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Two Distinct K^+ Channels in Lamprey (*Lampetra fluviatilis*) Erythrocyte Membrane Characterized by Single Channel Patch Clamp

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Abstract. Two channels, distinguished by using single-channel patch-clamp, carry out potassium transport across the red cell membrane of lamprey erythrocytes. A small-conductance, inwardly rectifying K^+-selective channel was observed in both isotonic and hypotonic solutions (osmolarity decreased by 50%). The single-channel conductance was 26 ± 3 pS in isotonic (132 mM K^+) solutions and 24 ± 2 pS in hypotonic (63 mM K^+) solutions. No outward conductance was found for this channel, and the channel activity was completely inhibited by barium. Cell swelling activated another inwardly rectifying K^+ channel with a larger inward conductance of 65 pS and outward conductance of 15 pS in the on-cell configuration. In this channel, rectification was due to the block of outward currents by Mg^{2+} and Ca^{2+} ions, since when both ions were removed from the cytosolic side in inside-out patches the conductance of the channel was nearly ohmic. In contrast to the small-conductance channel, the swelling-activated channel was observed also in the presence of barium in the pipette. Neither type of channel was dependent on the presence of Ca^{2+} ions on the cytosolic side for activity.

Key words: Lamprey — *Lampetra fluviatilis* — Erythrocyte — Ion transport — K^+ channel — Volume regulation

Introduction

Lamprey (*Lampetra fluviatilis*) red cells regulate their cell volume after osmotic swelling by extruding KCl and osmotically obliged water (Nikinmaa, Tufts & Boutilier, 1993; Virkki & Nikinmaa, 1995). In most red cells, regulatory volume decrease (RVD) occurs mainly via swelling-activated KCl cotransport (for a review, *see* Lauf et al., 1992), although a contribution from a chloride-independent potassium transport pathway has been observed in some fish erythrocytes (*see* Cossins & Gibson, 1997). In lamprey red cells, K^+ and Cl^- ions appear to be transported through separate conductive pathways during RVD (Virkki & Nikinmaa, 1995). Our data, obtained using radioisotopes (Virkki & Nikinmaa, 1995), suggested that potassium transport would occur via two different pathways: one active in isotonic conditions and another activated by hypotonicity.

In our previous study (Virkki & Nikinmaa, 1996) we have characterized, using the whole-cell patch-clamp technique, the major charge-carrying pathway of lamprey erythrocytes in isotonic conditions. The pathway is a K^+-selective, Ba^{2+}-sensitive channel that shows strong inward rectification. The channel saturates at a low concentration of external K^+ (half-maximal inward conductance produced by only 12 mM external K^+) and is not dependent on intracellular Ca^{2+} ions for activity.

In this study, we set out to characterize the potassium transport pathways in osmotically swollen cells. The results confirm our conclusions, based on radioisotope fluxes, about the presence of two distinguishable potassium channels, one of which is activated by cell swelling. Using single-channel patch-clamp the properties of these two different channels could be elucidated.

Material and Methods

River lampreys (*Lampetra fluviatilis*, weight 25–70 g) were caught from the Kymijoki river in southern Finland during their spawning run and maintained in laboratory conditions (dechlorinated Helsinki tap water, 10–15°C) for a minimum of two weeks before experimentation.

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Blood samples were taken by dorsal puncture from anesthetized lampreys (MS-222, 1 g l^{-1} water, buffered with $NaHCO_3$) into heparinized syringes. Red cells and plasma were separated by centrifugation and the cells washed twice with the following saline (in mM): 110 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 20 HEPES, 5.5 glucose, 9 Na-pyruvate, pH 7.5. The cells were resuspended in the saline. The suspension was kept at +4°C and used the same day. The experiments were carried out at room temperature (24°C).

A small amount of red cell suspension was added to the experimental chamber containing the desired experimental solution and the cells were allowed to settle for a few minutes before the measurements were started. The bath was grounded through an agar-salt bridge. Patch electrodes were pulled from borosilicate glass capillaries with inner filament (GC150F, Clark Electromedical Instruments) on a two-step Narishige PP-83 puller (Narishige, Japan) and used without heat polishing. Pipette resistance was 6–10 MΩ when the pipette was filled with chloride-containing internal solution. After seal formation (seal resistance 5–50 GΩ), an excised patch in the inside-out-configuration could be obtained by briefly exposing the pipette tip with the attached cell to air. Continuous single-channel recordings were made using an EPC-9 amplifier (Heka electronics, Germany) driven by an Atari Mega ST^E computer. Currents were saved directly on hard disk at a sampling rate of 5–10 kHz after low-pass filtering at 1–2 kHz. The data were analyzed using the program Review (M2 Lab, Instrutech Corporation, Elmont, CA), or exported to PAT program (J. Dempster, Strathclyde Electrophysiology Software version 7.3). The voltages given in the graph and test are given as $-V_{pip}$ and are therefore the opposite of the actual pipette holding potential. Thus, positive values of $-V_{pip}$ correspond to depolarizing voltages (and vice versa) applied to the channel. Corrections were made for liquid junction potentials, which were calculated according to Barry and Lynch (1991) or, in the case of glucuronate solutions, measured (Neher, 1992).

The composition of the solutions used in the experiments was 127 KCl (or 122 NaCl and 5 KCl), 1 $CaCl_2$, 1 $MgCl_2$ and 10 HEPES (or 10 Tris in some experiments). To induce cell swelling, cells were subjected to hypotonic solution before they were attached to the pipette. Thereafter hypotonic solutions were used in the bath and the pipette throughout the experiment. Attempts to induce cell swelling after the cell was attached to a patch (by decreasing bath osmolarity at this point) resulted in bad seal quality precluding single-channel measurements and resulting in loss of the patch within a few minutes. In hypotonic solutions, the KCl (or NaCl) concentration was decreased from 127 to 58 mM. All solutions were adjusted to pH 7.5 with KOH or NaOH. To obtain low-Cl^- solutions, K-glucuronate or Na-glucuronate was equimolarly substituted for KCl or NaCl. Pipette solutions were filtered through Millipore filters (0.22 μm).

The glucuronate solutions were not completely free of Cl^- ions, since they contained $MgCl_2$ and/or $CaCl_2$. Thus, the solutions with glucuronate as the major anion contained also maximally 4 mM Cl^-. Buffering of 10 mM HEPES to pH 7.5 with KOH or NaOH was estimated to increase the K^+ or Na^+ concentration of the solution by 5 mM.

Solutions were changed and blockers added by bath perfusion. Statistical analysis of the data were carried out using the least significant difference test of the ANOVA/MANOVA module of Statistica Software (Statsoft, Tulsa, OK). Values in the text are given as means ± SD.

Results

It was relatively easy to obtain seals in the range of 5 to 50 GΩ on lamprey erythrocytes. When a successful patch was obtained, the quality of the seal and the patched membrane usually remained good for at least 15 min of recording in Ca^{2+}-containing solutions. Single-channel recordings were made in both on-cell and inside-out conformations. For the two channels described below, the small-conductance channel usually remained active in the on-cell configuration or inside-out patches for 10–15 min, whereas the swelling-activated channel often could be maintained for a substantially longer time period.

Small-conductance K Channel

With an isotonic solution in the bath and KCl or K-glucuronate solution in the pipette, inwardly conducting single-channel openings with well-defined rectangular current waveforms were observed in 13 out of 54 patches (24%; Fig. 1). No channel openings were observed with NaCl or Na-glucuronate in the pipette, indicating that the channel is K^+-selective. The inward slope conductance, measured from excised inside-out patches in symmetric KCl or K-glucuronate solutions was 26 ± 3 pS ($n = 6$). No channel openings carrying outward current could be detected in either the on-cell configuration or in excised inside-out patches bathed in symmetric K^+ solutions. The cause of this strong inward rectification is not clear. In some cell types, inward rectification of K^+ channels is due to block of outward conductance by internal Mg^{2+} ions (*see* e.g., Matsuda, 1991 for review). In lamprey red cells, however, removal of Mg^{2+} and Ca^{2+} ions and the addition of 1 mM EGTA to the extracellular bath in inside-out patches did not reveal any outwardly conducting single-channel openings for the small-conductance, constitutively active channel. Thus, inward rectification is not caused by block of outward current by these ions, but is possibly an intrinsic property of the channel. The channels might remain closed during depolarization, or the outwardly conducting channel openings are too small to be detected above the background noise, which was 120–140 fS (rms noise) in good recordings in our experiments.

The mean open probability of the inwardly rectifying K^+ channel as a function of the holding voltage (recorded in symmetric, isotonic K^+ solutions) is shown in Fig. 1*C*. The open probability appears slightly voltage dependent. The channel was not dependent on the presence of Ca^{2+} ions in the bath for activity, as the open probability (at −70 mV) was 0.89 ± 0.03 in a bath solution containing 1 mM Ca^{2+}, compared with 0.92 ± 0.02 ($n = 4$) in a bath solution containing 1 mM EGTA and no added Ca^{2+}.

In hypotonic solutions the small-conductance K^+ channel was observed in 54 out of 216 patches (25%). This value is similar to the value obtained in isotonic solutions (24%). The values for open probability was slightly but significantly smaller in hypotonic solutions

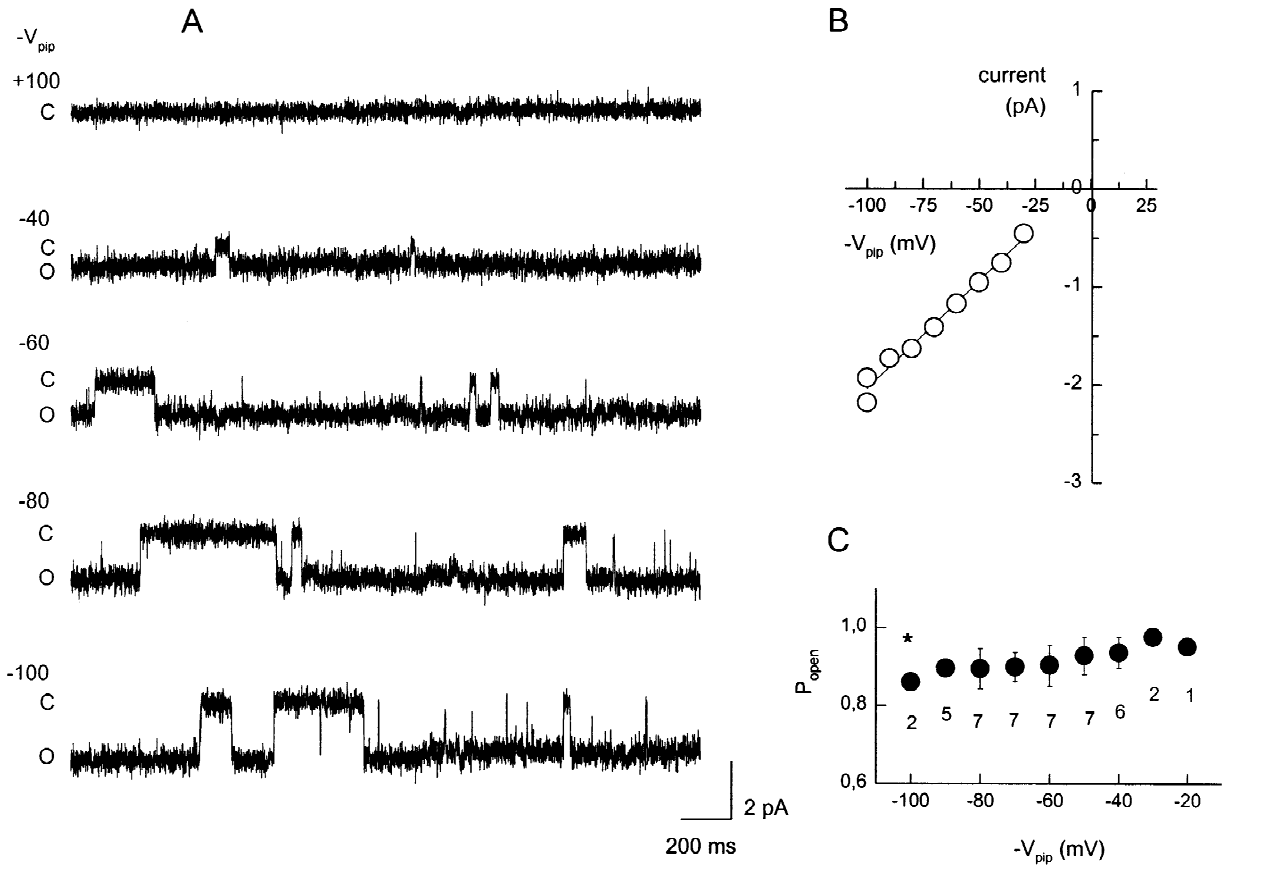

Fig. 1. (*A*) Representative original single-channel current trace for small-conductance K^+ channel in lamprey erythrocytes in the inside-out configuration at different holding potentials. Voltage is given as the opposite to the pipette potential ($-V_{pip}$). Positive (inward) current is shown as downward deflections from the closed level. At positive values of $-V_{pip}$ (i.e., outward currents) no channel openings were observed. The record was digitized at 5 kHz and filtered at 1 kHz. (*B*) Representative current-voltage relations for small-conductance K^+ channel in lamprey erythrocytes in the inside-out configuration. (*C*) The open probability (P_{open}) of the small-conductance K^+ channel, as recorded in inside-out patches in symmetric K^+ solutions. N values for individual data points are given in the graph. * indicates statistically significant difference ($P < 0.05$) to the value obtained at −40 mV. The graphs in *A* and *B* are from a recording on the same cell made with similar isotonic KCl solutions in the pipette and bath.

(0.76 ± 0.1, as recorded at −70 mV, $n = 7$) as compared to isotonic solutions (0.89 ± 0.03, $P < 0.01$). Thus, this channel may be somewhat inhibited by cell swelling.

The inward slope conductance of the channel was 24 ± 2 pS ($n = 8$) in hypotonic solutions, which did not differ significantly from the conductance recorded in isotonic solutions (26 pS). The difference in K^+ concentration between isotonic and hypotonic solutions is, however, more than twofold, being 63 mM in hypotonic solutions and 132 mM in isotonic solutions. Thus, it appears that the channel is saturated already at 63 mM K^+ concentration and thus an increase in K^+ concentration to 132 mM hardly increases the inward conductance. This result agrees well with earlier findings (Virkki & Nikinmaa, 1996), where the whole-cell K^+-selective conductance that was recorded in isotonic solutions showed half-maximal saturation at 12 mM. Thus, it is apparent that this channel is the basis of the whole-cell K^+ conductance described in Virkki and Nikinmaa (1996).

No channel openings of the volume-insensitive type were observed when the pipette solution contained 1–2 mM Ba^{2+}, indicating that this channel is completely blocked by Ba^{2+} ions at these concentrations ($n = 7$).

Swelling-activated K Channel

In hypotonic solutions (osmolarity decreased by 50%) with KCl or K-glucuronate solutions in the pipette, flickery single-channel openings, which occurred in bursts, were observed in 134 out of 238 patches (56%; Fig. 2*a*). Channel openings of this type could not be evoked in cells in isotonic solution by merely applying negative pressure in the pipette by suction, but appeared to require swelling of the whole cell for activation. This suggests that the pathway cannot be activated by mere stretching of the membrane, but that activation requires a more complex signalling sequence. Since the channel was

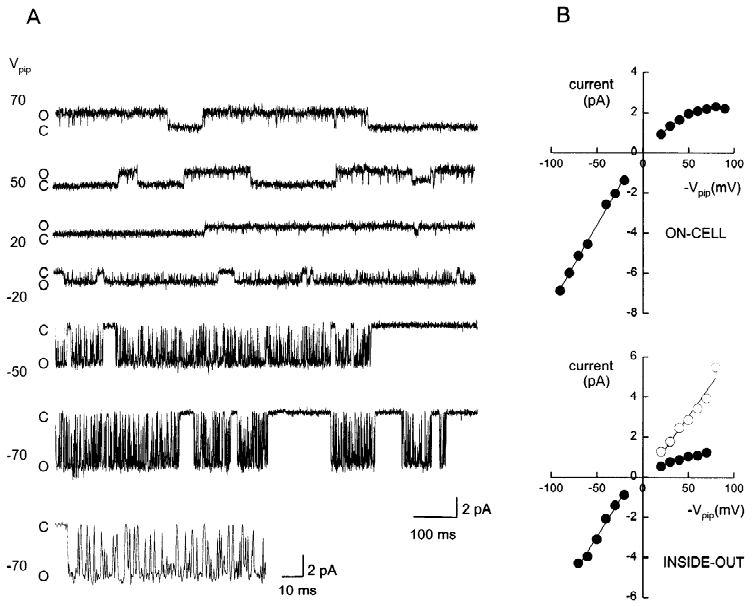

Fig. 2. (*A*) Representative original single-channel current trace for swelling-activated K^+ channel in the inside-out configuration in lamprey erythrocytes at different holding potentials. Voltage is given as the opposite to the pipette potential ($-V_{pip}$). Positive (inward) current is shown as downward deflections from the closed level. The record was digitized at 5 kHz and filtered at 1 kHz. (*B*) Representative current-voltage relations for swelling-activated K^+ channel in lamprey erythrocytes in the on-cell (top) and inside-out (bottom) configurations. Recordings were made with similar hypotonic K-glucuronate solutions in the pipette and bath. In the inside-out configuration, recordings for outward current was made with 1 mM Mg and 1 mM Ca in the bath (closed circles), and with 1 mM EGTA and no divalent cations added to the bath (open circles). All graphs are from a recording on the same cell.

usually not seen in inside-out patches suggests that a change in protein concentration at the cytosolic face of the membrane (e.g., Colcalsure & Parker, 1992) is not alone a sufficient condition for channel activation. Inward conductance was observed when the pipette solution contained K^+ ions but not when the pipette solution contained only Na^+ ions. Outward conductance was observed in the on-cell configuration, and also in excised inside-out patches if K^+ ions were present in the bath. The channel showed inward rectification in both on-cell and inside-out (symmetric K^+ solutions) configurations (Fig. 2*b*). In isotonic conditions, channel opening of this type were observed only in 3 patches out of 58 (5%), as compared to 56% in hypotonic conditions. Thus, the channel can be characterized as a swelling-activated, inwardly rectifying K^+ channel. The open probability of the channel was very variable, in our recordings we observed values between 0.001 and 0.5. Frequently, more than one and up to 4–5 channels could be observed in the same patch.

The inward slope conductance of the swelling-activated K^+ channel in the on-cell configuration was 65 ± 10 pS and outward conductance 15 ± 7 pS (n = 4–6). In inside-out patches in symmetric hypotonic solutions, the inward slope conductance was 79 ± 5 pS (n = 8), and outward slope conductance was 16 ± 3 pS in the presence of 1 mM Mg^{2+} and 1 mM Ca^{2+} in the bath and pipette (n = 7). The rectification of the swelling-activated K^+-selective channel in the inside-out configuration was due to block of outward currents by Mg^{2+} and Ca^{2+} ions. When both ions were removed from the bath solution in inside-out patches, the channel showed nearly ohmic conductance (Fig. 2*b*). Outward currents in the presence of 1 mM Mg^{2+} was 16 ± 4 pS (n = 3), and 20 ± 4 pS (n = 3) in the presence of 1 mM Ca^{2+} in the bath, showing that both ions reduced outward conductance. Omission of Mg^{2+} and/or Ca^{2+} ions from the bath or the pipette had no effect on inward currents.

On the basis of the data on single-channel conductance (26 pS) and open probability (0.89 at −70 mV) of the small-conductance channel, and taking the value for whole-cell conductance in isotonic solutions obtained in previous studies (3000 ± 1600 pS; Virkki & Nikinmaa, 1996), it is possible to roughly estimate the number of active ion channels in the lamprey red cell membrane in isotonic solutions to around 130 per cell. Since the approximate area of lamprey erythrocyte membrane is 284 μm^2 (Virkki & Nikinmaa, 1994) and the approximate area of the tip of the patch pipette ca. 1 μm^2, a probability of 24% indicates around 70 channels per cell, somewhat less than the 130 estimated from the data above. For the swelling-activated channel, no data on the whole-cell conductance is available. The probability of 56% to hit a channel when patching the cell indicates more than 200 channels of the swelling-activated type in the cell membrane.

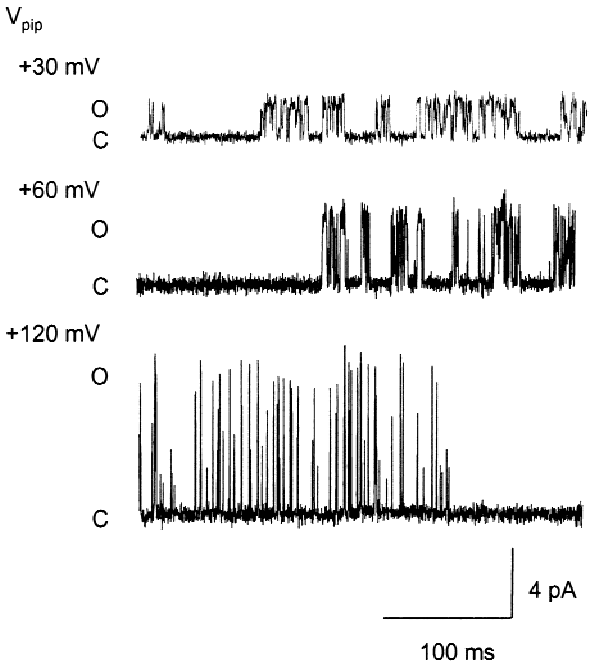

Fig. 3. Representative original single-channel current trace for swelling-activated K^+ channel in the inside-out configuration in lamprey erythrocytes at different holding potentials. Voltage is given as the opposite to the pipette potential ($-V_{pip}$). Positive (inward) current is shown as downward deflections from the closed level. Recordings were made with hypotonic KCl solution and 1 mM Ba^{2+} in the pipette, and solution containing 122 mM NaCl and 5 mM KCl in the bath. The record was digitized at 10 kHz and filtered at 1 kHz (top record) or filtered at 1.5 kHz (two bottom records).

Effects of Ba on the Swelling-activated Potassium Channels

In the presence of 1–2 mM Ba^{2+} in the pipette, channel openings of the swelling-activated channel were observed in 3 out of 7 patches. Thus, external Ba^{2+} ions did not completely inhibit the swelling-activated channel, in contrast to the constitutively active channel. A recording of the swelling-activated channel in the presence of barium is given in Fig. 3. Although barium did not fully inhibit the swelling-activated channel, our present results cannot exclude the possibility that some inhibition would occur—the number of observations was too small for definitive analysis of the behavior of the channel in the presence of barium, and there were no recordings from the same channel in the presence and in the absence of barium.

Discussion

This study demonstrates the presence of two inwardly-rectifying K^+ channels in lamprey erythrocytes, one which is constitutively active (and possibly inhibited by cell swelling) and one which is activated by cell swelling.

The constitutively active K^+ channel described here on the single-channel level is apparently the same channel that underlies the whole-cell K^+ conductance we described earlier using whole-cell patch-clamp, since both are inhibited by Ba^{2+} and show saturation by external K^+ (Virkki & Nikinmaa, 1996). Also, neither is dependent on internal Ca^{2+} for activity. It is also apparent that this channel is identical to the pathway mediating Ba^{2+}-sensitive K^+ transport described earlier in several flux studies (Kirk, 1991; Gusev, Sherstobitov & Skulskii, 1992; Virkki & Nikinmaa, 1995). In the study by Kirk (1991), unidirectional, Ba^{2+}-sensitive K^+ influx (as measured using ^{86}Rb as a tracer) showed saturation with increasing extracellular K^+ concentration. Furthermore, depletion of intracellular Ca^{2+} did not significantly affect unidirectional K^+ efflux (Virkki & Nikinmaa, 1995) or influx (Kirk, 1991).

At present the physiological significance of the constitutive, strongly inwardly rectifying K^+ channel is unclear. The functional significance of the fact that no channel openings carrying outward current were observed is apparently to minimize leak of K^+ out of the cell via this pathway. The channel may further be important in regulating the K^+ homeostasis of the animal—any increase in extracellular K^+ concentration will tend to increase K^+ influx via the pathway, thus stabilizing the extracellular K^+ concentration. It has been suggested that, in teleosts, circulating erythrocytes could buffer accumulation of K^+ in plasma caused by strenuously exercising muscle (Nielsen & Lykkeboe, 1992*a,b;* Nielsen, 1997). This idea is supported for lampreys by the fact that the whole-cell K^+ conductance of lamprey erythrocytes described in Virkki and Nikinmaa (1996) was dependent on the presence of K^+ ions in the extracellular bath for activity—decreasing $[K^+]_e$ below 10 mM depolarized the cell. However, the effects of exercise on erythrocyte and plasma ion levels have not been measured in lamprey in vivo. Also, the inwardly rectifying K^+ channel active in isotonic conditions may reduce the energy expenditure required for maintaining the transmembrane K^+ gradient in erythrocytes by the sodium pump. The savings in energy may be significant for the lamprey which does not feed from the onset of migration (in August–September) to spawning (in the following May–June).

Despite the lack of single-channel openings carrying outward current in the present studies, on the basis of the whole-cell conductance data obtained earlier (Virkki & Nikinmaa, 1996), it is possible that some K^+ efflux is carried via the constitutively-active K^+ channel: The whole-cell K^+ conductance was shown to be dependent on external K^+ ions for activity, and decreasing the external K^+ concentration below 10 mM also decreased

outward current, in addition to the inward current. In the present single channel study, the channel openings carrying outward current would have remained unnoticed, if they were too small to be observed above the background noise (i.e., less than 2 pS).

In addition to Ba^{2+}, the small-conductance K^+ channel is probably to some degree inhibited by amiloride. In a study by Kirk (1991), the ouabain-insensitive unidirectional K^+ fluxes (as measured using radioactive isotopes) in isotonic conditions were inhibited by amiloride after a 1-hr preincubation, and the whole-cell inward conductance was reduced by 30% in the presence of amiloride in isotonic medium (Virkki & Nikinmaa, 1996). This unique property of K^+ transport in lamprey erythrocytes was, however, not tested in the present study.

The results obtained in this study agree well with our previous data on K^+ transport obtained using radioactive isotopes (Virkki & Nikinmaa, 1995). Those results suggested that there are two pathways for conductive K^+ efflux present in the lamprey red cell membrane: one that is sensitive to Ba^{2+} and inhibited by cell swelling and another that is less sensitive to Ba^{2+} and is activated by cell swelling. This is in good agreement with data obtained in this study: no single-channel openings were observed for the 26 pS K^+ channel when the pipette contained 1–2 mM Ba^{2+}, and the open probability of the channel was somewhat decreased by cell swelling. Cell swelling activated a new type of channel, which was less sensitive to external Ba^{2+} ions than the 26 pS channel, had a larger single-channel conductance, and carried significant outward current. The swelling-activated K^+ channel is likely to play a role in RVD. Our previous studies (Virkki & Nikinmaa, 1995) have shown that the swelling-activated K^+ and Cl^- transport pathways are separate, i.e., KCl cotransport does not contribute to RVD. At a modest (0–30%) reduction of osmolarity, it appears that only the unidirectional Cl^- efflux increases, but that at the 50% reduction of osmolarity, also the K^+ efflux is increased (Virkki & Nikinmaa, 1995).

So far, we have not found an effective inhibitor of the swelling-activated K^+ pathway. Similar to the observations made earlier, in which the K^+ transport was less inhibited by Ba^{2+} in swollen cells (as measured using radioactive isotopes; Virkki & Nikinmaa, 1995), the swelling-activated K^+ channel was less sensitive to Ba^{2+} than the channel operating in isotonic conditions. Also amiloride and tetrapentylammonium bromide are less potent inhibitors of K^+ efflux in hypotonic conditions, as compared to isotonic conditions (Virkki & Nikinmaa, 1995).

Interestingly, neither channel depended on calcium for activity. Among amphibians, a swelling-activated K^+ conductance has recently been demonstrated in the aquatic salamander (*Necturus maculosus*) red cells (Bergeron, Stever & Light, 1996). However, in the salamander, channel activity was dependent on Ca^{2+}. Also in other cell types in which separate K^+ and Cl^- channels are involved in RVD, Ca^{2+} appears to be required for volume regulation (*see*, e.g., Sarkadi & Parker, 1991; McCarty & O'Neil, 1992 for review).

Thus, it appears that RVD in lamprey erythrocytes after cell swelling differs from RVD in the erythrocytes of most other species studied so far, and also from other cell types utilizing superficially the same mechanism. In most erythrocytes, RVD occurs mainly via swelling-activated KCl cotransport (*see* Lauf et al., 1992 for review), but in lamprey it occurs via separate conductive pathways for potassium and chloride. Although coupled conductive potassium and chloride transport is utilized in RVD also in many other cells, at least the swelling-activated potassium channel does not require calcium for activity, in contrast to swelling-activated channels of other cell types.

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