The Journal of Experimental Biology 201, 1405–1419 (1998)
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JEB1415

CELL VOLUME REGULATION IN PROXIMAL RENAL TUBULES FROM TROUT (*SALMO TRUTTA*)

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Accepted 12 February; published on WWW 20 April 1998

Summary

Regulatory volume decrease (RVD) following hypo-osmotic stimulation was studied using videometric methods in isolated proximal renal tubules from trout (*Salmo trutta*). The relative tubule diameter increased by 132.0±4.8 % (maximum swelling within 1 min at 15 and 25 °C and within 4 min at 10 °C) following a change from iso-osmotic (290 mosmol kg^{-1}) to hypo-osmotic (160 mosmol kg^{-1}) Ringer's solution. The tubule diameter subsequently decreased to approximately one-quarter of the maximal value. Ouabain (1 mmol l^{-1}) reduced cell swelling and inhibited the RVD response by 28.0±10.5 %. Furthermore, increasing the bath K^+ concentration by 30 mmol l^{-1} inhibited RVD by 76.5±3.6 %. The K^+ channel blocker quinine, but not Ba^{2+} (1 and 2 mmol l^{-1}), significantly decreased the RVD response (by 25.0±5.4 and 72.3±5.1 % at 0.1 and 0.5 mmol l^{-1}, respectively). Similarly, increasing the Cl^- concentration in the bath from 47 to 102 mmol l^{-1} induced a significant reduction (45.2±7.9 %) in RVD. The RVD response was also markedly reduced (by 54.7±5.3 %) by the Cl^- channel blocker indacrinone (MK-196; 0.5 mmol l^{-1}), but only marginally by 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB; 1, 5, 8 and 10 μmol l^{-1}). Addition of the K^+/Cl^- symport inhibitor furosemide (0.1 mmol l^{-1}) resulted in a 39.8±3.9 % inhibition of RVD. This inhibition could be completely overcome by simultaneous administration of 1 μmol l^{-1} tributyltin (anion exchanger) and furosemide.

Chelation of either extracellular (1 mmol l^{-1} EGTA) or both extra- and intracellular Ca^{2+} (1 mmol l^{-1} EGTA, 10 μmol l^{-1} A23187) had no effect on this RVD process. Furthermore, as measured using the fluorescent dye Fura-2/AM, there was no increase in the intracellular free Ca^{2+} concentration upon hypo-osmotic stimulation. Administration of the 5-lipoxygenase antagonist ETH 615-139 (20 μmol l^{-1}), however, induced a 60 % inhibition of RVD. Simultaneous addition of ETH-615 and either the K^+ ionophore gramicidin (0.5 mmol l^{-1}) or the anion exchanger tributyltin (1 μmol l^{-1}) could not reverse the ETH 615-139 inhibition. Finally, administration of the cycloxygenase inhibitor indomethacin had only a small, but significant, effect on RVD.

We conclude that RVD following hypo-osmotic swelling is in these cells a temperature- and ouabain-sensitive process that appears to be the result of K^+ efflux through quinine-sensitive, Ba^{2+}-insensitive K^+ channels and Cl^- efflux through an MK-196- and furosemide-sensitive Cl^- conductance that is relatively unaffected by NPPB. This KCl efflux seems to be regulated by eicosanoids produced by the 5-lipoxygenase. Arachidonic acid metabolites from the cycloxygenase pathway are not involved in this process. Similarly, neither extra- nor intracellular Ca^{2+} appears to be important for the signalling of RVD.

Key words: proximal renal tubule, teleost, trout, cell volume regulation, KCl efflux, K^+ channel, Cl^- channel, quinine, barium, MK-196, NPPB, Ca^{2+}, Fura-2, arachidonic acid, eicosanoid, 5-lipoxygenase, leukotriene, *Salmo trutta*.

Introduction

Almost all cells possess the ability to regulate their volume upon osmotic perturbations originating from changes in the osmolality of the extracellular medium, imbalances in the influx and efflux rates of osmotically active solutes across the plasma membrane or variations in intracellular metabolism. To maintain both function and volume relatively undisturbed, cells have developed mechanisms that sense and oppose volume changes. It has been shown in proximal renal tubules from rabbit that, if the osmotic changes occur relatively slowly, the cells are able to maintain a fairly constant volume over a wide osmotic range (Lohr and Grantham, 1986). When subjected to faster and larger osmotic changes, the cells are unable to withstand the perturbation. When the cell volumes depart from the 'set-point' by a significant degree, regulatory volume changes are activated and the cell volume is restored by compensatory fluxes of osmotically active particles.

Many cells respond to swelling with an efflux of KCl and an osmotically obligated water efflux (Davis and Finn, 1987;

Dellasega and Grantham, 1973; Grinstein *et al.* 1983; Hoffmann *et al.* 1984; Terreros *et al.* 1990; Kristensen and Folke, 1984). Since the relatively high intracellular K^+ concentration found in most cells is dependent on the activity of Na^+/K^+-ATPase, it is not surprising that the osmoregulatory KCl efflux in many tissues seems to depend on this enzyme (Gagnon *et al.* 1982; Linshaw and Grantham, 1980). None the less, the manner in which the regulatory volume decrease (RVD)-associated KCl efflux occurs varies among species and among tissues. In some cells, K^+ and Cl^- efflux occur through separate conductive pathways (Hoffmann *et al.* 1984; Welling and O'Neil, 1990; Macri *et al.* 1993; De Smet *et al.* 1995; Banderali and Roy, 1992). There is emerging evidence that the Cl^- conductance could be a rather non-specific anion channel that is also permeable to amino acids (Kirk *et al.* 1992; Sanchez-Olea *et al.* 1991; see also Kirk, 1997), and it has recently been found that upon osmotic swelling a Cl^--independent channel opens through which both K^+ and amino acids may pass (Bursell and Kirk, 1996). In other cells, RVD is due to furosemide-sensitive K^+/Cl^- symporters, K^+/H^+ and Cl^-/HCO_3^- exchangers or K^+ channels and Cl^-/HCO_3^- exchangers (Lauf, 1985; Cala, 1980; Terreros *et al.* 1990).

The signalling mechanism for this RVD response is not completely understood. Early experiments in several systems indicated that RVD was activated following Ca^{2+} influx (Neufield *et al.* 1983; McCarty and O'Neil, 1991). More recent work has shown that an increase in cytosolic $[Ca^{2+}]$ parallels hypo-osmotic cell swelling (Beck *et al.* 1991; Raat *et al.* 1995; Sardini *et al.* 1995; Ehrenfeld *et al.* 1994); this increase may be partly or wholly dependent on the availability of extracellular Ca^{2+} (McCarty and O'Neil, 1991; Ross and Cahalan, 1995; Rothstein and Mack, 1990; Raat *et al.* 1995; Sardini *et al.* 1995). However, other studies have shown that volume regulation can operate in opossum, rabbit and MDCK renal cells incubated in Ca^{2+}-free medium (Ubl *et al.* 1988*a*; Breton *et al.* 1992; Roy and Sauve, 1987). A similar relative or complete extracellular Ca^{2+}-independence has also been observed in hepatocytes, lymphocytes and other non-renal cells (Corasanti *et al.* 1990; Grinstein *et al.* 1982; Hoffmann *et al.* 1984; Margalit *et al.* 1993; vom Dahl *et al.* 1991).

Moreover, studies in Ehrlich ascites tumour cells and human lymphocytes indicate that neither intra- nor extracellular Ca^{2+} is required for RVD (Harbak and Simonsen, 1995; Grinstein and Smith, 1990); other work has suggested that loss of K^+ during RVD is not affected by Ca^{2+}, nor does Ca^{2+} affect RVD-associated ion currents (Harbak and Simonsen, 1995; Beck *et al.* 1991; Best *et al.* 1996).

Metabolites of arachidonic acid and other polyunsaturated fatty acids (i.e. eicosanoids) have, during the last few years, received attention as possible second messengers for the activation of RVD-associated KCl and taurine efflux (for a review, see Lambert, 1994). Of these eicosanoids, it has been found that the synthesis of leukotrienes is stimulated following hypo-osmotic cell swelling (Lambert *et al.* 1987). Specifically, leukotriene-D_4 (LTD_4) accelerated RVD by stimulation of K^+, Cl^- and taurine efflux. Furthermore, decreasing the intracellular content of LTD_4 with 5-lipoxygenase inhibitors (inhibitors of arachidonic acid metabolism) blocked RVD (Lambert *et al.* 1987). Findings from other tissues also indicate a role for arachidonic acid metabolites in RVD signalling. For example, inhibition of phospholipase A_2 (which catalyzes phospholipid metabolization to arachidonic acids), 5-lipoxygenase or cycloxygenase decreases the osmoregulatory response associated with RVD in several cell types (Fatherazi *et al.* 1994; Ling *et al.* 1992; Fugelli *et al.* 1995; Thoroed and Fugelli, 1994). (Eicosanoids are also produced in fish; see Rowley, 1991.)

In the present study, RVD following hypo-osmotic stimulation of proximal renal tubules from trout (*Salmo trutta*) was studied using videometric techniques and the fluorescent dye Fura-2/AM. It was observed that RVD in these cells is a temperature- and ouabain-sensitive KCl efflux process. Following hypo-osmotic swelling, osmoregulatory shrinkage seemed to be due to K^+ efflux through quinine-sensitive K^+ channels that were relatively insensitive to Ba^{2+}. Cl^- efflux appeared to occur *via* MK-196- and furosemide-sensitive but NPPB-insensitive Cl^- conductive pathways. The RVD process was independent of extra- and intracellular Ca^{2+}. Likewise, no increase in the intracellular Ca^{2+} activity was found upon hypo-osmotic stimulation. Administration of the 5-lipoxygenase antagonist ETH 615-139, but not the cycloxygenase inhibitor indomethacin, however, inhibited RVD to a major degree. This inhibition probably occurred at the level of both the K^+ and the Cl^- conductance. Thus, in these trout proximal renal tubules, it appears that KCl efflux associated with hypo-osmotically stimulated RVD is a Ca^{2+}-independent process that is regulated by arachidonic acid metabolites from the 5-lipoxygenase pathway.

Materials and methods

General methodology

Trout (*Salmo trutta*, 50–100 g, presmolt) obtained from Ims Biological Station, Norwegian Institute for Nature Research, Stavanger, Norway, were kept at 12 °C in standard freshwater aquarium conditions. At the time of the experiments, the fish were decapitated and their renal tissue removed and placed in an iso-osmotic (290 mosmol kg^{-1}) fish Ringer's solution, similar in composition and pH to trout plasma (Table 1). Renal tubules were dissected manually and identified as proximal (Fig. 1) by the presence of a glomerulus. Following dissection, individual proximal tubules were transferred to iso-osmotic Ringer's solution in a glass-bottomed chamber located on the temperature-adjustable stage of an inverted microscope. Peristaltic pumps allowed continuous exchange of bath solution through inlet and outlet ports in the chamber. Each tubule end was crimped and held in place by applying suction to two glass pipettes mounted on micromanipulators. The tubular lumens were closed in all experiments; thus, only the basolateral aspect was studied. Unless otherwise indicated, the experiments were carried out at 15 °C. Experiments were video-taped using a charge-coupled-device (CCD) camera,

Table 1. *Composition of solutions*

	Iso-osmotic control	Hypo-osmotic control	Iso-osmotic high-[K^+]	Hypo-osmotic high-[K^+]	Iso-osmotic control for high-[Cl^-]	Hypo-osmotic control for high-[Cl^-]	Hypo-osmotic 77 mmol l^{-1} Cl^-	Hypo-osmotic 102 mmol l^{-1} Cl^-
NaCl	140	70	110	40	110	40	70	95
KCl	3	3	3	33	3	3	3	3
$NaHCO_3$	7	7	7	7	7	7	7	7
$MgSO_4$*	5	5	5	5	5	5	5	5
Glucose	5	5	5	5	5	5	5	5
$CaCl_2$	2†	2†	2	2	2	2	2	2
NaH_2PO_4	1	1	1	1	1	1	1	1
Mannitol	0	0	60	0	60	60	0	0
pH	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8
Osmolality	290	160	290	160	290	160	160	200

All solutions were bubbled with compressed 1% CO_2 in air.
*Substituted with $MgCl_2$ in experiments with $BaCl_2$.
†0.5 mmol l^{-1} in experiments with EGTA or EGTA and A23187.
Concentrations are given in mmol l^{-1} or mosmol kg^{-1}.

attached to both the optical port of the microscope and a video recorder.

The tubules were incubated for 3–5 min in the iso-osmotic Ringer's solution with or without test substances (see below). The bath solution was then rapidly exchanged with hypo-osmotic (160 mosmol kg^{-1}) Ringer's solution (Table 1) and stimulated for 15 min with or without test substances. The solutions were made hypo-osmotic by omission of 70 mmol l^{-1} NaCl. For each experiment with a test substance, a control experiment was performed in parallel. Tubular diameters were measured on the screen of a television monitor by replaying the video-taped experiments. The diameters were used as a cell volume indicator, and the data were expressed as relative diameter change (compared with the diameter measured at the time of solution change, i.e. time 0).

Effects of temperature and Na^+/K^+-ATPase on regulatory volume decrease

To test the effects of temperature on RVD and to find both a functional and practical temperature for the experiments, proximal renal tubules were exposed to iso-osmotic then hypo-osmotic control solutions (as explained above) at 10, 15 or 25 °C. These temperatures range from 2–3 °C below aquarium temperature up to room temperature. Five experiments were performed at each temperature. All other experiments in this study were performed at 15 °C. Further, to determine the role of the Na^+/K^+-ATPase in RVD in these tubules, 1 mmol l^{-1} (final concentration) ouabain was added to both the iso-osmotic and hypo-osmotic solutions (seven experiments).

Role of K^+ efflux during regulatory volume decrease

To determine the role of K^+ efflux during RVD in trout proximal renal tubules, the K^+ efflux gradient in the hypo-osmotic solution was reduced by increasing the K^+ concentration from 3 to 33 mmol l^{-1} (iso-osmotic replacement of mannitol for KCl; Table 1). In the iso-osmotic solutions of both the control experiments (N=5) and the experiments with an increased K^+ concentration (N=5), 30 mmol l^{-1} NaCl was exchanged with 60 mmol l^{-1} mannitol (KCl concentration was kept normal, Table 1). To study whether a potential RVD-associated K^+ efflux occurred through K^+ channels, the K^+ channel inhibitors $BaCl_2$ (1 and 2 mmol l^{-1}, three and five experiments, respectively), quinine (0.1 and 0.5 mmol l^{-1}, three and five experiments, respectively) or $BaCl_2$ (2 mmol l^{-1}) and quinine (0.5 mmol l^{-1}, five experiments) were added to the iso-osmotic and hypo-osmotic solutions. In the experiments with $BaCl_2$, the $MgSO_4$ in the iso-osmotic and hypo-osmotic solutions was substituted with $MgCl_2$.

Role of Cl^- efflux during regulatory volume decrease

Similar experiments were performed in which the Cl^- concentration of the hypo-osmotic solution was increased from 47 mmol l^{-1} to 77 and 102 mmol l^{-1} by increasing the NaCl concentration (five experiments each; Table 1). In these experiments, 60 mmol l^{-1} mannitol (substituted for 30 mmol l^{-1} NaCl) of the control hypo-osmotic solution was replaced by 70 and 95 mmol l^{-1} NaCl, respectively. The osmolality of the high-[Cl^-] (102 mmol l^{-1}) hypo-osmotic solution was increased to 200 mosmol kg^{-1}, while the other hypo-osmotic osmolalities remained as in the control experiments (160 mosmol kg^{-1}). The iso-osmotic solutions in all these experiments contained 60 mmol l^{-1} mannitol substituted for 30 mmol l^{-1} NaCl (Table 1). To determine whether a potential Cl^- efflux during RVD could occur through Cl^- channels, the Cl^- channel inhibitors 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) (1, 5, 8 and 10 µmol l^{-1}; N=5, 5, 2 and 3, respectively) or MK-196 (indacrinone) (0.5 mmol l^{-1}, five experiments) were added to both the iso-osmotic and hypo-osmotic solutions. Following the demonstration that MK-196 could inhibit RVD, proximal

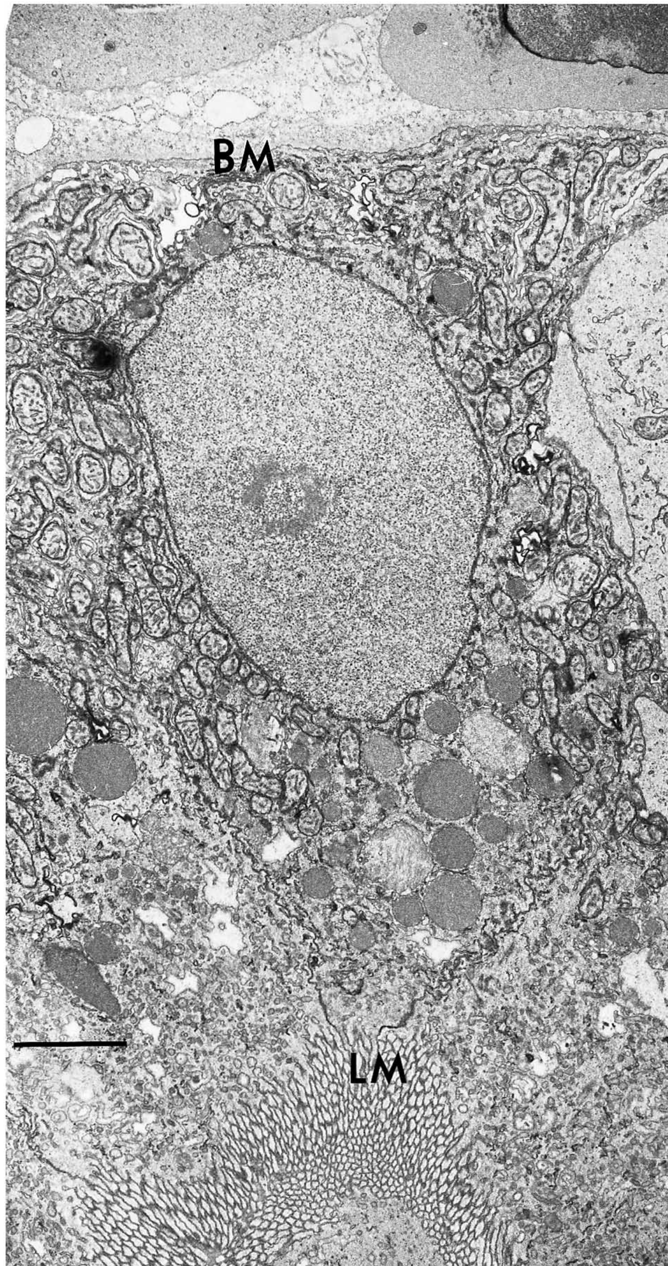


Fig. 1. Electron micrograph of a proximal renal tubule cell from trout (*Salmo trutta*). As in their mammalian counterpart, the luminal membrane has numerous microvilli, the nucleus occupies approximately two-thirds of the cell and the cytosol contains pinocytotic vesicles. In contrast to proximal renal tubules of mammals, but like those in other teleosts, mitochondria in the trout cells can be found throughout the cytoplasm, and the trout basolateral membrane contains relatively few infoldings. LM, luminal membrane; BM, basolateral membrane. Scale bar, 2 μm.

renal tubules were exposed to both iso-osmotic and hypo-osmotic solutions containing both MK-196 (0.5 mmol l^{-1}; five experiments) and the anion exchanger tributyltin (TBT; 1 μmol l^{-1}) (Wieth and Tosteson, 1979; Wulf and Byington, 1975).

In addition, furosemide (0.1 mmol l^{-1}) was added to both the iso-osmotic and hypo-osmotic Ringer's solutions to investigate whether part of the RVD-associated KCl efflux could occur through K^+/Cl^- symporters (five experiments). Five experiments were also performed to determine whether a potential furosemide inhibition of RVD occurred at the level of Cl^- efflux only. In these experiments, furosemide (0.1 mmol l^{-1}) and TBT (1 μmol l^{-1}) were administered simultaneously to the iso-osmotic and hypo-osmotic solutions.

Role of Ca^{2+} and arachidonic acid metabolites in regulatory volume decrease

To determine whether extracellular Ca^{2+} could be an important signalling factor for RVD, trout proximal renal tubules were exposed to iso-osmotic (290 mosmol kg^{-1}) then hypo-osmotic (160 mosmol kg^{-1}) Ringer's solutions in which 2 mmol l^{-1} $CaCl_2$ had been replaced by 1 mmol l^{-1} EGTA and 0.1 mmol l^{-1} $CaCl_2$ (five experiments). Control experiments were performed with similar solutions except for the omission of EGTA. Other experiments were performed in which both the extracellular and intracellular Ca^{2+} concentrations were reduced to zero by the addition of 1 mmol l^{-1} EGTA and 10 μmol l^{-1} A23187 (0.1 mmol l^{-1} $CaCl_2$) to both the iso-osmotic and hypo-osmotic solutions (five experiments).

The possible participation of arachidonic acid metabolites in the activation of RVD was tested by using the pharmacological agent ETH 615-139 (20 μmol l^{-1}, five experiments) to inhibit the 5-lipoxygenase pathway of arachidonic acid metabolism. To determine which of the RVD fluxes (K^+ or Cl^-) had been inhibited by this agent, ETH 615-139 (20 μmol l^{-1}) was added to both the iso-osmotic and hypo-osmotic solutions simultaneously with either the K^+ ionophore gramicidin (0.5 mmol l^{-1}) or the anion exchanger tributyltin (TBT; 1 μmol l^{-1}) (five experiments each). In five experiments, the cycloxygenase inhibitor indomethacin (10 μmol l^{-1}; an antagonist of the metabolism of arachidonic acid into, among other products, the prostaglandins) was added to both the iso-osmotic and hypo-osmotic solutions.

Measurements of variations in intracellular [Ca^{2+}]

To confirm the results with Ca^{2+} chelation, experiments were performed using the acetoxymethyl ester form (AM) of the fluorescent intracellular Ca^{2+} indicator Fura-2 (Grynkiewicz *et al.* 1985; Sanna *et al.* 1994). In these experiments, proximal renal tubules were placed on pieces of coverslip coated with CellTak to improve tissue adhesion to the glass. The coverslips were placed in a glass-bottomed chamber filled with iso-osmotic (290 mosmol kg^{-1}) Ringer's solution to which 10 μmol l^{-1} Fura-2/AM was added to a final concentration of 1 μmol l^{-1}. Tubules were incubated in this solution for approximately 40 min in the dark at room temperature (25 °C). It had previously been determined that RVD at this temperature is identical to that at 15 °C. Following incubation, excess Fura-2/AM was removed by rinsing the chamber three times with Fura-free iso-osmotic Ringer's solution. The chambers containing the proximal tubules were then transferred to the stage of a Nikon Diaphot 300 inverted microscope fitted with EPI-fluorescence attachments and a

colour-chilled CCD camera and controller (C5310-11, Hamamatsu, Japan). The intensity of the fluorescence signal was measured in the iso-osmotic solutions at excitation wavelengths of 340 and 380 nm (emission wavelength 509 nm). The solution in the chamber was rapidly exchanged with the hypo-osmotic (160 mosmol kg^{-1}) Ringer's solution, and the fluorescence intensity was measured as with the iso-osmotic solution. The ratio (R) of the fluorescence intensity (F) at 340 and 380 nm ($R=F_{340}/F_{380}$) was used as a measure of the relative Ca^{2+} concentration in the cells (determined using Image Pro Plus software, version 2.0, Media Cybernetics, MD, USA). The value of R of the Ca^{2+} response in the hypo-osmotic solution was expressed as a percentage of that in the iso-osmotic solution (iso-osmotic R set to 100 %).

Chemicals

MK-196 was a gift from MSD, Oslo, Norway. Tributyltin chloride was purchased from Aldrich Chemicals, Germany, and NPPB was purchased from the SMS group, Hørsholm, Denmark. ETH 615-139 was kindly donated by Dr E. Petersen, Leo Pharmaceutical Products Ltd. All other chemicals were purchased from Sigma Chemical Co., St Louis, MO, USA.

Statistics

All data are expressed as means ± standard error (S.E.M.). Significance was established using Student's *t*-tests. Differences were considered significant if $P<0.05$. One-way analysis of variance (ANOVA) and *post-hoc* comparisons of means (Duncan test) were performed using the Statistica software program (version 5.1, StatSoft, Inc., Tulsa, OK, USA).

Results

Role of temperature and Na^+/K^+-ATPase in regulatory volume decrease

To determine whether the RVD response in trout proximal renal tubules was temperature-sensitive, tubules were incubated with iso-osmotic solutions and stimulated with hypo-osmotic solutions at 10, 15 or 25 °C. The relative diameter change (and relative volume change) did not vary significantly in the iso-osmotic solutions (Fig. 2A). Following a change to hypo-osmotic solution (time 0), the cells swelled rapidly as the result of water influx, and at 15 and 25 °C the cells reached a maximum relative tubule diameter of 132.0±4.8 % within 1 min. At 10 °C, the cell volumes did not reach their maximum until 4 min after hypo-osmotic stimulation. Following the initial swelling, cell diameters at all temperatures decreased gradually (RVD phase) by approximately 65–75 % as measured 15 min after hypo-osmotic stimulation (Fig. 2A; percentage relative diameter decrease from maximum cell swelling: 74.3±6.3, 74.5±4.5 and 74.5±4.8 at 10, 15 and 25 °C, respectively). All other experiments in this study were performed at 15 °C (see the Discussion), and percentage relative volume regulation for all the controls was set to 100 %.

In the presence of 1 mmol l^{-1} ouabain, cell volumes reached a maximum at approximately the same time as in the control experiments (Fig. 2B). Both the amount of cell swelling and the RVD response were, however, reduced ($P<0.05$) by this Na^+/K^+-ATPase antagonist (percentage relative diameter decrease: 72.0±10.5, controls: 100; Table 2; Fig. 2B).

K^+ efflux during regulatory volume decrease

To determine whether K^+ efflux could play a role during the RVD phase in proximal renal tubules the K^+ efflux

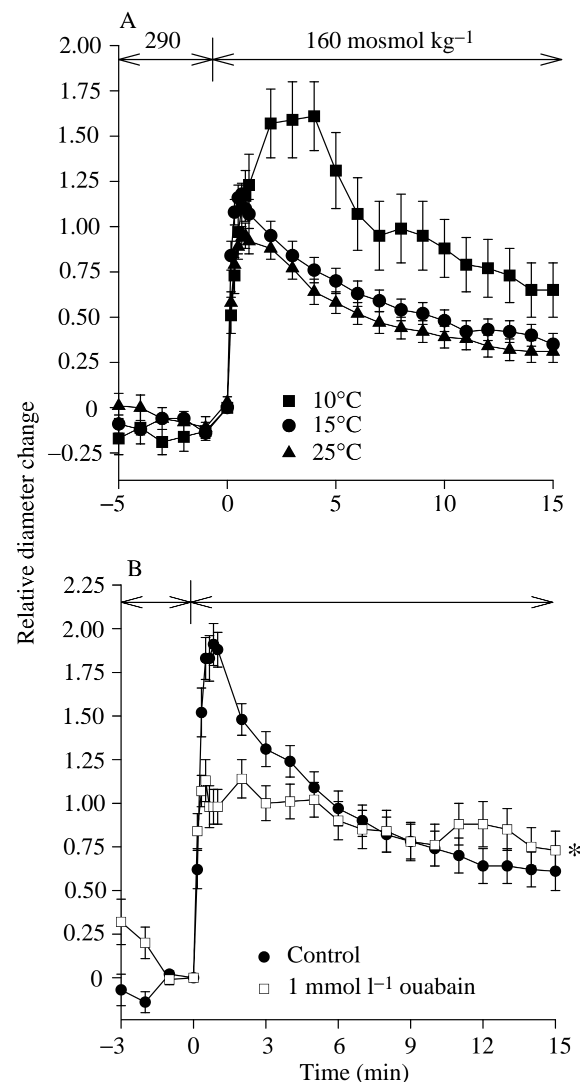


Fig. 2. (A,B) Effects of temperature (A) and ouabain (B) on the regulatory volume decrease (RVD) in trout proximal renal tubules. Relative diameter change is used as a measure of relative volume change. Isolated tubules were exposed to iso-osmotic (290 mosmol kg^{-1}) trout Ringer's solution for 5 min prior to hypo-osmotic (160 mosmol kg^{-1}) stimulation (time 0). Experiments were performed at solution temperatures of 10, 15 and 25 °C (N=5). In a different set of experiments, tubules were exposed to 1 mmol l^{-1} ouabain for 3 min in iso-osmotic solution and for 15 min in hypo-osmotic solution (15 °C, N=7). Parallel control experiments were performed without ouabain (N=7). Values are means ± S.E.M. An asterisk indicates a significant difference in RVD from the control value ($P<0.05$).

Table 2. *Summary of results*

Experiment	Percentage volume regulation (compared with controls)
Control	100
Ouabain	72.0±10.5*
High [K^+]	23.5±3.6***
Quinine (0.1 mmol l^{-1})	75.0±5.4*
Quinine (0.5 mmol l^{-1})	27.7±5.1***
Ba^{2+} + quinine	49.8±3.9**
High [Cl^-] (102 mmol l^{-1})	54.8±7.9*
NPPB (8 μmol l^{-1})	71.8±8.3*
MK-196	45.3±5.3***
MK-196 + TBT	74.7±9.5*
Furosemide	60.2±3.9***
ETH 615-139 (20 μmol l^{-1})	40.6±4.3***
ETH 615-139 + gramicidin (0.5 mmol l^{-1})	35.0±7.5***
ETH 615-139 + TBT (1 μmol l^{-1})	58.2±5.2*
Indomethacin (10 μmol l^{-1})	84.2±4.2*

Temperature experiments are not included since these were used to establish the control value (100 %).

The magnitude of regulatory volume decrease was almost identical at 10, 15 and 25 °C (see text).

Only results that are significantly different from controls are shown: *P<0.05; **P<0.01; ***P<0.001.

NPPB [5-nitro-2-(3-phenylpropylamino)benzoic acid] is a Cl^- channel inhibitor; MK-196 (indacrinone) is a Cl^- channel inhibitor; ETH 615-139 is a 5-lipoxygenase inhibitor; TBT (tributyltin) is an anion exchanger.

Values are means ± S.E.M., N=3–10.

gradient was decreased by increasing the K^+ concentration in the hypo-osmotic solution from 3 to 33 mmol l^{-1} (iso-osmotic exchange of mannitol for KCl). Increased K^+ concentration resulted in a significant (P<0.001) inhibition of RVD (Fig. 3A; percentage relative volume change 23.5±3.6; Table 2). To determine whether this K^+ efflux could occur through K^+ channels, the K^+ channel inhibitor quinine was added to both the iso-osmotic and hypo-osmotic solutions (Fig. 3B). This inhibitor reduced RVD at 0.1 mmol l^{-1} (P<0.05; percentage relative volume regulation: 75.0±5.4; Table 2) and more strongly at 0.5 mmol l^{-1} (P<0.001; percentage relative volume regulation: 27.7±5.1; Table 2). In contrast, no significant inhibition was found in the presence of either 1 or 2 mmol l^{-1} $BaCl_2$ (data not shown; percentage relative volume regulation: 74.7±10.0 and 95.1±4.3, respectively). To determine whether Ba^{2+} and quinine could exert an additive inhibition of RVD, 2 mmol l^{-1} $BaCl_2$ and 0.5 mmol l^{-1} quinine were added simultaneously to both the iso-osmotic and hypo-osmotic Ringer's solutions. It was found (data not shown) that the inhibitory effect on RVD was no greater with these two compounds administered together than with quinine alone (percentage relative volume decrease: 49.8±3.9; Table 2; P<0.01).

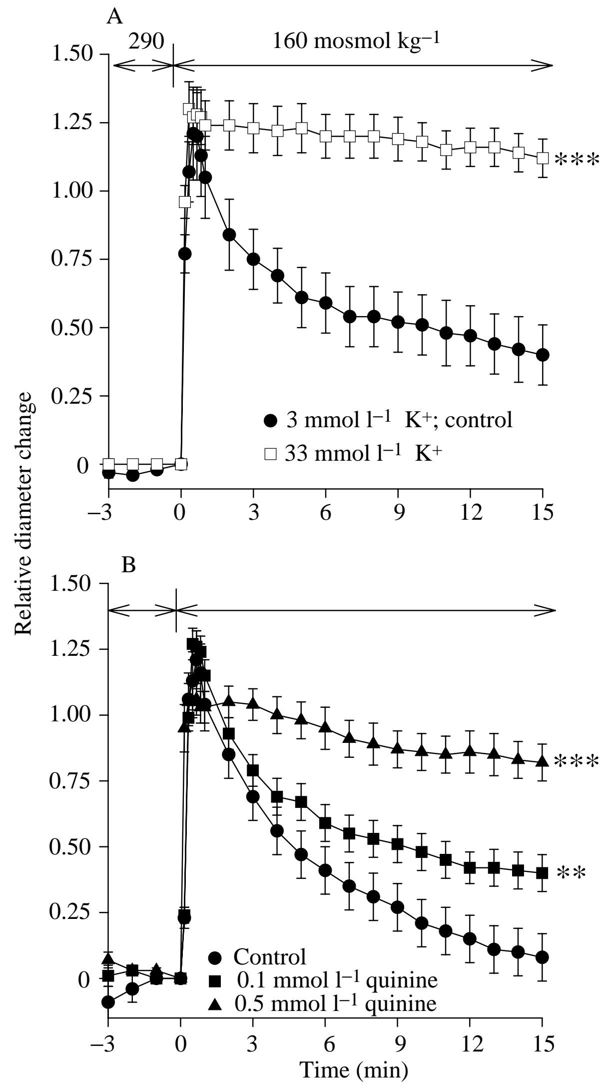


Fig. 3. (A,B) Effects of a high [K^+] in the bath (A) or the K^+ channel blocker quinine (B) on regulatory volume decrease (RVD) in trout proximal renal tubules. Tubules were exposed for 3 min to an iso-osmotic (290 mosmol kg^{-1}) Ringer's solution with normal K^+ concentration (3 mmol l^{-1}). At time 0, the solution was exchanged with a hypo-osmotic (160 mosmol kg^{-1}) solution in which 60 mmol l^{-1} mannitol had been replaced by 30 mmol l^{-1} KCl (final K^+ concentration 33 mmol l^{-1}) (N=5). Iso-osmotic and hypo-osmotic solutions with normal K^+ concentration (3 mmol l^{-1}) were used in the control experiments (N=5). In a different set of experiments, tubules were preincubated for 3 min in the iso-osmotic Ringer's solution containing either 0.1 mmol l^{-1} (N=5) or 0.5 mmol l^{-1} (N=3) quinine chloride. At time 0, the bath solution was exchanged with hypo-osmotic control Ringer's solution also containing either 0.1 or 0.5 mmol l^{-1} quinine. Control experiments (N=7) were performed without quinine. Values are means ± S.E.M.. An asterisk indicates a significant difference from the control value at 15 min after transfer to hypo-osmotic solution; **P<0.01; ***P<0.001.

Cl^- efflux during regulatory volume decrease

To determine whether Cl^- efflux was important for RVD, the Cl^- efflux gradient was decreased (Table 1) by increasing the Cl^- concentration in the hypo-osmotic solution from 47 to

77 mmol l^{-1} (iso-osmotic addition of NaCl for mannitol; final osmolality 160 mosmol kg^{-1}) or 102 mmol l^{-1} (addition of NaCl for mannitol; final osmolality 200 mosmol kg^{-1}). It was observed that increasing the Cl^- concentration of the hypo-osmotic solution by 30 mmol l^{-1} had no significant influence on RVD (Fig. 4A) (percentage relative volume change: 79.4±8.7). Increasing the Cl^- concentration by 55 mmol l^{-1}, however, resulted in a significant inhibition ($P<0.05$) of RVD (Fig. 4A) (percentage relative volume regulation: 54.8±7.9; Table 2). Since, in the latter experiments, the osmolality of the hypo-osmotic solution was increased by 40 mosmol kg^{-1} compared with the control hypo-osmotic solution, the osmotic difference between the iso-osmotic and the hypo-osmotic solutions was smaller and, therefore, the amount of water influx and cell swelling were reduced compared with the control experiments. The magnitude of RVD in these experiments, thus, cannot be directly compared with the control experiments without introducing some error. Therefore, to study further the possible participation of Cl^- in RVD, the Cl^- channel inhibitor MK-196 (0.5 mmol l^{-1}) was added to the iso-osmotic and hypo-osmotic solutions. MK-196 significantly ($P<0.001$) decreased RVD (Fig. 4B; percentage relative volume decrease: 45.3±5.3; Table 2). To confirm that this effect was due to a decreased Cl^- efflux, experiments were performed in which MK-196 (0.5 mmol l^{-1}) was added to the iso-osmotic and hypo-osmotic solutions together with the anion exchanger TBT. It was found that the presence of TBT resulted in a partial recovery of RVD (Fig. 4B; percentage relative volume regulation: 74.7±9.5; Table 2). In contrast, the Cl^- channel antagonist NPPB (1, 5, 8 and 10 μmol l^{-1}) had no such negative effect on RVD (data not shown). In these experiments, it was observed that RVD was slightly, though significantly ($P<0.05$), inhibited only at 8 μmol l^{-1} NPPB (percentage relative volume regulation: 71.8±8.3; Table 2).

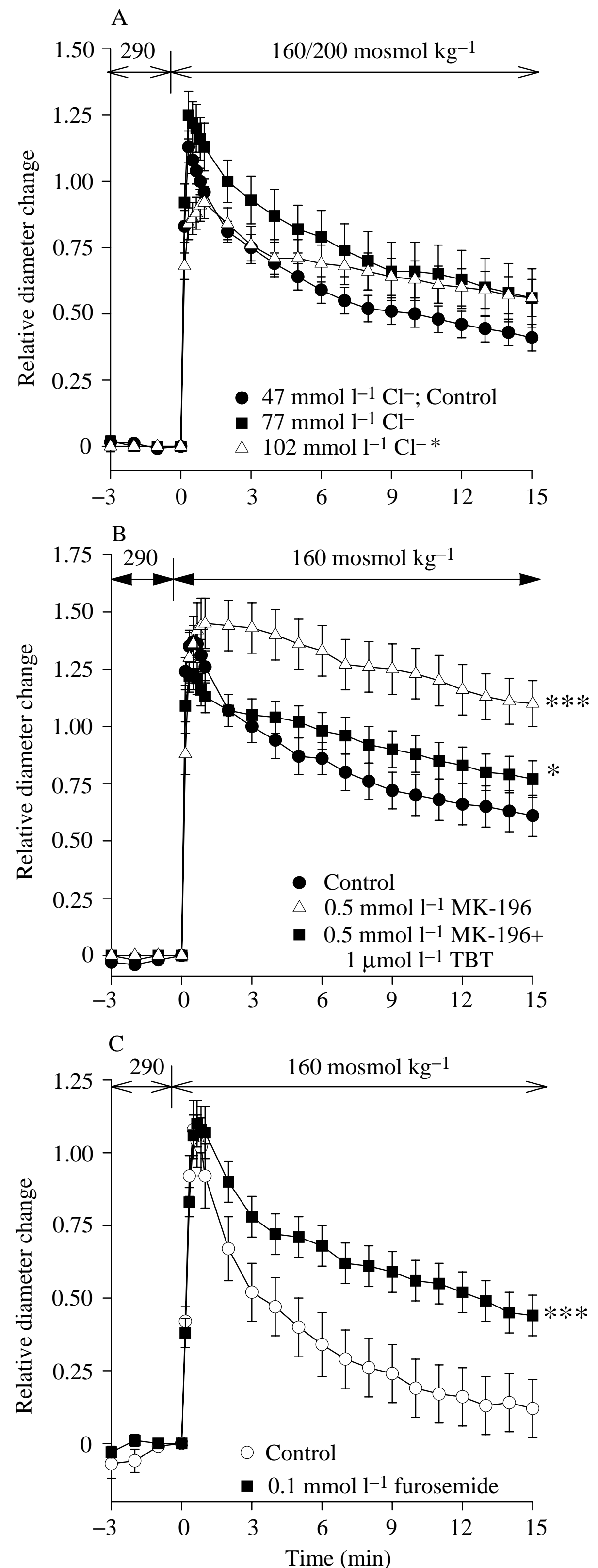
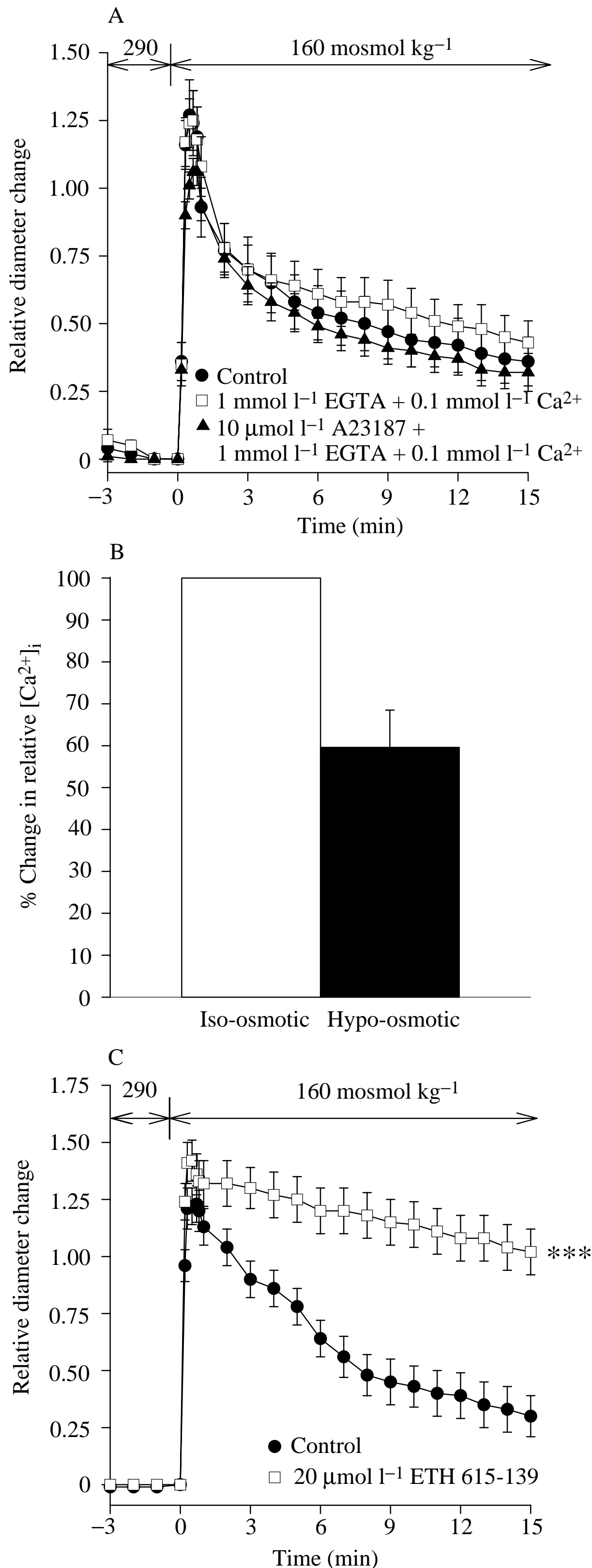


Fig. 4. (A–C) Effects of increasing bath Cl^- concentration (A) or adding the Cl^- channel blocker MK-196 (B) or the K^+/Cl^- cotransport inhibitor furosemide (C) on regulatory volume decrease (RVD). Proximal renal tubules from trout were exposed for 3 min to iso-osmotic solutions (290 mosmol kg^{-1}) in which 30 mmol l^{-1} NaCl had been substituted with 60 mmol l^{-1} mannitol. At time 0, the bath solution was exchanged with a solution similar to the control hypo-osmotic solution but in which 30 mmol l^{-1} NaCl had been exchanged with mannitol (160 mosmol kg^{-1}; total Cl^- concentration 47 mmol l^{-1}, N=10), a control hypo-osmotic solution (160 mosmol kg^{-1}; total Cl^- concentration 77 mmol l^{-1}) or a control hypo-osmotic solution to which 25 mmol l^{-1} NaCl had been added (200 mosmol kg^{-1}; total Cl^- concentration 102 mmol l^{-1}). In a different set of experiments, proximal renal tubules were exposed to 0.5 mmol l^{-1} MK-196 added to both the iso-osmotic (290 mosmol kg^{-1}) and hypo-osmotic (160 mosmol kg^{-1}) solutions. To determine whether the effect of MK-196 could be related to Cl^- efflux, proximal renal tubules in another set of experiments were exposed to iso-osmotic and hypo-osmotic solutions to which both 0.5 mmol l^{-1} MK-196 and the anion exchanger tributyltin (TBT, 1 μmol l^{-1}) had been added. Control experiments were performed without MK-196. In yet other experiments, tubules were exposed to 0.1 mmol l^{-1} furosemide for 3 min in iso-osmotic solution (290 mosmol kg^{-1}). At this time, the solution was exchanged (time 0) with a hypo-osmotic control Ringer's solution containing 0.1 mmol l^{-1} furosemide. Values are means ± S.E.M. Unless otherwise indicated, N=5. An asterisk indicates a significant difference from the control value at 15 min after transfer to hypo-osmotic solution; *$P<0.05$; ***$P<0.001$.

Finally, to study whether some of the KCl efflux associated with the RVD process could occur through the K^+/Cl^- symporter, 0.1 mmol l^{-1} furosemide was added to both the iso-osmotic and hypo-osmotic solutions. This inhibitor caused a significant (P<0.001) inhibition of the volume regulatory response (Fig. 4C; percentage relative volume regulation: 60.2±3.9; Table 2). This inhibition could be reversed by the addition of 1 μmol l^{-1} TBT to the furosemide-containing solutions (data not shown, percentage relative volume regulation: 89.4±6.4).



Signalling of regulatory volume decrease

To study whether Ca^{2+} influx from the extracellular medium was necessary for activation of RVD, proximal renal tubules were incubated in iso-osmotic (290 mosmol kg^{-1}, 3 min) and stimulated with hypo-osmotic (160 mosmol kg^{-1}) Ringer's solutions in which $[Ca^{2+}]$ had been chelated towards zero by the addition of 1 mmol l^{-1} EGTA (0.1 mmol l^{-1} Ca^{2+}). No significant change in RVD was induced by this chelation (percentage relative volume regulation: 92.1±8.2; Fig. 5A). Similarly, chelation of both intra- and extracellular Ca^{2+} (10 μmol l^{-1} A23187, 1 mmol l^{-1} EGTA, 0.1 mmol l^{-1} Ca^{2+}) had no significant effect on RVD (percentage relative volume regulation: 86.8±8.0; Fig. 5A). These results were confirmed using the intracellular Ca^{2+} indicator Fura-2/AM. No increase in intracellular Ca^{2+} activity was observed following hypo-osmotic stimulation (Fig. 5B).

The role of arachidonic acid metabolites in the signalling of

Fig. 5. (A–C) Effect of reducing extracellular and both extra- and intracellular Ca^{2+} activity on regulatory volume decrease (RVD) (A) and the role of intracellular Ca^{2+} (B) and the 5-lipoxygenase pathway (C) in RVD in proximal renal tubules from trout. Tubules were exposed to an iso-osmotic (290 mosmol kg^{-1}) Ringer's solution containing either 1 mmol l^{-1} EGTA and 0.1 mmol l^{-1} Ca^{2+} or 10 μmol l^{-1} A23187, 1 mmol l^{-1} EGTA and 0.1 mmol l^{-1} Ca^{2+}. After 3 min (time 0), the solution was exchanged with hypo-osmotic Ringer's containing similar concentrations of A23187, EGTA and Ca^{2+}. Parallel control experiments were performed without A23187 and EGTA. In additional experiments, changes in intracellular free Ca^{2+} concentration were measured upon hypo-osmotic stimulation (B). Proximal renal tubules of trout were exposed to iso-osmotic (290 mosmol kg^{-1}) Ringer's solution preloaded with the fluorescent intracellular Ca^{2+} indicator Fura-2/AM (N=3). The bath solution was then exchanged with hypo-osmotic Ringer's solution (160 mosmol kg^{-1}). The intensity of the fluorescence signal was measured at excitation wavelengths of 340 and 380 nm (emission wavelength 509 nm). The ratio (R) of the fluorescence intensity (F) at 340 and 380 nm ($R=F_{340}/F_{380}$) was used as a measure of the relative Ca^{2+} concentration in the cells. The value of R of the Ca^{2+} response in the hypo-osmotic solution is expressed as a percentage of that in the iso-osmotic solution (iso-osmotic R set to 100 %). Finally, to test whether arachidonic acid metabolites from the 5-lipoxygenase pathway could be involved in the signalling of RVD in these tubules, the 5-lipoxygenase inhibitor ETH 615-139 (20 μmol l^{-1}) was added to iso-osmotic (290 mosmol kg^{-1}) and hypo-osmotic (160 mosmol kg^{-1}) Ringer's solutions. Control experiments were performed without the inhibitor. Values are means ± S.E.M. Unless otherwise indicated, N=5. An asterisk indicates a significant difference from the control value at 15 min after transfer to hypo-osmotic solution; ***P<0.001.

RVD in these tubules was then examined. Inhibition of 5-lipoxygenase (the enzyme that catalyzes the production of leukotrienes) with 20 μmol l^{-1} ETH 615-139 induced a strong inhibition ($P<0.001$) of RVD (Fig. 5C; percentage relative volume regulation: 40.6±4.3; Table 2). This inhibition could not be overcome using either the K^+ ionophore gramicidin (0.5 mmol l^{-1}; percentage relative volume regulation: 35.0±7.5; Table 2; data not shown) or the Cl^-/OH^- exchanger tributyltin (1 μmol l^{-1}; percentage relative volume regulation: 58.2±5.2; Table 2; data not shown). Inhibition of cycloxygenase (the enzyme that catalyzes arachidonic acid breakdown into prostaglandins) by 10 μmol l^{-1} indomethacin induced only a small, though significant ($P<0.05$), decrease in RVD (percentage relative volume regulation: 84.2±4.2; data not shown).

Discussion

In terrestrial species, cell volume regulatory mechanisms have developed mainly in response to variations in the internal environment. For aquatic species, however, cell volume regulation is critical in dealing both with variations in the external environment and with changes in the composition of extracellular body fluids. In fish, both the kidneys and the gills are important in maintaining the composition of extracellular body fluid relatively constant. Most saltwater teleosts are exposed to salt loading and water loss and, therefore, potential cell shrinkage. These fish excrete excess salt through the gills and faeces, while urine output is kept to a minimum. In contrast, the typical freshwater teleost is threatened by dilution of extracellular body fluid and cell swelling due to water influx. In such fish, excess water is excreted as hypo-osmotic urine. Thus, for the trout used in the present study, the physiological meaning of the regulatory volume decrease response is probably in maintaining a relatively constant cell volume during osmotic variations induced both by changes occurring within the animal itself (for example, changes in metabolism) and by the external environment (fresh water).

The role of temperature and the Na^+/K^+-ATPase in regulatory volume decrease

The cell volumes of the proximal renal tubules remained relatively constant in iso-osmotic (290 mosmol kg^{-1}) solutions (Fig. 2A). This lack of change is to be expected as there should be no significant difference in osmolality between the intra- and extracellular media. Upon hypo-osmotic (160 mosmol kg^{-1}) stimulation, these water-permeable cells swelled rapidly due to water influx. This swelling was followed by an RVD phase in which the cell diameters decreased by approximately 65–75 %. The rate of cell swelling appeared to be nearly the same at both 15 and 25 °C. At 10 °C, however, the time from hypo-osmotic stimulation to maximum cell swelling was longer than that at 15 and 25 °C (4 min at 10 °C compared with 40 s at 15 °C and 50 s and 25 °C). Thus, the time of onset of the RVD phase was delayed by approximately 3 min at 10 °C compared with the higher temperatures, resulting in an elevated maximum cell volume. These results may be explained by the observations that the plasma membrane fluidity and the activity of transport molecules, e.g. the Na^+/K^+-ATPases (Charnock *et al.* 1971; Esmann and Skou, 1988), are temperature-sensitive in many tissues. Furthermore, it is also possible that, as observed in isolated rabbit proximal renal tubules, hypothermia inhibits RVD through an increase in Na^+ accumulation (Grantham *et al.* 1977). The remainder of the experiments in the present study were performed at 15 °C, a temperature at which the RVD response was rapid. In addition, this temperature is near that of the aquarium.

Since Na^+/K^+-ATPase, which is necessary to maintain the intracellular K^+ concentration, is important in cell volume regulation in iso-osmotic solutions (Gagnon *et al.* 1982; Grantham *et al.* 1977; Linshaw and Grantham, 1980), the role of this transporter in hypo-osmotically induced volume regulation was studied by the addition of the cardiac glycoside ouabain. Ouabain was found both to reduce the amount of cell swelling upon hypo-osmotic stimulation and to inhibit RVD (Fig. 2B). Volume regulation in proximal renal tubules has been found to be inhibited by ouabain in other studies (Gagnon *et al.* 1982; Kirk *et al.* 1987; Linshaw and Grantham, 1980). Some researchers have attributed this effect to the importance of Na^+ and water efflux during RVD (Gagnon *et al.* 1982). It has, however, been noted in proximal straight tubules that the cells initially shrink upon exposure to ouabain (Kirk *et al.* 1987; Linshaw *et al.* 1977). This shrinkage is followed by cell swelling to a steady-state volume. As a consequence of the high K^+ permeability of the basolateral membrane, the initial ouabain-induced shrinking was thought to be because K^+ efflux exceeded Na^+ influx (Grantham *et al.* 1977; Welling *et al.* 1985). This theory corresponds well with the observation that the intracellular K^+ activity is quickly reduced upon ouabain administration, the effect occurring within minutes after addition (Biagi *et al.* 1981). In the present study, cell shrinkage in the iso-osmotic solution, but no subsequent swelling, was also observed upon ouabain administration (Fig. 2B). The lack of cell swelling in the iso-osmotic solution is probably due to the short exposure to ouabain, only 3 min, prior to hypo-osmotic stimulation. In comparison, Gagnon *et al.* (1982) observed cell swelling only after approximately 20 min of exposure to ouabain. Therefore, if indeed ouabain induces K^+ efflux in iso-osmotic solutions, it is to be expected that a short (minutes) incubation in ouabain followed by hypo-osmotic stimulation would lead to (i) a cell swelling that is less than that in the control preparation due to the pre-shrunken state of the cells (result of K^+ efflux), and (ii) a compromised RVD due to a reduction in the intracellular concentration of the osmoeffector K^+ (discussed below). In addition, as has been proposed by Kirk *et al.* (1987), it is likely that, owing to the coupling between the pump and the K^+ leak, the K^+ permeability might have been decreased indirectly upon inhibition of Na^+/K^+-ATPase.

Mechanism of osmoregulatory KCl efflux during regulatory volume decrease

K^+ efflux

As observed in tissues of a variety of species, K^+ and Cl^- efflux followed by the efflux of osmotically obligated water

also appear to be important for RVD in trout proximal renal tubules (Hoffmann *et al.* 1984; Macri *et al.* 1993; Terreros *et al.* 1990). As others have found in renal cells (Kirk *et al.* 1987; Rothstein and Mack, 1990), we found that RVD was reduced when the efflux gradient for K^+ was decreased by increasing the peritubular K^+ concentration (Fig. 3A). It appears that much of this K^+ efflux occurs through K^+ channels since administration of the K^+ channel inhibitor quinine (Cook, 1988) inhibited the trout proximal tubule RVD response by 70 % (Fig. 3B). Interestingly, it was found that the K^+ channel inhibitor Ba^{2+} (Cook, 1988) had little effect on RVD. This result was initially surprising since Ba^{2+}-sensitive K^+ loss during RVD has been described in other tissues (Haddad and Graf, 1989; Lambert *et al.* 1984; Lau *et al.* 1984; Terreros *et al.* 1990; Welling *et al.* 1985). A similarly slight effect of Ba^{2+} has, however, been documented in hepatocytes where quinidine blocked RVD-associated K^+ efflux more effectively than Ba^{2+} (Haussinger *et al.* 1990). Furthermore, hypo-osmotically stimulated K^+ loss that is more sensitive to quinine/quinidine than to Ba^{2+} has been documented in other cells (De Smet *et al.* 1995; Illek *et al.* 1992). In MDCK cells, Ba^{2+} is unable to inhibit RVD-associated K^+ loss significantly (Simmons, 1991). Contrary to observations in proximal renal tubules from rabbit (Lapointe and Duplain, 1991), in the present study addition of both Ba^{2+} ($2\,mmol\,l^{-1}$) and quinine ($0.5\,mmol\,l^{-1}$) induced no additional inhibitory effect on volume regulation compared with each of the inhibitors alone.

Cl^- efflux

It appears that Cl^- efflux is important for RVD in the trout proximal renal tubules used in the present study. Increases in extracellular Cl^- concentration (from 47 to 77 and $102\,mmol\,l^{-1}$), to reduce the electrochemical gradient for Cl^- efflux, induced a slight inhibition of RVD (Fig. 4A). Administration of the Cl^- channel blocker MK-196, however, resulted in a 55 % inhibition of RVD (Fig. 4B). It was confirmed that this effect was due to inhibition of the Cl^- conductance because simultaneous addition of MK-196 and the anion carrier tributyltin almost completely reversed this repression (Fig. 4B). Interestingly, the Cl^- channel blocker NPPB (Wangemann *et al.* 1986), which has been reported to inhibit swelling-activated Cl^- efflux (Best *et al.* 1996; Grunewald *et al.* 1993; Kubo and Okada, 1992), had only minor effects on RVD in the present study. Of the four concentrations of NPPB tested (1, 5, 8 and $10\,\mu mol\,l^{-1}$), only $8\,\mu mol\,l^{-1}$ significantly inhibited RVD. Although the IC_{50} of Cl^- channel inhibition by NPPB is $8\times10^{-8}\,mol\,l^{-1}$ in thick ascending limb sections of rabbit nephrons (Wangemann *et al.* 1986), and $1\,\mu mol\,l^{-1}$ NPPB has been used in cell volume regulation studies in other proximal renal tubules (Völkl and Lang, 1988), the NPPB concentration used for various tissues in several different studies varies a great deal and is frequently much higher than the IC_{50} noted above. Thus, even though a slight effect was noted at $8\,\mu mol\,l^{-1}$ in the present study, it may still be that this inhibitor could have been more effective at another concentration. At any rate, if NPPB-inhibitable Cl^- channels are involved to a significant degree in RVD in these trout proximal renal tubules, these channels do not belong to the most NPPB-sensitive group. Furthermore, it should be noted that NPPB at high concentrations inhibits swelling-activated K^+ channels in HT-29/B6 cells (human colon adenocarcinoma origin; Illek *et al.* 1992). The concentrations necessary to induce this effect (IC_{50} $114\,\mu mol\,l^{-1}$) were much higher than those used in the present study; however, it has been suggested that as 'little' as $10\,\mu mol\,l^{-1}$ NPPB may interfere with the activity of other types of K^+ channels (Illek *et al.* 1992). Thus, it is not known whether some of the slight effect of $8\,\mu mol\,l^{-1}$ NPPB in the present study may have been due to inhibition of RVD-activated K^+ channels.

Finally, hypo-osmotic stimulation was performed in the presence of $0.1\,mmol\,l^{-1}$ furosemide to exclude the possibility that K^+ and Cl^- efflux during RVD in these proximal renal tubules may occur through a furosemide-sensitive K^+/Cl^- symporter, which has been localized to the basolateral cell membrane in mammalian (Greger and Schlatter, 1983) and *Amphiuma means* (Guggino, 1986) renal cells. It was observed that furosemide inhibited RVD by approximately 40 %; thus, it is possible that the K^+/Cl^- symporter functions in conjunction with osmoregulatory K^+ and Cl^- channels (Fig. 4C). However, it could be that most of this effect is due to inhibition of the Cl^- conductance only, since simultaneous addition of TBT and furosemide resulted in an almost complete restoration of RVD. Indeed, Cl^- channels that are directly or indirectly furosemide-sensitive have been observed in a variety of tissues (Uchida *et al.* 1995; Ishikawa and Cook, 1993; Evans *et al.* 1986).

Signalling of regulatory volume decrease

Role of extra- and intracellular Ca^{2+}

It appears that the RVD process in proximal renal tubules is not dependent on Ca^{2+} influx from the extracellular medium since chelation of bath Ca^{2+} with EGTA ($0.1\,mmol\,l^{-1}$ Ca^{2+}) had no effect on hypo-osmotically induced volume recovery (Fig. 5A). The tubules were exposed to EGTA for only 3 min in the iso-osmotic solution before hypo-osmotic stimulation (also in the presence of EGTA); thus, the treatment should not have interfered with intracellular Ca^{2+} stores to a significant degree (Ross and Cahalan, 1995). This result is in agreement with findings from both renal (Ubl *et al.* 1988*a*; Breton *et al.* 1992; Roy and Sauve, 1987) and non-renal (Corasanti *et al.* 1990; Hoffmann *et al.* 1984; Margalit *et al.* 1993; vom Dahl *et al.* 1991) cells. Nevertheless, cells apparently vary in their need for extracellular Ca^{2+} influx during RVD. For example, in the absence of extracellular Ca^{2+}, a complete regulatory volume decrease following hypo-osmotic stimulation does not occur in some renal cells in culture (Ehrenfeld *et al.* 1994; Suzuki *et al.* 1990).

However, it appears that the signal for RVD in the renal tubules used in the present study is not Ca^{2+} released from intracellular stores either, since intracellular Ca^{2+} chelation with the Ca^{2+} ionophore A23187 and extracellular EGTA ($0.1\,mmol\,l^{-1}$ Ca^{2+}) did not change the RVD response (Fig. 5A). This result was confirmed by the lack of increase in

the intracellular free Ca^{2+} concentration as observed with the Ca^{2+} indicator Fura-2/AM (Fig. 5B). These findings were initially surprising since they are in contrast with several studies in which chelation of intracellular Ca^{2+} or inhibition of Ca^{2+} release from intracellular stores results in a reduced or completely inhibited RVD (Hoffmann *et al.* 1984; Grinstein *et al.* 1982; Terreros *et al.* 1990). Similarly, the results are not in agreement with the participation of Ca^{2+}-activated K^+ channels proposed to be activated during the RVD process (Kawahara *et al.* 1991; Neufield *et al.* 1983; Terreros *et al.* 1990). In addition, increases in the intracellular Ca^{2+} activity paralleling cell swelling have been documented using fluorescent dyes in several renal cell types (Rothstein and Mack, 1990; Beck *et al.* 1991; McCarty and O'Neil, 1991; Raat *et al.* 1995; Ishii *et al.* 1996) and in some non-renal cells (Sardini *et al.* 1995; Ross and Cahalan, 1995). There are, however, some studies reporting no apparent increase in intracellular free $[Ca^{2+}]$ upon hypo-osmotic stimulation (Harbak and Simonsen, 1995; Thomas-Young *et al.* 1993; Grinstein and Smith, 1990; Rink *et al.* 1983). In addition to these observations, no effect of Ca^{2+} on RVD-associated ion currents has been observed in some cells (Beck *et al.* 1991; Best *et al.* 1996) and, moreover, it has been found that the RVD-associated K^+ loss known to occur through a K^+ channel could not be blocked by several inhibitors of Ca^{2+}-activated K^+ channels (Harbak and Simonsen, 1995; Thomas-Young *et al.* 1993). Thus, emerging evidence indicates that intracellular Ca^{2+} may not be a requirement for RVD in all cells. In interpreting these results, it should be kept in mind that it is possible that increases in $[Ca^{2+}]_i$ are too localized to be registered by our apparatus (as discussed by Foskett, 1994). In addition, although the Fura-2/AM concentration used in the present study is within the range normally used to study increases in intracellular $[Ca^{2+}]$, the presence of the intracellular dye may itself buffer Ca^{2+} to such an extent that normal cell function is disrupted (Kanli *et al.* 1992; Rink *et al.* 1983). Furthermore, it cannot be excluded that the volume expansion itself may induce changes in the fluorescence signals. These artefacts seem mostly to be noted as increases in the intracellular free Ca^{2+} concentration (Botchkin and Matthews, 1993; Ishii *et al.* 1996) and are, therefore, of no consequence for the results with trout proximal tubules. In fact, in the present study, a decrease in the relative amount of free intracellular Ca^{2+} was observed upon hypo-osmotic stimulation (Fig. 5B), which is consistent with the expected result of cytoplasmic dilution. It seems relatively certain that, owing to the lack of effect of intracellular Ca^{2+} chelation on volume recovery, increases in the free intracellular Ca^{2+} concentration, either due to influx from the extracellular medium or due to release from intracellular stores, is not responsible for the signalling of RVD in these cells.

Role of arachidonic acid metabolites

Application of the 5-lipoxygenase inhibitor ETH 615-139 induced a 60 % inhibition of RVD in trout proximal renal tubules (Fig. 5C). This inhibitor reduces the amount of arachidonic acid that is metabolized into the hydroperoxy eicosatetraenoic acids, which give rise to the signal molecules of the leukotriene family. Only arachidonic acid is discussed here as being the origin of the eicosanoids; however, several fatty acids, e.g. eicosatrienoic acid and eicosapentaenoic acid, may also give rise to several of the prostaglandins and leukotrienes (Rowley, 1991). We are aware that direct evidence for the importance of eicosatetraenoic acids in fish is lacking; however, clear evidence exists for an eicosanoid-generating potential in many trout organs (Knight *et al.* 1995). It should be noted, however, that it is not known whether the 5-lipoxygenase inhibitor used in the present study is as specific in trout proximal renal tubules as it reportedly is in other tissues. It has been found in other studies that this lipoxygenase is important for RVD (Lambert *et al.* 1987). Furthermore, of the eicosanoids produced, LTD_4 was shown to be able to stimulate KCl efflux. In human platelets, the lipoxygenase product hepoxilin A_3 has been identified as a signalling molecule for RVD-associated K^+ fluxes (Margalit *et al.* 1993). In addition, RVD in *Carassius auratus* proximal renal tubules can be inhibited by 5-lipoxygenase antagonists (Fugelli *et al.* 1995), and it has been found that the RVD-associated taurine efflux in fish red blood cells is greatly reduced by similar lipoxygenase inhibitors (Thoroed and Fugelli, 1994). Thus, it appears that there is a role for arachidonic acid metabolites as signals for volume recovery following cell swelling in cell types from both mammalian and non-mammalian vertebrates. It is not surprising, therefore, that arachidonic acid metabolites have been found to regulate both mammalian (Ando and Asano, 1995; Besseghir, 1985; Kinoshita *et al.* 1989) and fish (Gupta *et al.* 1985; Wales and Gaunt, 1986) renal function.

To determine whether the effect of 5-lipoxygenase inhibition on RVD was a result of a decrease in K^+ efflux, trout proximal renal tubules were exposed to iso-osmotic and hypo-osmotic solutions to which both ETH 615-139 and the K^+ ionophore gramicidin had been added. No partial or full recovery of RVD could be observed in the presence of gramicidin. ETH 615-139 and the anion exchanger TBT were then added to the Ringer's solutions to determine whether the 5-lipoxygenase effect could be attributed to an inhibition of Cl^- efflux. Since no recovery of osmoregulatory shrinkage was found in this instance either, it appears that arachidonic acid metabolites from the 5-lipoxygenase pathway are important for the activation of both K^+ and Cl^- efflux during RVD. A similar result has been obtained in Ehrlich ascites tumour cells, where it has been found that LTD_4 can activate both the K^+ and the Cl^- conductances (Lambert, 1994). It is not known at present which of the 5-lipoxygenase products regulates the osmoregulatory KCl efflux in these proximal renal tubules.

Arachidonic acid metabolic products from the cycloxygenase pathway have also been reported to play a role in RVD. In some renal tissue, cycloxygenase inhibition slightly reduces hypo-osmotically induced efflux of the osmoeffector taurine (Fugelli *et al.* 1995), while a similar treatment reduces activation of hypo-osmotically induced K^+ channels in collecting tubule cells (Ling *et al.* 1992). No such effect on

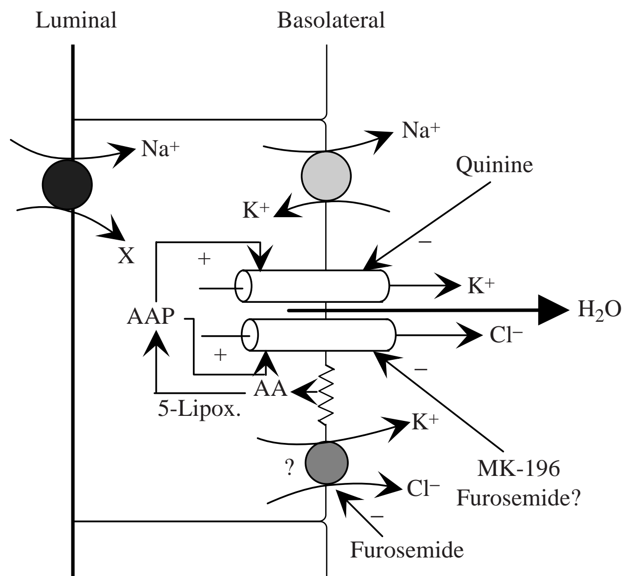


Fig. 6. Speculative model of signalling of regulatory volume decrease (RVD) in trout proximal renal tubule cells. Na^+, cotransported with other substrates (X) into the cell from the lumen, is transported out of the cell *via* the basolateral Na^+/K^+-ATPase. Upon cell swelling, KCl efflux occurs through quinine-inhibitable K^+ channels and MK-196-sensitive (a Cl^- channel blocker) and possibly furosemide-sensitive Cl^- conductances. Osmotically obligated water follows. A minor component of the osmoregulatory KCl efflux may occur through furosemide-sensitive K^+/Cl^- symporters. Both the K^+ and the Cl^- conductances appear to be regulated by arachidonic acid metabolic products from the 5-lipoxygenase pathway. It is possible that plasma membrane stretch during cell swelling somehow activates the phospholipase A_2 that produces arachidonic acid from membrane phospholipids. Arachidonic acid metabolites from the cycloxygenase pathway (i.e. prostaglandins) appear not to be involved in the RVD signalling process. Furthermore, neither extra- nor intracellular Ca^{2+} appears to play a role in the signalling of osmoregulatory shrinkage. AA, arachidonic acid; AAP, arachidonic acid metabolic product; 5-Lipox., 5-lipoxygenase.

RVD was observed upon cycloxygenase inhibition in trout proximal renal tubules, which is in accordance with studies on Ehrlich cells (Lambert, 1994).

The signal for swelling-induced increased breakdown of membrane phospholipids into arachidonic acid and its metabolites is not yet understood. One could speculate that the enzymes responsible for metabolization of membrane phospholipids are stretch-sensitive and, thus, activated upon plasma membrane stretch secondary to cell swelling (Lambert, 1994). In human platelets, it has been suggested that mechanical stress activates a G-protein localized in the plasma membrane (Margalit *et al.* 1993). The dissociated G-protein will, in turn, activate phospholipase A_2, and thus induce an increase in arachidonic acid production. Furthermore, it has been determined that membrane stretch can activate the production of 1,4,5-trisphosphate and tetrakisphosphate in cardiomyocytes (Dassouli *et al.* 1993). The possibility also exists that the channels/transporters responsible for RVD are themselves directly stretch-activated (Filipovic and Sackin, 1992; Ubl *et al.* 1988*b*; Christensen, 1987). However, the exact nature of the sensing mechanism of cell swelling remains to be resolved.

In conclusion, a tentative model of RVD in trout proximal renal tubules can be proposed (Fig. 6). As a result of cell swelling, a temperature- and ouabain-sensitive regulatory volume decrease mechanism is activated, possibly in response to stretch of the plasma membrane, resulting in increased K^+ and Cl^- efflux through separate conductances. (Na^+ entering from the lumen is transported out of the cell *via* basolateral Na^+/K^+-ATPases.) Osmotically obligated water follows. The K^+ channel is inhibited by quinine, but is relatively insensitive to Ba^{2+}. The Cl^- conductance, in contrast, is sensitive to the Cl^- channel blocker MK-196 but is not very responsive to NPPB inhibition. It also appears that the Cl^- conductance is furosemide-inhibitable. A minor participation of the furosemide-sensitive K^+/Cl^- cotransporter during RVD cannot be excluded. Arachidonic acid metabolites from the 5-lipoxygenase pathway seem to activate both the K^+ and the Cl^- conductances during RVD, while arachidonic acid metabolites from the cycloxygenase pathway appear to play a minor role in this KCl efflux. It cannot be ruled out, however, that in this system other fatty acids may have given rise to the eicosanoids. Neither extra- nor intracellular Ca^{2+} appears to regulate RVD.

Thanks are extended to Mrs Tove Bakar at the Electron Microscopy Laboratory, Department of Biology, University of Oslo, for technical assistance with the electron microscopy work and to Mr Gunnar Kinn at the Norwegian Radiation Protection Authority for his assistance with the Fura-2/AM measurements. We are also indebted to Mr Erik Mørk for his help with the computer-assisted drawing and to Jørn Olsen and Torstein Kvernstuen for designing and manufacturing the cooling system. We wish to thank Professor Daniel A. Terreros for introducing us to the videometric techniques, Professor Kjell Fugelli, Division of General Physiology, University of Oslo, for helpful discussions in preparing this paper and Kristin Kardel M.S. and Ellen Roll M.S. for critically reviewing the manuscript. This work was supported by the Norwegian Research Council and the Nansen Foundation.

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