Differential Regulation of Cell Volume and Shape in Confluent Rat Hepatocytes Under Hypertonic Stress

Heidrun Olsen¹, Frank ter Veld², Ulrike Herbrand³, Mohammad R. Ahmadian¹, Rolf K.-H. Kinne¹ and Frank Wehner¹

¹Max-Planck-Institut für molekulare Physiologie, Dortmund, ²Deutsches Diabetes-Zentrum, Universität Düsseldorf, ³Zentrum für medizinische Biotechnologie, Universität Duisburg-Essen

Key Words

Rat hepatocytes • Cell volume • Cell shape • Regulation • Cation channel • Exocytosis • PLC • Tyrosine kinase • G protein • Stress fibres • Rho GTPases

Abstract

In confluent primary cultures of rat hepatocytes, hypertonic stress leads to cell shrinkage and activates non-selective cation channels as the main mechanism of regulatory cell volume increase. The process is found to employ the exocytotic insertion of channels into the plasma membrane and (in addition to PKC) PLC, tyrosine kinases and G proteins, but not PI 3-kinase are part of the signalling network. Furthermore, hypertonic stress leads to the formation of stress fibres and significantly alters the activity of RhoA, Rac and Cdc42. These latter effects, however, are likely to reflect the restoration of cell shape rather than the regulation of cell volume, both most probably converging at the level of focal adhesions and integrins.

Introduction

Rat hepatocytes in confluent monolayer-culture exhibit a variety of physiological characteristics very much resembling those of the *in vivo* system. Among these is the formation of bile canaliculi at contact sites of adjacent cells [1], the reestablishment of electric cell-to-cell coupling via gap junctions [2-4], as well as an active volume control under hypertonic stress, e.g. a sizeable regulatory volume increase (RVI) in response to cell shrinkage [5-7]. In addition, only under these experimental conditions, primary rat hepatocytes exhibit the hypertonic activation of non-selective cation channels [8-10] which, whenever a given preparation is studied in a quantitative fashion, have proven to be the main mechanism of RVI [11, 12].

In the confluent monolayer-system, the relative contribution of cation channels, $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ symport and Na^+/H^+ antiport to rat hepatocyte RVI is approx. 4:1:1 [6, 7]. Concerning regulation, it could be shown recently that the hypertonic activation of cation channels and $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ symport, but not that of Na^+/H^+ antiport is mediated

Copyright © 2007 S. Karger AG, Basel

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2007 S. Karger AG, Basel
1015-8987/07/0196-0259\$23.50/0

Accessible online at:
www.karger.com/cpb

Frank Wehner

Abteilung Systemische Zellbiologie, Max-Planck-Institut für molekulare Physiologie, Otto-Hahn-Strasse 11, 44227 Dortmund (Germany)
Tel. +49 231/133-2225, Fax +49 231/133-2699
E-Mail frank.wehner@mpi-dortmund.mpg.de

by PKC [13]. In line with this, the cation channel and $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ symport exhibit profiles of osmotic sensitivity that are virtually identical and very different from the one of $\text{Na}^+\text{/H}^+$ antiport [7].

Here, the signalling elements involved in the hypertonic activation of cation channels in rat hepatocyte monolayers are characterised in more detail. Special emphasis is given to exocytosis, PLC, tyrosine kinases, G proteins, the actin cytoskeleton, as well as to the small GTPases RhoA, Rac and Cdc42. It is found that, in addition to its effect on cell volume and cation channel activation, hypertonic stress also impinges on rat hepatocyte cell shape. Most likely, the regulation of both is achieved via separate pathways which, however, appear to converge at the level of focal adhesions.

Materials and Methods

Cell culture

Harvesting of rat hepatocytes and preparation of confluent monolayer cultures have been described in detail previously [5-7].

Electrophysiology

Cable-analysis and current-clamp recordings with low Na^+ pulses are intracellular micro-electrode techniques and were done as reported earlier [5-7, 13, 14].

Briefly, in a cable-analysis experiment, current pulses were injected into a cell and the resultant changes in membrane voltage were recorded with a second micro-electrode at 35, 100, 200 and 400 μm from the point of current injection. These measurements were performed consecutively on four sheets of monolayers cut out from one single Petri-dish. From the profile of voltage vs. distance between electrodes, the electric cell-to-cell coupling resistance as well as the specific conductance of cell membranes can be derived in a highly quantitative fashion. In the present study, for instance, the latter equalled $141.7 \pm 2.1 \mu\text{S}/\text{cm}^2$ under isotonic control conditions. Since the average surface of a rat hepatocytes in a confluent monolayer is $19.2 \cdot 10^{-6} \text{ cm}^2$ [7], this gives $2.72 \pm 0.04 \text{ nS}$ for the membrane of a single cell.

In the current-clamp experiments cell membrane voltage and input resistance was determined and a step-like reduction of extra-cellular Na^+ (from 144 to 4 mmol/l, in exchange for choline) was performed rapidly changing the Nernst-equilibrium for Na^+ across the membrane. This maneuver results in an instantaneous hyperpolarization of membrane voltage, yielding a semi-quantitative estimate of cell membrane Na^+ conductance. In a series of Cl^- substitution experiments, extra-cellular Cl^- was 100-fold reduced (in exchange for gluconate) and from the resultant depolarization the Cl^- conductance of the cell membrane was computed [5].

Immuno-precipitation and pull-down assays

Concerning PLC γ 1, the same antibody (Santa Cruz, Heidelberg, Germany) was used for immuno-precipitation and Western blotting. Four culture dishes were employed for every single sample.

The binding domain of the respective downstream effector (expressed as a GST-fusion protein and immobilized to sepharose) was used to pull down the active form of each of the small GTPases that was tested for. This was GST-Rhotekin-RBD for RhoA and GST-PBD (p21 binding domain) of Pak1 for Rac1 and Cdc42 [15]. RhoA, Rac1 and Cdc42 proteins were detected by Western blotting using the 12C4 monoclonal antibody (Santa Cruz), the 23A8 monoclonal antibody (Upstate Biotechnology, NY, USA) and P1 polyclonal antibodies (Santa Cruz), respectively. Three culture dishes were employed for every single sample.

Phosphorylation profiling

The phosphorylation status of FAK and Src was investigated using the Customised KineteworksTM Signal Transduction Protein Profiling Service offered by the Kinexus Bioinformatics Corporation (Vancouver, Canada). This screen had the advantage that, at a high temporal resolution, all phosphorylation sites could be analysed within one single preparation of rat hepatocytes. Three culture dishes were pooled for each data point and all cells were from one single preparation.

Fluorescence of G and F-actin

For visualisation and quantification of G and F-actin, cells were fixed with para-formaldehyde (3.75%) and permeabilised with Triton X-100 (0.1%), for 10 min each. Next, they were incubated with 12 mg/ml DNaseI-Alexa 488 (Molecular Probes, Leiden, Netherlands) and 0.1 mg/ml phalloidin-TRITC (Sigma-Aldrich, Deisenhofen, Germany) for 20 min. In addition, nuclei were stained for 5 min with DAPI (Molecular Probes) at a final concentration of 25 ng/ml. For the *qualitative* analysis, epifluorescence images were recorded on an AxioPhot microscope with a Plan-Neofluar lens (63 x, 1.25 oil), that was equipped with an AxioCam and driven by the AxioVision software, version 2 (Carl Zeiss, Jena, Germany). Specification of filters (absorbance/emission) was 495 nm/519 nm (green, for Alexa 488), 544 nm/572 nm (red, for TRITC) and 358 nm/461 nm (blue, for DAPI). In the *quantitative* measurements, fluorescence was determined in areas of $8 \times 8 \text{ mm}^2$ of confluent cells by use of a Fluoroskan Ascent FL micro-plate reader (Thermo Labsystems, Helsinki, Finland). Here, filters were 485 nm/538 nm (Alexa 488), 530 nm/590 nm (TRITC) and 355 nm/460 nm (DAPI). Fluorescence values of Alexa 488 and TRITC were computed with reference to DAPI, normalizing the amounts of G and F-actin to the actual number of cells.

Solutions

The isotonic control solution (300 mosmol/l, pH 7.4) contained (in mmol/l): NaCl, 144; KCl, 2.7; MgCl_2 , 1.1; CaCl_2 , 1.8; NaH_2PO_4 , 0.4, HEPES, 2.5, Na-HEPES, 2.5; glucose, 5.6. Hypertonicity was achieved by the addition of 100 mosmol/l sucrose

Fig. 1. Pharmacology of hypertonicity-induced cation channel activation. (A) Effects of hypertonic stress (300 → 400 mosmol/l) on the membrane conductance (g_m) of a single rat hepatocyte in confluent monolayer culture, determined by means of cable-analysis (representative experiment). (B) Hypertonicity-induced changes of membrane conductance (Δg_m) under control conditions and with blockers of exocytosis (nocodazole = NOC and tannic acid = TANN), PLC (U73122), PI 3-kinase (wortmannin = WRTM and LY294002), G proteins (pertussis toxin = PTX) as well as tyrosine kinases (genistein = GEN; $n=4-6$). (C) The instantaneous change of membrane voltage (V_m) in response to low Na^+ pulses is significantly increased under hypertonic (δ_{hyper}) when compared to isotonic conditions (δ_{iso}). Typical current-clamp experiment (see text for details). (D) Hypertonicity-induced changes in the voltage response to low Na^+ pulses ($\Delta V_m = \delta_{\text{hyper}} - \delta_{\text{iso}}$) under control conditions and with the same blockers as they were used in (B) ($n=4-7$).

and the osmolality of experimental solutions was routinely checked (Knauer, Berlin, Germany). In the cable-analysis experiments, 0.5 mmol/l quinine was used to block K^+ conductance [6, 7]. Experiments were performed at $36.5 \pm 0.5^\circ\text{C}$.

Tannic acid, U73122, LY294002, pertussis toxin and H-1152 was purchased from Calbiochem (Darmstadt, Germany), nocodazole was from Tocris (Balwin, MO, USA), wortmannin from ICN Biochemicals (Eschwege, Germany) and Genistein from Sigma-Aldrich.

Statistical analysis

Data are presented as means $\pm$ s.e.m. with n denoting the number of preparations tested. For the statistical analysis, Student's t -tests for paired and unpaired data were applied as appropriate. In the figures, "*", "**" and "***" indicate a $p < 0.05$, 0.01 and 0.001, respectively.

Results and Discussion

Pharmacology of cation-channel activation

In the present study, two complementary techniques were applied to monitor the effects of hypertonic stress on rat hepatocyte Na^+ conductance. This is, first, cable-analysis yielding absolute values on total membrane conductance at a time resolution of 10 seconds [5-7, 13] and, second, current-clamp recordings with low Na^+ pulses from which the actual changes of Na^+ conductance can be estimated in a semi-quantitative manner [5, 13, 14]. Because of the high degree of electric cell-to-cell coupling, patch-clamp experiments on confluent monolayers are not feasible.

As was determined by means of cable-analysis, changing extracellular osmolality from 300 to 400 mosmol/l increased the membrane conductance of rat hepatocytes from 2.72 ± 0.04 to 3.20 ± 0.04 nS, i.e. by 0.49 ± 0.04 nS ($n=6$; $p < 0.01$; Fig. 1A). The effect evolved in a monotonic fashion and typically conductance was stabilizing at a constant elevated level after some 2 to 4 min of hypertonic stress. Since all cable-analysis experiments were performed in the continuous presence of 0.5 mmol/l quinine to exclude possible effects of osmotic stress on K^+

conductance [6, 7] and because in previous current-clamp recordings with low Cl^- pulses no changes in Cl^- conductance upon hypertonicity were detectable [5, 6] this increase in membrane conductance reflects the activation of non-selective cation channels [5-7, 16] that have also been shown to be amiloride-sensitive [5, 14, 17]. Quinine per se has no effect on the basal volume of rat hepatocytes [6, 7].

In current-clamp recordings, a step-like reduction of extracellular Na^+ (from 144 to 4 mmol/l) led to an instantaneous hyperpolarization of membrane voltage that equalled -2.2 ± 0.4 mV ($n=7$; $p<0.01$), under isotonic conditions (Fig. 1C). This voltage response results from the experimental reduction of the Nernst-equilibrium for Na^+ and, since it is also amiloride-sensitive [5, 14, 17], it is a reliable measure of the actual Na^+ conductance of the plasma membrane. At 5 min of hypertonic stress, the instantaneous membrane hyperpolarization was significantly increased to -6.7 ± 0.7 mV, i.e. by -4.4 ± 0.8 mV ($p<0.01$) reflecting the hypertonic activation of cation channels. To some extent, the voltage response may be limited by the rather low selectivity of hypertonicity-induced cation channels in rat hepatocytes exhibiting an actual $P_{\text{Na}}/P_{\text{K}}$ of 1.4 [16]. The depolarization of membrane voltage in the late phase of a low Na^+ pulse is a secondary effect brought about by the inhibition of Na^+/H^+ antiport and the pH dependence of K^+ conductance [5, 6, 14].

The hypertonic activation of Na^+ conductance in rat hepatocytes may employ the exocytotic insertion of cation channels into the plasma membrane. To test for this hypothesis, cable-analysis experiments in the presence of 30 $\mu\text{mol/l}$ nocodazole and 0.5% tannic acid were performed. Nocodazole is a disruptor of microtubules and it is commonly used to block the activation of membrane transporters that are supposed to occur via exocytotic vesicles [18, 19]. Tannic acid inhibits exocytosis by stabilizing cell membranes from their extra-cellular leaflet [20, 21]. As is depicted in Fig. 1B, nocodazole as well as tannic acid completely blocked the hypertonic activation of cation channels. The apparent reduction of membrane conductance in the presence of both compounds was non-significant (with $p=0.43$ and $p=0.27$ for nocodazole and tannic acid, respectively).

In rat hepatocytes, PKC is one of the key elements in the hypertonic activation of cation channels [13]. In order to identify the actual regulators further upstream to PKC we were, first, focussing on PLC. As is shown in Fig. 1B, channel activation was completely blocked in the presence of the specific PLC inhibitor U73122 (applied at 5 $\mu\text{mol/l}$). Furthermore, in immuno-precipitation

Fig. 2. Hypertonic activation of PLC γ . Changes in the activity of PLC γ were determined in immuno-precipitation experiments at various times of hypertonic stress ($n=4-6$).

experiments, hypertonic stress led to a sizeable activation of PLC γ (Fig. 2), the most likely isoform to be coupled to the proposed tyrosine kinases further upstream in the signalling network (see below) [22].

Next, we tested for a possible role of PI 3-kinase in the activation process. As Fig. 1B clearly shows, however, neither wortmannin nor LY294002 (at 100 nmol/l and 50 $\mu\text{mol/l}$, respectively) had any detectable effect on hypertonic channel activation. This clearly shows that PI 3-kinase is *not* employed in the hypertonic activation of cation channels in rat hepatocytes.

Finally, the putative contribution of G proteins and tyrosine kinases to the activation process was analysed. As is depicted in Fig. 1B, genistein (at 50 $\mu\text{mol/l}$) reduced channel activation to some 35% of the control value, already suggestive of the significant role of tyrosine kinases in the signalling network (see below).

In the presence of pertussis toxin (50 ng/ml), the membrane response to hypertonic stress was not only completely blocked, rather, there was an actual *decrease* of membrane conductance by $63.3 \pm 2.0\%$ (Fig. 1B), the only negative shift of this series that was per se signifi-

cant ($n=4$, $p<0.01$). To test for possible additional effects of the compound on Cl^- conductance a series of ion substitution experiments was performed in which Cl^- was 100-fold reduced in exchange for gluconate (not shown). With the toxin present, the relative Cl^- conductance of the cell membrane was $27 \pm 2\%$ under hypertonic stress, which was not significantly different from the isotonic value ($31 \pm 2\%$, $n=5$). In the control recordings, relative Cl^- conductance equalled $37 \pm 4\%$ and $35 \pm 3\%$ ($n=5$) under isotonic and hypertonic conditions, respectively. Since these data are not significantly different from each other (and with K^+ conductance blocked by quinine) the effects of pertussis toxin on membrane conductance most likely reflect an actual interference with cation channel activation. This may include some impact on the overall set-point of the regulatory system, in addition to merely blocking the hypertonic stimulation of the channel.

The current-clamp recordings with low Na^+ pulses yielded a pharmacological profile that is virtually identical to the one obtained by means of cable-analysis (Fig. 1B and D) giving strong additional support to the conclusions derived so far. The only possible exception may be the series with nocodazole where, in the ion substitution experiments, the inhibitory effect of the compound remained short off significance (with $p=0.16$).

Taken together, the electrophysiological approaches outlined above clearly show that, in confluent rat hepatocytes, the hypertonic activation of cation conductance employs tyrosine kinases, G proteins, as well as PLC. Furthermore, the exocytotic insertion of channels into the plasma membrane is very likely part of the response. In contrast, PI 3-kinase is *not* an element of the signalling machinery in charge.

Recently, a hypertonic activation of non-selective cation channels was reported from rat hepatoma HTC cells [23]. Interestingly, in this system, PI 3-kinase appears to be one of the mediators of channel activation in addition to (and independent of) Src kinase. Also of note, hypertonic stress was found to increase exocytosis although channel activation was clearly insensitive to colchicine, a compound that is supposed to inhibit the formation of microtubules in a way very similar to that of nocodazole [23]. It should be emphasized, however, that those experiments have been performed on detached cells, i.e. under conditions where hepatocytes lack, both, contact to their neighbours as well as to their extracellular matrix. Furthermore, given the enormous complexity of signalling networks in general, a certain impact of cell immortalization on such a network would not be surprising.

Fig. 3. Time course of hypertonicity-induced changes in the phosphorylation of FAK and Src. The phosphorylation status of FAK was determined at Y397 (auto-phosphorylation site) and Y576 (A), that of Src at Y529 (B). Single-shot Kineteworks™ screen (see text for details).

Focal-adhesion signalling

Focal adhesions are the sites at which the actin cytoskeleton is coupled to the extra-cellular matrix, and FAK and Src are the tyrosine kinases most frequently in charge of focal adhesion signalling. In the following, we monitored time-dependent changes in the phosphorylation of FAK and Src in response to hypertonic stress by means of a customised screen (see *Materials and Methods*). For FAK, auto-phosphorylation site Y397 and Src-phosphorylation site Y576 were examined. For Src, Y529 was tested; since this tyrosine is located in an auto-inhibitory domain of the enzyme, any dephosphorylation is equivalent to an actual activation of Src. It was found that, at 1

minute of hypertonicity, auto-phosphorylation of FAK Y397 was increased to approx. 170% of the isotonic reference; thereafter, it was stabilizing at some 130% above control (Fig. 3A). In contrast, FAK Y576 did not change its status of phosphorylation until 5 min of hypertonic stress when it was transiently increasing to approx. 140%. Src Y529, on the other hand, exhibited a tri-phasic behaviour with a transient decrease of phosphorylation to 75 and 63% at 1 and 5 min of hypertonic stress, respectively, and with a final increase, at 7 min, to 147% of the control value (Fig. 3B). According to these data, the following pattern of hypertonicity-induced tyrosine kinase activation is proposed: The initial event is the auto-phosphorylation of FAK at Y397, which allows the priming of FAK and Src for interaction [24]. Subsequently, Src phosphorylates FAK at Y576, resulting in the maximal (sustained) activation of FAK.

Interestingly, in rat hepatoma HTC cells, a monophasic increase of Src activity was observed under *hypotonic* stress [25].

Rho GTPases

In general terms, there is a close correlation between the shape of a given cell and the actual organization of its actin cytoskeleton. In particular, this holds for epithelial structures like, as for instance, the palisade-like organisation of hepatocytes in the liver. Since RhoA, Rac1 and Cdc42 are key-players in the organization of the actin texture, various pull-down assays were employed (see *Materials and Methods*) to test for time-dependent changes in the activity of these small GTPases. As a matter of fact, the amount of active RhoA was almost doubled from 3 min of hypertonic stress onwards, with no change of total RhoA detectable (Fig. 4A). The amount of Rac-GTP, on the other hand, was significantly decreased to some 50% of control, however, only at 3 min of hypertonicity. In addition, a continuous slight decrease of total Rac was detectable with increasing time of hypertonic stress (Fig. 4B). Cdc42 exhibited a biphasic response with a decrease of the active form to some 50 and 40% of control at 3 and 7 min of anisotonicity, respectively, and with little if any change of its total amount over time (Fig. 4C).

Taken together, these experiments show that hypertonic stress changes the activity of all three GTPases studied and that there is a significant shift in activity from Rac and Cdc42 towards RhoA. It has been reported for macrophages that Rac and Cdc42 are required for the formation of focal adhesion sites [26]. Furthermore, integrin-mediated adhesion of fibroblasts leads to Rac and

Fig. 4. Hypertonicity-induced changes in the activity of small Rho-like GTPases. The activation status of RhoA (A), Rac1 (B) and Cdc42 (C) was determined by means of various pull down-assays (see text for details; n=4 - 6).

Fig. 5. Fluorescence microscopy on hepatocyte F and G-actin. F-actin was labelled with phalloidin-TRITC and G-actin was stained with DNase1-Alexa 488, both under isotonic conditions and at 5 min of hypertonic stress (representative experiment).

Cdc42 activation and subsequent cell spreading [27]. Accordingly, the inactivation of these GTPases, as it is observed in the present study, may well reflect the opposite effect, namely, the disassembly of adhesion sites and a retraction of cells from their substratum. Also of note, the activation of RhoA appears to be important for the detachment of leukocytes during migration [28] whereas inhibition of RhoA was found to cause the opposite effect in fibroblasts, i.e. an enhancement of cell spreading [29]. We conclude that the partial detachment of hepatocytes from their substratum relieves the mechanical tension that is exerted via cell/matrix-interaction sites and actually caused by the hypertonic shrinkage of cells. Concerning time courses, a minimum of 3 min appears to be required for the observed changes in the activities of all three GTPases. Thus, the initial auto-phosphorylation of FAK and the first transient activation of Src (see Fig. 3) will be fast enough to trigger these effects, but not the maximal (sustained) activation of FAK.

The actin cyto-skeleton

Counteraction of mechanical forces and maintenance of tissue integrity are achieved by the formation of stress fibres, contractile elements composed of myosin I and F-actin. The assembly of stress fibres is concomitant with actin polymerisation, a process triggered by the activation of RhoA. As shown in Fig. 5, hypertonic stress led to the distinct formation of stress fibres that appear to be located in close vicinity to the cell membrane. There were no detectable changes in the amount of G-actin.

To study the effect of hypertonicity on the actin cytoskeleton in a more quantitative fashion, a fluorometric analysis was performed (see *Materials and Methods*).

Fig. 6. Fluorometry of changes of hepatocyte F and G-actin content after various times of hypertonic stress. Experiments were performed under control conditions (A) and in the presence of the ROCK inhibitor H-1152 (B) (n=3 - 6).

Fig. 7. ROCK inhibitor has no effect on the hypertonic activation of cation conductance in rat hepatocytes. Cable-analysis experiments are summarised in (A), current-clamp recordings with low Na^+ pulses in (B) ($n=4-6$).

In the control measurements, a biphasic increase of the F-actin content was observed, with the first phase occurring in the range of 0.5 to 1 min and with the second rise at 5 min of hypertonic stress (Fig. 6A). There were no major changes in the amount of G-actin detectable.

Cell volume vs. shape

To elucidate whether these increases of hepatocyte F-actin are related to RhoA signalling additional experiments were conducted in the presence of (100 nmol/l) of the Rho kinase inhibitor H-1152. Rho kinase (ROCK) is a downstream mediator of Rho activation and its enzymatic activity is crucial for the RhoA-induced formation of stress fibres [30]. Under these conditions, the first phase in the formation of F-actin remained unchanged; the second phase, however, was completely missing (Fig. 6B). There were no significant changes in the amount of G-actin in this series of measurements. Also of note, ROCK inhibitor did not alter the isotonic amount of cell F and G-actin (data not shown). These experiments show that the second rise of F-actin is triggered via RhoA, whereas the first one clearly is not.

Besides balancing mechanical forces and adjusting cellular shape, the actin cytoskeleton is of central importance for a variety of signal transduction processes. This raises the question of whether the hypertonic formation of stress fibres via RhoA is involved in the activation of Na^+ channels, the main mechanism of RVI in primary rat hepatocytes. As shown in Fig. 7, however, neither in cable-analysis nor in current-clamp recordings with low Na^+

Fig. 8. PLC is not employed in the hypertonicity-induced formation of F-actin. Experiments were performed under control conditions (A) and in the presence of the PLC inhibitor U73122 (B) ($n=4$).

Fig. 9. Working hypothesis on the hypertonic regulation of cell volume and cell shape in rat hepatocytes (see text for details).

pulses there were any detectable effects of ROCK inhibitor on cation channel activation.

Furthermore, in an additional series of measurements, a possible contribution of PLC to the hypertonicity-induced formation of F-actin was tested for. In the absence of U73122, the amount of F-actin was increased to 142.2 ± 3.9 % of control at 5 min of hypertonic stress

($n=4$, $p<0.001$; Figure 7A). With the blocker present, F-actin was $158.7 \pm 17.2\%$ ($n=4$, $p<0.01$; Figure 7B) which is virtually identical to the reference value. These data show that PLC as a mayor element in the hypertonic activation of cation channels, is clearly not employed in the formation of F-actin; or in other words, these results provide strong additional evidence for a distinct regulation of cell volume and cell shape in rat hepatocytes.

Working model

From these findings a model can be established, in which hypertonic stress evokes a bidirectional signalling between focal adhesions and the cytoskeleton (see Fig. 9). Cell shrinkage exerts mechanical tension on cell-matrix adhesion sites, initiating focal adhesion signalling. This is mediated via small GTPases of the Rho family towards the formation of actin stress fibres that are anchored in focal adhesion sites, thereby transmitting tension. The consequence would be the regulation of cell shape by

enhancing the intracellular scaffold on the one hand, and adjustment of the cell-matrix interaction forces on the other, both leading to the maintenance of tissue integrity.

Quite independent from this pattern of cell shape regulation, focal adhesion signalling also triggers the activation of PLC and PKC, mediating - via exocytosis - the insertion of cation channels into the hepatocyte plasma membrane. Conductive import of Na^+ is enhanced, cell Na^+ (plus Cl^-) is increasing and this is paralleled by the influx of osmotically obliged water until the process of RVI is actually completed (Fig. 9).

Acknowledgements

The invaluable technical assistance of G. Beetz is gratefully acknowledged. The authors also wish to thank C. Pieczka and M. Wilkesmann for help with the artwork.

References

- Petzinger E, Föllmann W, Acker H, Hentschel J, Zierold K, Kinne RKH: Primary liver cell cultures grown on gas permeable membrane as source for the collection of primary bile. *In Vitro Cell Dev Biol* 1988;24:491-499.
- Wehner F, Guth D: Electric properties of rat liver cell-cultures on gas-permeable membranes. *Am J Physiol* 1991;261:G407-G416.
- Wehner F, Beetz G, Rosin-Steiner S: Osmolarity reduction transiently increases K^+ conductance of confluent rat hepatocytes in primary culture. *Am J Physiol* 1992;263:G913-G919.
- Wehner F, Rosin-Steiner S, Beetz G, Sauer H: The anion transport inhibitor DIDS increases rat hepatocyte K^+ conductance via uptake through the bilirubin pathway. *J Physiol (Lond)* 1993;471:617-635.
- Wehner F, Sauer H, Kinne RKH: Hypertonic stress increases the Na^+ conductance of rat hepatocytes in primary culture. *J Gen Physiol* 1995;105:507-535.
- Wehner F, Tinel H: Role of Na^+ conductance, $\text{Na}^+\text{-H}^+$ exchange, and $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ symport in the regulatory volume increase of rat hepatocytes. *J Physiol (Lond)* 1998;506:127-142.
- Wehner F, Tinel H: Osmolyte and Na^+ transport balances of rat hepatocytes as a function of hypertonic stress. *Pflugers Arch* 2000;441:12-24.
- Corasanti JG, Gleeson D, Boyer JL: Effects of osmotic stresses on isolated rat hepatocytes. I. Ionic mechanisms of cell volume regulation. *Am J Physiol* 1990;258:G290-G298.
- Gleeson D, Corasanti JG, Boyer JL: Effects of osmotic stresses on isolated rat hepatocytes. II. Modulation of intracellular pH. *Am J Physiol* 1990;258:G299-G307.
- Kirschner U, van Driessche W, Werner A, Wehner F: Hypertonic activation of phospholemman in solitary rat hepatocytes in primary culture. *FEBS Lett* 2003;537:151-156.
- Wehner F, Bondarava M, ter Veld F, Endl E, Nürnberger HR, Li T: Hypertonicity-induced cation channels. *Acta Physiol* 2006;187:21-25.
- Wehner F, Olsen H, Tinel H, Kinne-Saffran E, Kinne RKH: Cell volume regulation: osmolytes, osmolyte transport, and signal transduction. *Rev Physiol Biochem Pharmacol* 2003;148:1-80.
- Heinzinger H, van den Boom F, Tinel H, Wehner F: In rat hepatocytes, the hypertonic activation of Na^+ conductance and $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ symport - but not $\text{Na}^+\text{-H}^+$ antiport - is mediated by protein kinase C. *J Physiol (Lond)* 2001;536:703-715.
- Böhmer C, Wagner CA, Beck S, Moschen I, Melzig J, Werner A, Lin JT, Lang F, Wehner F: The shrinkage-activated Na^+ conductance of rat hepatocytes and its possible correlation to rENaC. *Cell Physiol Biochem* 2000;10:187-194.

- 15 Reid T, Bathoorn A, Ahmadian MR, Collard JG: Identification and characterization of hPEM-2, a guanine nucleotide exchange factor specific for Cdc42. *J Biol Chem* 1999;274:33587-33593.
- 16 Böhmer C, Wehner F: The epithelial Na⁺ channel (ENaC) is related to the hyper-tonicity-induced Na⁺ conductance in rat hepatocytes. *FEBS Lett* 2001;494:125-128.
- 17 Wehner F, Tinel H, Kinne RKH: Pharmacology of volume activated Na⁺ conductance in rat hepatocytes. *Physiologist* 1997;40:A-4.
- 18 Moriya M, Linder MC: Vesicular transport and apotransferrin in intestinal iron absorption, as shown in the Caco-2 cell model. *Am J Physiol* 2006;290:G301-G309.
- 19 Karylowski O, Zeigerer A, Cohen A, McGraw TE: GLUT4 is retained by an intracellular cycle of vesicle formation and fusion with endosomes. *Mol Biol Cell* 2004;15:870-882.
- 20 Zaidi ZF, Matthews MR: Exocytotic release from neuronal cell bodies, dendrites and nerve terminals in sympathetic ganglia of the rat, and its differential regulation. *Neuroscience* 1997;80:861-891.
- 21 Newman TM, Tian M, Gomperts BD: Ultrastructural characterization of tannic acid-arrested degranulation of permeabilized guinea pig eosinophils stimulated with GTP-gamma-S. *Eur J Cell Biol* 1996;70:209-220.
- 22 Rebecchi MJ, Pentyala SN: Structure, function, and control of phosphoinositide-specific phospholipase C. *Physiol Rev* 2000;80:1291-1335.
- 23 Feranchak AP, Kilic G, Wojtaszek PA, Qadri I, Fitz JG: Volume-sensitive tyrosine kinases regulate liver cell volume through effects on vesicular trafficking and membrane Na⁺ permeability. *J Biol Chem*. 2003;278:44632-44638.
- 24 Ruest PJ, Roy S, Shi E, Mernaugh RL, Hanks SK: Phosphospecific antibodies reveal focal adhesion kinase activation loop phosphorylation in nascent and mature focal adhesions and requirement for the autophosphorylation site. *Cell Growth Differ* 2000;11:41-48.
- 25 Barfod ET, Moore AL, Melnick RF, Lidofsky SD: Src regulates distinct pathways for cell volume control through Vav and phospholipase C gamma. *J Biol Chem* 2005;280:25548-25557.
- 26 Allen WE, Jones GE, Pollard JW, Ridley AJ: Rho, Rac and Cdc42 regulate actin organization and cell adhesion in macrophages. *J Cell Sci* 1997;110:707-720.
- 27 Price LS, Leng J, Schwartz MA, Bokoch GM: Activation of Rac and Cdc42 by integrins mediates cell spreading. *Mol Biol Cell* 1998;9:1863-1871.
- 28 Alblas J, Ulfman L, Hordijk P, Koenderman L: Activation of RhoA and ROCK are essential for detachment of migrating leukocytes. *Mol Biol Cell* 2001;12:2137-2145.
- 29 Arthur WT, Burridge K: RhoA inactivation by p190RhoGAP regulates cell spreading and migration by promoting membrane protrusion and polarity. *Mol Biol Cell* 2001;12:2711-2720.
- 30 Hall A: Rho GTPases and the actin cytoskeleton. *Science* 1998;279:509-514.