ELSEVIER

Biochimica et Biophysica Acta 1239 (1995) 99–102

BBA Biochimica et Biophysica Acta

Rapid Report

Activation of a Cl^--conductive pathway in primary cultures of rat inner medullary collecting duct (IMCD) cells under hypotonic stress

Heinrich Sauer [1], Rolf K.H. Kinne, Frank Wehner *

Max-Planck-Institut für molekulare Physiologie, Abteilung Epithelphysiologie, Postfach 102664, 44026 Dortmund, Germany

Received 27 April 1995; accepted 13 June 1995

Abstract

In intracellular recordings with conventional microelectrodes on rat IMCD cells, we find that hypotonic stress depolarizes membrane voltage and decreases cell input resistance. Ion substitution experiments reveal that these effects are largely due to the activation of a prominent Cl^- conductance. After block of this conductance with dideoxyforskolin a smaller concomitant increase in K^+ conductance becomes detectable.

Keywords: Inner medullary collecting duct; Volume regulation; Chloride conductance; Potassium ion conductance; Dideoxyforskolin; Hypotonic stress; (Rat)

In renal medullary cells regulatory volume decrease (RVD) is mainly achieved by a release of organic osmolytes such as sorbitol, inositol, betaine, glycerophosphorylcholine, and various amino acids [1–4]. Grunewald et al. [5], however, reported additional effects of hypotonic stress on intracellular ion content in isolated IMCD cells of the rat. In their electron microprobe study, RVD was accompanied by a net loss of Na^+ and Cl^- while cell K^+ remained virtually unchanged. This was discussed in terms of K^+ and Cl^- exit in concert with activation of Na^+/K^+-ATPase. In the present study, we report that hypotonic stress leads to a considerable increase of Cl^- conductance in primary cultures of rat IMCD cells. Most likely, this effect is paralleled by a smaller, but significant increase in cell membrane K^+ conductance.

Isolation of IMCD cells and primary cell culture were the same as previously described [6,7]. Cells formed confluent monolayers within 3 days and were used from day 4 through day 7 after preparation.

For the intracellular recordings, sheets of gas-permeable membranes of approx. 1 cm^2 covered with confluent cell monolayers were cut from the bottom of Petriperm® cell culture dishes (Bachofer, Reutlingen, Germany). The sheets were then transferred onto a glass plate forming the bottom of the superfusion chamber. They were clamped to this glass plate with the upper part of the chamber, consisting of a Perspex block with a circular aperture which opened upward at an angle of 55°. The total fluid volume above the monolayers was 0.1 ml. The cells were continuously superfused by means of a multichannel peristaltic pump (PLG; Desaga, Heidelberg, Germany) at a rate of 5 ml/min. Changes of experimental solutions were achieved by use of a 4-way valve (ms 131 da; Whitey, Highland Heights, OH, USA) close to the chamber. As monitored with Trypan-blue stained solutions, changes in the superfusate were virtually complete within 15 s. All storage vessels, the superfusion lines and the chamber were water-jacketed and maintained at 36.0 ± 0.5°C. Two-channel microelectrodes were pulled from 1.5 mm o.d. 'Thick-Septum-Theta' glass capillaries (WPI, New Haven, CT, USA) on a Kopf vertical puller (750; David Kopf Instruments, Tujunga, CA, USA). One channel was used to measure voltage, the second to inject constant current pulses. The channels were filled with 0.5 mol/l KCl and had resistances of 80–130 MΩ with the electrode immersed in standard solution. An Ag-AgCl wire in series with an agar-KCl (0.5 mol/l) bridge was used as the reference electrode except in Cl^- substitution experiments, where a custom-made 3 mol/l KCl flowing junction was employed to avoid liquid junction potentials. The respective reference electrode was placed in an additional 1 ml compart-

* Corresponding author. Fax: +49 231 1206494.

[1] Institut für Neurophysiologie der Universität zu Köln, Robert-Koch-Str. 39, 50931 Köln, Germany.

SSDI 0005-2736(95)00149-2

ment connected to the chamber via a hole, 1.5 mm in diameter and 15 mm in length, through which all superfusates were removed to exclude any contamination of the preparation by KCl leakage. In low Na^+ solutions (see below) a liquid junction potential of 1.7 mV evolved at the agar-KCl bridge (measured with the KCl flowing junction as the reference). All membrane voltages in low Na^+ were corrected by this amount (except those shown in the original trace of Fig. 2B). There were no measurable liquid junction potentials in the K^+ substitution experiments. The experimental chamber was mounted on the stage of an inverted microscope (IM35; Zeiss, Oberkochen, Germany). Cell impalements were performed at an angle of 45° on a custom-made vibration-damped table under 320 × magnification using a piezo-manipulator (PM 500-20; Frankenberger, Germering, Germany). Cell membrane potentials and input resistances were determined by means of a high-impedance electrometer combined with a current-injection unit (Frankenberger). Current pulses of 1 s duration and 0.1 to 0.3 nA, as appropriate, were injected every 10 s. After A/D-conversion (DT 2811; Data Translation, Marlboro, MA, USA) voltages were displayed and stored on a personal computer (PS 2/30, IBM). Criteria for successful impalements were as follows: (1) An abrupt change in voltage upon impalement that was monophasic or sometimes followed by a slow increase of some 5–8 mV within the first 3 min. This time-dependent change was always paralleled by an increase in input resistance and, consequently, is likely to reflect a sealing of the cell membrane around the electrode tip. (2) Stable potentials within ±3 mV. (3) A return of measured voltages to 0 ± 3 mV upon withdrawal of the microelectrode. The standard solution (600 mosmol/l) contained (in mmol/l): 118 NaCl, 10 Na^+-*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Na^+-Hepes), 10 Hepes, 3.2 KCl, 2.5 $CaCl_2$, 1.8 $MgSO_4$, 1.8 KH_2PO_4, 5 glucose, 300 sucrose (pH 7.4). 600 mosmol/l is close to the average extracellular osmolarity for these cells, which, however, may vary considerably between 200 mosmol/l (diuresis) and 1400 mosmol/l (antidiuresis) [8]. Reduction of osmolarity was achieved by omission of sucrose. In the ion substitution experiments, K^+ was isosmotically increased 10-fold in exchange for Na^+. Na^+ was reduced 20-fold by exchange with either choline or *N*-methyl-D-glucamine (NMDG). Cl^- was reduced 10-fold in exchange with gluconate. Data are given as mean values ± S.E. with n denoting the number of experiments. Student's t-test for paired and unpaired data was applied as appropriate. A value of $P < 0.05$ was considered significant.

In continuous intracellular recordings, reducing bath osmolarity from 600 to 300 mosmol/l, first, led to a transient hyperpolarization of membrane voltage from −36.0 ± 1.5 to −39.4 ± 2.0 mV ($n = 9$; $P < 0.001$; Fig. 1). Thereafter, membrane voltage slowly depolarized to −25.8 ± 2.7 mV, which is 10.2 ± 1.5 mV more positive than the initial control value ($P < 0.001$). Under normosmotic conditions, cell input resistance equalled 101 ± 7 MΩ; under hypotonic stress, this parameter continuously decreased to 34 ± 3 MΩ ($P < 0.001$). Upon return to 600 mosmol/l, cell membranes transiently depolarized by 2.6 ± 0.6 mV ($P < 0.005$). Thereafter membrane voltage slowly hyperpolarized to −35.8 ± 2.0 mV and cell input resistance increased to 103 ± 8 MΩ, so that these values are not significantly different from the initial values.

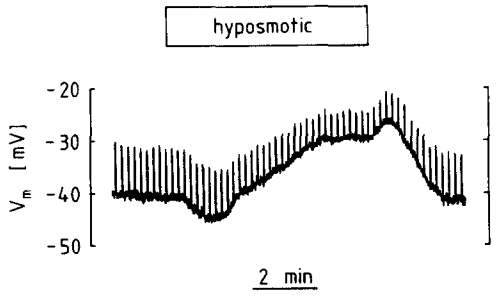

Fig. 1. Effects of hypotonic stress (−300 mosmol/l sucrose for the time indicated) on membrane voltage (V_m) and cell input resistance. Vertical deflections result from injected current pulses of 0.1 nA.

The above experiments suggest that hypotonic stress leads to considerable changes in the membrane conductance of IMCD cells. These changes in conductance were further characterized in three sets of ion substitution experiments.

Increasing K^+ concentration from 2.7 to 27 mmol/l depolarized the cells by 8.3 ± 1.3 mV under control conditions (Fig. 2A). In hyposmotic solutions, the effect of increasing K^+ was reduced to 3.0 ± 0.5 mV ($n = 5$; $P < 0.01$). After return to 600 mosmol/l, K^+-induced membrane depolarization equalled 6.0 ± 1.1 mV, which is not significantly different from the initial value.

Replacing Na^+ by 95% with NMDG (or choline) hyperpolarized membrane voltage by 9.7 ± 1.6 mV under control conditions (Fig. 2B). In 300 mosmol/l, the voltage response was changed to a depolarization of 1.9 ± 1.1 mV ($n = 7$; $P < 0.001$). After return to 600 mosmol/l, Na^+ substitution hyperpolarized the cells by 11.9 ± 1.4 mV, which is not significantly different from the initial value.

In principle, the above ion substitution experiments could reflect a parallel decrease in K^+ and Na^+ conductance. This, however, is opposite to what one would expect from the about 3-fold increase in overall membrane conductance. Consequently, activation of an alternate conductive pathway would be a more likely explanation for the observed reduction in the voltage responses to high K^+ and low Na^+ solutions. When bath Cl^- was replaced by 90% with gluconate membrane voltage depolarized by 8.6 ± 1.4 mV in normosmotic solutions (Fig. 2C). In 300 mosmol/l, this effect was increased to 19.8 ± 1.9 mV ($n = 7$; $P < 0.001$). After return to 600 mosmol/l, membrane depolarization equalled 5.9 ± 1.6 mV, which is significantly lower than under control conditions ($P < 0.01$).

The 2.3-fold increase in the voltage response to low

Cl^- solutions and the parallel decrease of cell input resistance to 34% strongly suggest activation of a Cl^--conductive pathway in rat IMCD cells under hypotonic stress. In this system, intracellular Cl^- activity is approximately 30 mV above electrochemical equilibrium (92 ± 13 mmol/l with 276 mmol/l external Cl^- [5]) probably due to Na^+-K^+-$2Cl^-$ cotransport [9]. Consequently, distinct membrane depolarizations are to be expected from increases in Cl^- conductance. The increase of Cl^- conductance readily explains the decrease of Cl^- content in freshly isolated IMCD cells under hypotonic stress that was observed by Grunewald et al. [5].

In addition to the sustained membrane depolarization, a transient hyperpolarization of 3.4 mV occurs upon reduction of osmolarity. This hyperpolarization may reflect an increase in K^+ conductance, but due to the low time resolution of our ion substitution experiments and due to the prominent activation of Cl^- conductance, this effect could not be directly characterized.

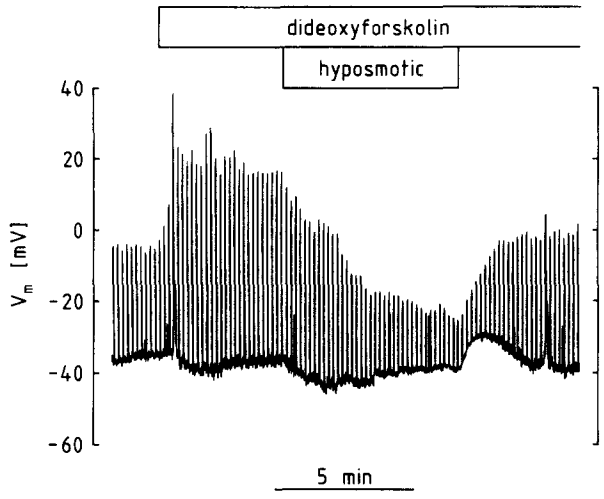

Fig. 3. Effects of hypotonic stress on membrane voltage and input resistance in the presence of 0.1 mmol/l dideoxyforskolin. Note the increase in input resistance upon addition of the compound.

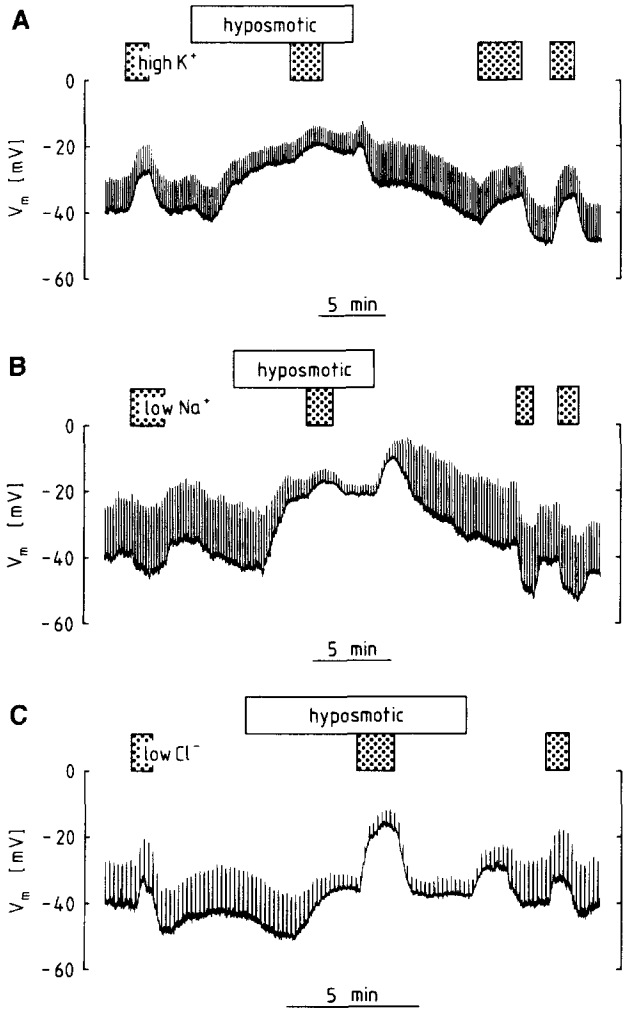

Fig. 2. Membrane effects of different ion substitutions in normosmotic solutions, after reduction of osmolarity and after return to normosmotic conditions. (A) K^+ was increased from 2.7 to 27 mmol/l in exchange for Na^+, (B) Na^+ was reduced 20-fold by replacement with choline, and (C) 90% of Cl^- was substituted for gluconate.

We, therefore, tried a variety of potential Cl^- channel blockers to unmask possible effects of hypotonic stress on K^+ conductance. SITS (4-acetamino-4′-isothiocyanatostilbene-2,2′-disulfonic acid) and DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid) in concentrations as high as 0.5 mmol/l did not significantly affect the membrane depolarization nor the decrease in cell input resistance upon hypotonic stress (data not shown). NPPB (5-nitro-2-(3-phenylpropylamino)benzoate) and niflumic acid at concentrations of 0.1 and 0.5 mmol/l, respectively, exhibited considerable side effects: as ion substitution experiments revealed, both drugs significantly decreased cell membrane K^+ conductance under normosmotic conditions (data not shown). We, therefore, turned to dideoxyforskolin which appears to be the most potent blocker of volume-activated anion channels in rat glioma cells [10], human endothelial cells [11], HeLa cells [12] as well as in the human lung cancer cell line S1 [13]. Addition of 0.1 mmol/l dideoxyforskolin per se had variable effects on membrane voltage, including a transient membrane hyperpolarization of some 7 mV in two out of four experiments (Fig. 3). After 4 min exposure to the drug, membrane voltages equalled −30.0 ± 2.3 mV, which is not significantly different from the control value, i.e. −31.5 ± 3.1 mV. In contrast, cell input resistance considerably increased from 141 ± 6 to 273 ± 25 MΩ ($P < 0.02$). In the continuous presence of dideoxyforskolin, reducing osmolarity from 600 to 300 mosmol/l significantly *hyperpolarized* the cells to −43.7 ± 3.6 mV ($P < 0.05$). In parallel, cell input resistance decreased to 164 ± 41 MΩ (remaining short off significance). Upon return to 600 mosmol/l (with dideoxyforskolin still present) membrane voltages transiently depolarized by 13.9 ± 0.9 mV and, thereafter,

(as well as cell input resistances) slowly returned to prestimulus values.

The above experiments with dideoxyforskolin corroborate the hypothesis that hypotonic stress leads to a prominent increase of Cl^- conductance in rat IMCD cells. In addition, they provide evidence for a parallel increase in cell membrane K^+ conductance. In the control experiments after about 5 min in hypotonic solution, membrane voltage remains constantly depolarized by about 10 mV. Consequently, at that time, activation of Cl^- conductance must exceed the concomitant increase in K^+ conductance which will tend to hyperpolarize the cell. In this context, the membrane hyperpolarization in the presence of dideoxyforskolin is apparently high. In the presence of the drug, however, the K^+ transference number will be increased and, consequently, membrane voltage will be more sensitive to changes in K^+ conductance. Moreover, with the Cl^- conductance blocked cell volume regulation will be impaired, which may provide a stronger stimulus for the activation of K^+ conductance.

In the control experiments, there was a transient membrane depolarization by 2.6 mV upon return to normosmotic solution. Because at the time when this effect becomes maximal, cell volume will almost have returned to the control value [7], while input resistance is still low (cf. Fig. 1) this voltage change could be due to an additional transient increase in the driving force for conductive Cl^- exit (via concentration of cell Cl^-). In the presence of dideoxyforskolin, however, this membrane depolarization augments to 13.9 ± 0.9 mV. Consequently, this transient voltage change more likely reflects a rapid decrease in K^+ conductance. As it is evident from the initial membrane hyperpolarization upon hypotonic stress in the control experiments, K^+ conductance appears to adapt more rapidly to changes in cell volume than Cl^- conductance.

In principle, quasi electroneutral release of K^+ and Cl^- would be the most effective mechanism of KCl exit via conductive pathways. This raises the question whether the observed prominent activation of Cl^- conductance plays a role in addition to Cl^- release. Recent studies suggest that in some systems a variety of organic osmolytes leave the cell via a single transport pathway that appears to be a swelling-activated non-selective anion channel [10,13–15].

Acknowledgements

We wish to thank Sigrid Rosin-Steiner and Alexander Giffey for invaluable technical assistance and Daniela Mägdefessel for excellent secretariat help. Part of the experiments presented here have been published in abstract form [16].

References

[1] Grunewald, R.W. and Kinne, R.K.H. (1989) Pflügers Arch. 414, 178–184.
[2] Garcia-Perez, A. and Burg, M.B. (1991) Physiol. Rev. 71, 1081–1115.
[3] Beck, F.X., Schmolke, M., Guder, W.G., Dörge, A. and Thurau, K. (1992) Am. J. Physiol. 262, F849–F856.
[4] Kinne, R.K.H., Czekay, R.-P., Grunewald, J.M., Mooren, F.C. and Kinne-Saffran, E. (1993) Renal Physiol. Biochem. 16, 66–78.
[5] Grunewald, J.M., Grunewald, R.W. and Kinne, R.K.H. (1993) Kidney Int. 44, 509–517.
[6] Stokes, J.B., Grupp, C. and Kinne, R.K.H. (1987) Am. J. Physiol. 253, F251–F262.
[7] Tinel, H., Wehner, F. and Sauer, H. (1994) Am. J. Physiol. 267, F130–F138.
[8] De Rouffignac, C. (1990) Comp. Physiol. 2, 31–102.
[9] Grupp, C., Pavenstädt-Grupp, I., Grunewald, R.W., Bevan, C., Stokes III, J.B. and Kinne, R.K.H. (1989) Kidney Int. 36, 201–209.
[10] Jackson, P.S. and Strange, K. (1993) Am. J. Physiol. 265, C1489–C15OO.
[11] Nilius, B., Oike, M., Zahradnik, I. and Droogmans, G. (1994) J. Gen. Physiol. 103, 787–805.
[12] Díaz, M., Valverde, M.A., Higgins, C.F., Rucareanu, C. and Sepúlveda, F.V. (1993) Pflügers Arch. 422, 347–353.
[13] Kirk, K. and Kirk, J. (1993) FEBS Lett. 336, 153–158.
[14] Banderali, U. and Roy, G. (1992) Am. J. Physiol. 263, C12OO–C1207.
[15] Kirk, K., Ellory, J.C. and Young, J.D. (1992) J. Biol. Chem. 267, 23475–23478.
[16] Sauer, H., Kinne, R.K.H. and Wehner, F. (1993) Biol. Chem. Hoppe-Seyler 374, 151.