. Jpn. J. Physiol. 44(Suppl.):77–79.

Ross, P.E., and M.D. Cahalan. 1995. Ca2+ influx pathways mediated by swelling or stores depletion in mouse thymocytes. J. Gen. Physiol. 106:415–444.

Ross, P.R., S.S. Garber, and M.D. Cahalan. 1994. Membrane chloride conductance and capacitance in Jurkat T lymphocytes during osmotic swelling. Biochim. Biophys. Acta. 1209:176.

Schless, F., R. Sinning, R. Fischer, C. Schmalenbach, and D. Haussinger. 1996. Calcium-dependent activation of ERK-1 and ERK-2 after hypo-osmotic astrocyte swelling. Biochem. J. 320:167–171.

Schwebert, E.M., M.E. Egan, T.H. Hwang, S.B. Fulmer, S.S. Allen, G.R. Cutting, and W.B. Guggino. 1995. CFTR regulates outwardly rectifying chloride channels through an autocrine mechanism involving ATP. Cell. 81:1063–1073.

Sölk, C.K., and J.J. Wine. 1991. Swelling-induced and depolarization-induced Cl− channels in normal and cystic fibrosis epithelial cells. Am. J. Physiol. 261:F658–F674.

Sorota, S. 1992. Swelling-induced chloride-sensitive current in canine atrial cells revealed by whole-cell patch-clamp method. Circ. Res. 70:679–687.

Sorota, S. 1995. Tyrosine protein kinase inhibitors prevent activation of cardiac swelling-induced chloride current. Pflugers Arch. 431:178–185.

Tilly, B.C., N. van den Berghe, L.G. Tertoolen, M.J. Edizhoven, and H.R. de Jonge. 1993. Protein tyrosine phosphorylation is involved in osmoregulation of ionic conductances. J. Biol. Chem. 268:19919–19922.

Voets, T., V. Manolopoulos, J. Egermont, C. Ellory, G. Droogmans, and B. Nilius. 1998. Regulation of a swelling-activated chloride current in bovine endothelium by protein tyrosine phosphorylation and G proteins. J. Physiol. 506:341–352.